ASCEPT-MPGPCR

JOINT SCIENTIFIC MEETING

Melbourne Convention & Exhibition Centre

7–11 December 2014

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POSTER PROGRAM AND ABSTRACTS

Poster presentations

Monday 8 December, 9.30am - 10.30am and 12.30pm - 1.30pm

Posterboard number	Speakers Paper Title	Presenter	Paper reference	Speakers Theme Description
513	Structure-function relationships of human dimethylarginine dimethylaminohydrolase (DDAH) isoforms, DDAH-1 & DDAH-2: an approach to rational drug design	Joshua Bizilis	513	Drug Disposition and Response
514	Tamoxifen and its active metabolite 4-hydroxytamoxifen up-regulate UDP-glucuronosyltransferases (UGT) 2B15 and 2B17 in breast cancer cells: A novelmechanism of tamoxifen antiestrogenic activity	Apichaya Chanawong	514	Drug Disposition and Response
515	A mouse model to determine the effects of polypharmacy on adverse geriatric outcomes	Sarah Hilmer	515	Drug Disposition and Response
516	A validated mouse frailty index to assess longevity interventions: Impact of calorie restriction and resveratrol	Alice Kane	516	Drug Disposition and Response
517	Detection of Testosterone dosing in racing male greyhounds	Jayasree Leela Devi	517	Drug Disposition and Response
518	Study of a novel regulatory mechanism that controls UGT1A8 synergistic activation by Cdx2 (Caudal-related Homeodomain protein-2) and HNF4a (Hepatocyte Nuclear Factor-4 alpha)	Siti Nurul Mubarokah	518	Drug Disposition and Response
519	Xenobiotic metabolism in Australian marsupial koala, *Phascolarctos cinereus*: Molecular and biochemical perspectives	Suong Ngo	519	Drug Disposition and Response
520	Xenobiotic metabolism in Australian marsupial koala, *Phascolarctos cinereus*: Comparative PKs/PDs and drug disposition	Suong Ngo	520	Drug Disposition and Response
521	Shape of the ciprofloxacin and tobramycin concentration-time profile is critical for resistance prevention in Pseudomonas aeruginosa	Vanessa Rees	521	Drug Disposition and Response
522	Extremes of body size in the dosing of high-dose methotrexate chemotherapy fornon-Hodgkin lymphoma	Geeta Sandhu	125	Drug Disposition and Response
523	Characterisation of the UDP-glucuronosyltransferase (UGT) enzyme inhibition selectivity of dapagliflozin: Implications for drug-drug interactions	Attarat Pattanawongsa	594	Drug Disposition and Response
525	Psychotropic drug utilisation in older people in New Zealand from 2005 to 2013	Henry Ndukwe	130	Pharmacoepidemiology
526	Older peoples' attitudes and beliefs about statin use and their willingness to have statins deprescribed	Katie Qi	526	Pharmacoepidemiology
527	Comparing utilisation of mycophenolate, tacrolimus, cyclosporin, sirolimus and everolimus in Australia and Northern Europe	Christine Staatz	527	Pharmacoepidemiology
528	Analgesic use, pain and daytime sedation in people with and without dementia in aged care facilities	Edwin Tan	528	Pharmacoepidemiology
529	Prevalence of potential statin-drug interactions in frail and robust older inpatients	Michele Thai	129	Pharmacoepidemiology
530	Prevalence of statin-drug interactions in older people: A systematic review	Michele Thai	530	Pharmacoepidemiology
531	Polypharmacy in older people with cancer: How many medicines are too many?	Justin Turner	531	Pharmacoepidemiology
532	Identifying the impact of medication complexity on chronic disease	Basia Diug	532	Pharmacoepidemiology
533	Medication Regimen Complexity Index: An indicator of bleeding risk	Basia Diug	533	Pharmacoepidemiology
534	Medication-administration problems in older people in Sweden: A population-based case-control study	Barbara Wimmer	534	Pharmacoepidemiology
573	Effect of chemical enhancers on the *in vitro* percutaneous penetration of caffeine	Eman Abd	573	Clinical Pharmacology
574	Redefining normal variability of drug disposition	Hesham Al-Sallami	574	Clinical Pharmacology
575	Resolvin D2 has mitogenic activity in estrogen receptor positive breast cancer cell lines via activation of estrogen receptor	Nuha Al-Zaubai	575	Clinical Pharmacology
576	Safety and Pharmacokinetics of Metformin in liver disease patients	Hannah Braithwaite	576	Clinical Pharmacology
577	Domperidone, QT prolongation and sudden cardiac death - is there really a risk?	Pam Buffery	577	Clinical Pharmacology
578	Microglia-Derived BDNF Modulates Dopamine Circuitry in Opioid Dependent States	Catherine Cahill	578	Clinical Pharmacology
579	Audit of clinical adherence to National Health Medical Research Council (NHMRC) venous thromboprophylaxis (VTE) guidelines in acutely ill older medical inpatients	Melissa Chin	579	Clinical Pharmacology
580	Ongoing poor management of medicines in the older-aged living independently in a rental retirement village	Sheila Doggrell	580	Clinical Pharmacology
581	Metformin pharmacokinetics in haemodialysis	Suraj Gangaram	581	Clinical Pharmacology
582	Neprilysin inhibitors preserve renal function in heart failure - meta-analysisof randomised controlled trials	Ingrid Hopper	212	Clinical Pharmacology
583	Utilisation and outcomes of pharmacological venous thromboembolism (VTE) prophylaxis in older frail and robust medical inpatients	Daisy Howe	583	Clinical Pharmacology
584	Insights into the pathophysiology of hyperuricaemia	Diluk Kannangara	213	Clinical Pharmacology
585	Barriers to care in gout: A qualitative study of patients' and prescribers' understanding and management	Diluk Kannangara	585	Clinical Pharmacology
586	Factors associated with a high anticholinergic burden in elderly Australians with and without dementia	Karen Kerr	586	Clinical Pharmacology
587	Healthcare practitioners' perspectives on prescribing and deprescribing anticholinergic and sedative medications in older adults	Lisa Kouladjian	317	Clinical Pharmacology
588	The influence of imatinib on disease progression in patients with gastro-intestinal stromal tumour (GIST)	Shaun Kumar	214	Clinical Pharmacology
589	Declining intra-lymphocyte concentrations of mycophenolic acid correlate with the incidence of graft rejection in renal transplant recipients: Preliminary results of a prospective study	Zaipul Md Dom	589	Clinical Pharmacology
590	Response to antiplatelet drugs in frail and non-frail older inpatients with atrial fibrillation	Sarah Hilmer	590	Clinical Pharmacology
591	Clinical review and documentation of rationale for potentially inappropriate medication prescribing in older hospital in-patients	Ric Day	591	Clinical Pharmacology
592	Patterns of deprescribing amongst Geriatricians: The Influence of cognition, dependency and pill burden	Ric Day	592	Clinical Pharmacology
593	Rates of polypharmacy at admission and discharge: A retrospective hospital-based study	Ric Day	593	Clinical Pharmacology
595	*In vivo* evaluation of felodipine as an inhibitor of cytochrome P450 enzymes and P-glycoprotein	Thomas Polasek	595	Clinical Pharmacology
596	Emerging definition of deprescribing: Implications for research and practice in older adults	Emily Reeve	596	Clinical Pharmacology
597	Patient autonomy in deprescibing: A qualitative study of the views, attitudes and beliefs of older adults and non-paid carers of older adults	Emily Reeve	597	Clinical Pharmacology
598	Piperacillin pharmacokinetics in intensive care unit patients with anuria andacute kidney injury receiving sustained low efficiency diafiltration	Mahipal Sinnollareddy	215	Clinical Pharmacology
599	Assay for measurement of etoposide and selected anti-retrovirals in dried blood spots	Robert Matthew Strother	599	Clinical Pharmacology

Posterboard number	Speakers Paper Title	Presenter	Paper reference	Speakers Theme Description
600	Oxycodone and other analgesics prescribing on discharge from Christchurch Hospital	Jane Vella-Brincat	600	Clinical Pharmacology
601	Anti-*Helicobacter* activities of Kakadu Plum and colloidal silver	Michael Whitehouse	601	Clinical Pharmacology
602	The relationship between fractional urate clearance and renal transporter genotype in patients with gout	Dan Wright	602	Clinical Pharmacology
603	Novel approach to optimising synergistic carbapenem plus aminoglycoside combinations to combat carbapenem-resistant *Acinetobacter baumannii*	Rajbharan Yadav	603	Clinical Pharmacology
604	*In-vivo in-vitro* correlation (IVIVC) of topical salicylate esters	Shereen Yousef	604	Clinical Pharmacology
637	Characterisation of novel benzamide derivatives as P2X7 receptor antagonists	Melissa Barron	637	Drug Discovery
638	Assessment of Janus protein tyrosine kinases as potential regulators of epithelial-mesenchymal transition using a model of epidermal growth factor induced breast cancer epithelial-mesenchymal transition	John Bassett	638	Drug Discovery
639	Assessment of the consequences of silencing of the secretory pathway calcium ATPase 1 in MDA-MB-231 breast cancer cells	Lisa Chaw	639	Drug Discovery
640	Expression of copper transporters in colorectal cancer cells and its relation to oxaliplatin	Haigang Cui	640	Drug Discovery
641	Comparative anti-proliferative and pro-apoptotic actions of a novel w-3 fatty acid analogue in human breast cancer cell lines	Adam Hraiki	641	Drug Discovery
642	Genetically-encoded calcium indicators for the assessment of intracellular calcium signalling in MDA-MB-231 breast cancer cells	Ellen Janke	642	Drug Discovery
643	miR-126 regulation in a mouse model of laser-induced choroidal neovascularisation	Amy Lee	135	Drug Discovery
644	Tumour microenvironment factors and the promotion of epithelial to mesenchymal transition in MDA-MB-468 breast cancer cells	Marnie Mitchell	644	Drug Discovery
645	Cruciferous vegetables and cancer prevention - A critical review	Suong Ngo	645	Drug Discovery
646	Comparative investigation of plasma glucose lowering activity and effect on liver, kidney and antioxidant enzymes parameters of Palmatine and Palmatine - like alkaloid isolated from stem of Consinum Fenestratum plant on STZ induced diabetic rat model	Patrick Okechukwu	646	Drug Discovery
647	The expression of plasma membrane Ca^{2+} ATPase isoform, PMCA2, in breast tissue with lactational change and in breast cancer cells and the consequences of its silencing in MDA-MB-231 breast cancer cells	Amelia Peters	647	Drug Discovery
648	Exploring the structure activity relationships of a series of agonists targeting the $\alpha4\beta2$ nicotinic acetylcholine receptor	Vanda Rudner-Varga	648	Drug Discovery
649	Allosteric interactions of indole and benzimidazole derivatives at the human translocator protein	Eryn Werry	649	Drug Discovery
650	AMTB, a TRPM8 channel blocker also inhibits voltage gated sodium channels (Na_v) in breast cancer cells	Kunsala Yapa	650	Drug Discovery
653	High antioxidant Australian plant extracts: Anti-proliferative activities against CaCo2 and HeLa cell lines	Joseph Sirdaarta	653	Drug Discovery
654	Macadamia nut as an anti-Giardial agent	Joseph Sirdaarta	654	Drug Discovery
655	Biopharming *Carica papaya* compounds with anti-Proteus activity: The potential for the treatment and prevention of rheumatoid arthritis	Nathan Jamieson	655	Drug Discovery
656	*Tasmannia lanceolata* extracts: Anti-Proteus activity and potential for the treatment and prevention of rheumatoid arthritis	Ian Cock	656	Drug Discovery
657	Multi-dimensional IMAC: The capture of multiple bacterial metabolites from culture	Jiesi Gu	657	Drug Discovery
658	Isolating native doxorubicin from *Streptomyces peucetius* var. *caesius* culture using immobilised metal ion affinity chromatography	Isla Nakano	658	Drug Discovery
659	Inhibitor activity of *Urena lobata* leaf extract on Dipeptidyl Peptidase IV (DPP-IV)	Yudi Purnomo	659	Drug Discovery
660	New histone deacetylase inhibitors from innovations in chemical biology	Tomas Richardson-Sanchez	660	Drug Discovery
661	The potential role of nonulosonic acids analogues in drug discovery: The road to new antibacterial agents	Matthew Zunk	661	Drug Discovery
662	Structure-activity analysis of biased agonism at the adenosine A_3 receptor	Jo-Anne Baltos	131	Drug Discovery
663	Differential regulation of the μ-opioid receptor by distinct agonists	Arisbel Batista	663	Drug Discovery
664	Comparative pharmacology of allosteric modulators at the M_5 muscarinic acetylcholine receptor	Alice Berizzi	664	Drug Discovery
665	Identifying novel small molecules that enhance the bronchodilation and anti-inflammatory effect of Vasoactive Intestinal Peptide	Tony Cardno	665	Drug Discovery
666	Phase I metabolism study of bacterial NorA efflux pump inhibitors	Emanuele Carosati	666	Drug Discovery
667	Ryanodine receptors: A novel target for cigarette smoking-related lung disease	Chantal Donovan	133	Drug Discovery
668	Rosiglitazone induction of mouse brite adipocytes derived from subcutaneous white adipose tissue	Bronwyn Evans	668	Drug Discovery
669	Discovery and optimization of novel, highly selective M5 mAChR allosteric modulators	Patrick Gentry	669	Drug Discovery
670	Probing the structure and dynamics of a class A G protein-coupled receptor using small-angle X-ray scattering	Natalie Gunn	670	Drug Discovery
671	Identifying novel small molecules that enhance the insulinotrophic effect of glucagon-like peptide-1 (GLP-1)	Caroline Hick	671	Drug Discovery
672	A novel role for mammalian target of rapamycin complex 2 (mTORC2) in brown adipose tissue (BAT) glucose uptake mediated by beta-adrenoceptors (AR)	Dana Hutchinson	672	Drug Discovery
673	Monitoring angiotensin II receptor complexes using bioluminescence resonance energy transfer	Elizabeth Johnstone	673	Drug Discovery
674	Rapid selection and development of GPCR expressing Mammalian cell lines using novel ClonePix Technology	Scott Jones	674	Drug Discovery
675	The role of the extracellular vestibule in ß2 adrenoceptor orthosteric and allosteric ligand interactions	Erica Leonar	136	Drug Discovery
676	Expression and purifcation of human calcitonin receptor	Yi-Lynn Liang	676	Drug Discovery
677	Pharmacological characterization of a muscarinic acetylcholine M1 receptor positive allosteric modulator MIPS1463	Herman Lim	677	Drug Discovery
678	Investigating the molecular determinants of allosteric modulation at the adenosine A_1 receptor	Lauren May	678	Drug Discovery
679	Potent non-peptide agonists for human C3a receptors	Ranee Singh	679	Drug Discovery
680	Fluorescently labelled ligands targeting the dopamine D_2 receptor	Monika Szabo	680	Drug Discovery
681	Designed multiple ligands targeting the dopamine D_2 and muscarinic M_1 receptors	Monika Szabo	681	Drug Discovery
682	Non-selective opioid receptor modulation of Beta-endorphin1-11 in cAMP accumulation	Kate Wang	682	Drug Discovery
683	Modulation of the M4 muscarinic acetylcholine receptor regulation by allosteric ligands	Adriel Wen	683	Drug Discovery

Poster presentations

Tuesday 9 December, 9.30am - 10.30am and 12.30pm - 1.30pm

Posterboard number	Speakers Paper Title	Presenter	Paper reference	Speakers Theme Description
500	NSAIDS: How clarifying a question of toxicity added to the vocabulary of pharmacology (a memoir)	Michael Whitehouse	500	Toxicology
501	Activation of AMPK prevents drug induced mitochondrial and hepatocellular damage	Dong Fu	501	Toxicology
502	Auditing adulterants: Toxicological assessment of herbal medicines sold in Australia during 2014	Claire Hoban	331	Toxicology
503	Resveratrol and SIRT1 do not protect against paracetamol toxicity	Alice Kane	333	Toxicology
504	The effect of ageing on isoniazid pharmacokinetics and hepatotoxicity in Fischer 344 rats	John Mach	314	Toxicology
505	Isoniazid does not activate the intrinsic death pathway *in vivo* in young and old Fischer 344 rats	John Mach	505	Toxicology
506	Rapid therapeutic monitoring of atorvastatin and three metabolites in human plasma by LC/MS	Ahmed Mostafa	506	Toxicology
507	Detecting adulteration and contamination in traditional Chinese medicine (TCM): Combined DNA and toxicological audits	Ian Musgrave	507	Toxicology
508	The effect of Saxitoxin on the the differentiation of D3 embryonic stem cells into a neural lineage	Katie O'Neill	332	Toxicology
537	Inhibition of platelet aggregation by vanilloid-like agents is not mediated by transient receptor potential vanilloid-1 (TRPV1) channels or cannabinoid (CB1, CB2) receptors	Dom Geraghty	537	Cardiovascular
538	Evidence against a role for pannexin-1 channels in α1-adrenoceptor-mediated vasoconstriction in resistance arteries	Ashenafi Haileyesus Betrie	538	Cardiovascular
539	Glucagon-like peptide-1 receptor as a target in treatment of cardiovascular disease	Melita Brdar	539	Cardiovascular
540	Long chain omega-3 polyunsaturated fatty acids improve aortic relaxation in a mouse model of abdominal aortic aneurysm	Corinna Bürgin-Maunder	540	Cardiovascular
541	CC Chemokine receptor 2 Inhibition exacerbates stroke outcome in a mouse model of transient middle cerebral artery occlusion	Hannah Chu	321	Cardiovascular
542	VCP746: A cardioprotective adenosine receptor agonist with minimal haemodynamic effects	Chung Hui Chuo	542	Cardiovascular
543	Endogenous Annexin-A1 (ANX-A1) is cardioprotective against myocardial infarction (MI) in mice *in vivo*	Siobhan Finlayson	310	Cardiovascular
544	AT4 Receptor/Insulin Regulated Aminopeptidase (IRAP): A target for protection in cardiovascular disease	Huey Wen Lee	311	Cardiovascular
545	Major role of endothelial nitric oxide release in the responses to noradrenaline in arteries isolated from different regions of the rat cerebral circulation	Yohannes Mamo	545	Cardiovascular
546	Genetic deletion of the complement receptor C5aR1 has no effect on angiotensin II-induced hypertension and vascular remodelling	Tamara Paravicini	546	Cardiovascular
547	A flavonoid rich extract from *Carpobrotus rossii* improves glucose tolerance but not lipid profile	Adam Pirie	547	Cardiovascular
548	Phosphoinositide 3-kinase (p110α) gene therapy attenuates diabetic cardiomyopathy in mice	Darnel Prakoso	325	Cardiovascular
549	The DPP-4 inhibitor linagliptin and the GLP-1 receptor agonist exendin-4 prevent high glucose-induced impairment of endothelial function in rat mesenteric arteries	Salheen Salheen	323	Cardiovascular
550	Endothelium-dependent cAMP and cGMP responses to serelaxin in human smooth muscle cells: Role of nitric oxide and prostanoids	Mohsin Sarwar	550	Cardiovascular
551	Serelaxin signalling in human primary vascular cells: G-proteins and their location determines the shape of the concentration-response relationship	Mohsin Sarwar	316	Cardiovascular
552	Endogenous hydrogen sulfide (H_2S) production in resistance arteries of the rat	Christopher Seaman	552	Cardiovascular
553	Cardiac dysfunction and ischaemic intolerance in murine type II diabetes mellitus is reversed by sustained ligand-activated preconditioning	Louise See Hoe	326	Cardiovascular
554	Chronic NaHS treatment protects vascular function in streptozotocin-induced diabetes in mice	Gunes Suzan Yildiz	554	Cardiovascular
555	Th2-promoting cytokine treatment limits brain injury after cerebral ischemia in Th1-dominant mice	Shenpeng Zhang	555	Cardiovascular
556	Improving warfarin education in community patients	Basia Diug	556	Education
557	Assessing patient knowledge and education techniques in order to improve safety with warfarin therapy: A review of the literature	Basia Diug	226	Education
558	Improving student outcomes and perceptions by enhancing engagement	Ian Cock	558	Education
559	Development of a smartphone 'app' for converting brand names of drugs to approved drug names to assist trainee doctors in taking drug histories and in prescribing	Bevyn Jarrott	559	Education
560	The effectiveness of current and emerging pharmacotherapy in a new model of human prostatic smooth muscle tone	Basu Chakrabarty	233	Urogenital and Gastrointestinal
561	Pannexin-2 expression in the human intestine - possible roles in gastrointestinal motility and secretion	Erica Diezmos	561	Urogenital and Gastrointestinal
562	Calcium homeostasis modulator 1 as a potential ATP release conduit in human colonic epithelial cells	Stiliani Drimousis	562	Urogenital and Gastrointestinal
563	A polar compound present in stinging nettle leaf extract inhibits the purinergiccomponent of contractility in the rat prostate gland	Nicole Eise	232	Urogenital and Gastrointestinal
564	Gemcitabine enhances release of ATP from bladder urothelial cells but is selectively cytotoxic to bladder cancer cell lines	Russ Chess-Williams	564	Urogenital and Gastrointestinal
565	The α1-adrenoceptor antagonist prazosin enhances sensitivity of hypoxic prostate cancer cells to irradiation	Russ Chess-Williams	565	Urogenital and Gastrointestinal
566	The differing effects of doxorubicin treatment on neurogenic detrusor responses of young and old porcine bladders	Russ Chess-Williams	566	Urogenital and Gastrointestinal
567	Alterations in bladder urothelial acetylcholine, ATP, prostaglandin E2 and inflammatory cytokines by the chemotherapeutic agent epirubicin	Russ Chess-Williams	567	Urogenital and Gastrointestinal
568	KCl potentiates subsequent responses to noradrenaline of the porcine urethra by enhancing ROCK and Ca^{2+} release	Donna Sellers	568	Urogenital and Gastrointestinal
569	The role of Rho-kinase in phenylephrine and 5-HT-induced contractile activity of the porcine ureter	Donna Sellers	569	Urogenital and Gastrointestinal
570	Bladder sensory nerve activity is enhanced by the cytotoxic drugs cyclophosphamide and ifosfamide	Kylie Mills	315	Urogenital and Gastrointestinal

Posterboard number	Speakers Paper Title	Presenter	Paper reference	Speakers Theme Description
571	Comparative Evaluation of Different Polyphenols of Green Tea and to study the most Effective Polyphenol in Nanotechnology Based Drug Delivery system alone and in Combination with Sulfasalazine in experimental Inflammatory Bowel Disease in Rats	Bikash Medhi	571	Urogenital and Gastrointestinal
572	Loss of fenestrations in liver sinusoidal endothelial cells impairs hepatic insulin signaling and glucose homeostasis	Mashani Mohamad	572	Urogenital and Gastrointestinal
605	Effect of Beta-endorphin fragments on interleukin-1beta release	Naghmeh Asvadi	605	Inflammation/respiratory
606	Glucocorticoids induce skeletal muscle wasting in a mouse model of acute exacerbations of Chronic Obstructive Pulmonary Disease	Victoria Austin	606	Inflammation/respiratory
607	Targeting oxidative stress reduces cigarette smoke-induced lung inflammation and wasting in mice	Ivan Bernardo	607	Inflammation/respiratory
608	Biotransformed dynorphin A fragments attenuate lipopolysaccharide-stimulated activation of THP-1 cell-derived macrophages through inhibition of nuclear factor-kappa beta (NF-κB) translocation	Siti Sarah Fazalul Rahiman	608	Inflammation/respiratory
609	How does early life viral-induced pneumococcal infection impact the adult mouse lung?	Meaghan FitzPatrick	609	Inflammation/respiratory
610	Extracts of Australian native stingless bee (*Tetragonula carbonaria*) cerumen modulate dermal fibroblast proliferation and myofibroblast differentiation *in vitro*	Karina Hamilton	610	Inflammation/respiratory
611	Nanoparticles as potential novel mast cell modulating agents	Sai-Min Htay	611	Inflammation/respiratory
612	Glucocorticoid gene regulation differs in airway epithelial cells compared to hepatocytes	Christine Keenan	612	Inflammation/respiratory
613	Relaxin and rosiglitazone exert differential inhibition of airway contraction to methacholine and endothelin-1	Maggie Lam	613	Inflammation/respiratory
614	Targeting ALX/FPR2 in models of bacterial and viral co-infection	Bruce Ngo	614	Inflammation/respiratory
615	Morphine modulates IL-4- or breast cancer cell-induced pro-metastatic activation of macrophages	Samira Khabbazzi	615	Inflammation/respiratory
616	Poly I:C, a TLR3 ligand and a viral RNA mimic, and heat-inactivated respiratory syncytial virus (RSV) recapitulate the glucocorticoid insensitivity evoked by RSV infection	Asmaa Radwan	616	Inflammation/respiratory
617	Guanine Deaminase: Significance, assay, identification of inhibitors, and evidence for redox regulation	Tim Shaw	617	Inflammation/respiratory
618	Microenviromental determinants of glucocorticoid responsiveness in airway smooth muscle	Amanda Wong	230	Inflammation/respiratory
619	Elastase activates PAR_2/TRPV4 signaling complex and causes inflammatory pain	Peishen Zhao	619	Inflammation/respiratory
620	GHB activates a subset of $GABA_A$Rs expressed in Xenopus oocytes	Nathan Absalom	620	Neuro- and Behavioural Pharmacology
621	Muscarinic acetylcholine M4 receptor regulation of psychosis-like behaviours induced by a dopamine D1 receptor-selective agonist in mice	Amy Nae-Yng Chen	334	Neuro- and Behavioural Pharmacology
622	Effect of positive allosteric modulators of M_1 muscarinic receptors on psychosis-like behaviours in mice	Christopher Choy	622	Neuro- and Behavioural Pharmacology
623	Activity of illicit synthetic cannabinoids, UR-144, XLR-11 and 5-hydroxypentyl-UR-144 at human cannabinoid receptors and cannabinoid-sensitive ion channels	Mark Connor	623	Neuro- and Behavioural Pharmacology
624	Varenicline improves motor coordination and alters cytokine expression profiles in the YAC128 transgenic mouse model of Huntington's Disease	Gary D'Souza	624	Neuro- and Behavioural Pharmacology
625	Development of novel polymeric microparticles for sustained-release intrathecal delivery of analgesics	Felicity Han	625	Neuro- and Behavioural Pharmacology
626	Neurokinin 1 receptor signalling from endosomes: A key source of pain signalling	Dane Jensen	626	Neuro- and Behavioural Pharmacology
627	Type-1 interferon signalling regulates the glial-mediated neuroinflammatory response in models of Parkinson's disease	Bevan Main	627	Neuro- and Behavioural Pharmacology
628	Varenicline improves motor, cognitive and psychiatric symptoms in the YAC128 mouse model of Huntington's Disease	Gary D'Souza	628	Neuro- and Behavioural Pharmacology
629	Investigating the activity of opioid and non-opioid alkaloids at the Toll-like receptor 4	Sanam Mustafa	629	Neuro- and Behavioural Pharmacology
630	Generic substitution of antiepileptic drugs: Patient attitudes and perceptions	Suong Ngo	630	Neuro- and Behavioural Pharmacology
631	Functional expression of TRPV4 by satellite glial cells of mouse dorsal root ganglia	Pradeep Rajasekhar	336	Neuro- and Behavioural Pharmacology
632	Mechanism of $\alpha 4\beta 2$ nicotinic acetylcholine receptor modulation unravelled by x-ray crystallographic and functional studies	Thomas Balle	632	Neuro- and Behavioural Pharmacology
633	*CYP2C8* genotype significantly alters imatinib metabolism in Chronic Myeloid Leukaemia patients	Hannah Cox	633	Pharmacogenomics
634	Impact of *CYP2C8*1/*3* polymorphism on *in vitro* metabolism of imatinib to its active metabolite N-desmethyl imatinib	Muhammad Suleman Khan	634	Pharmacogenomics
635	Using forensic pharmacogenetics in psychiatry and improving public health	Yolande Lucire	635	Pharmacogenomics
636	Introduction of a multi-gene pharmacogenomic test and an electronic support system into aged persons mental health inpatient care	Leslie Sheffield	636	Pharmacogenomics

Poster presentations

Wednesday 10 December, 9.30am - 10.30am and 12.30pm - 1.30pm

Posterboard number	Speakers Paper Title	Presenter	Paper reference	Speakers Theme Description
684	Mechanistic insights into allosteric structure-function relationships at the M_1 muscarinic acetylcholine receptor	Alaa Abdul-Ridha	684	Molecular Pharmacology of GPCRs
685	Signal transduction pathways activated by insulin-like peptide 5 at relaxin family peptide receptor 4	Sheng Yu Ang	685	Molecular Pharmacology of GPCRs
686	Investigating the signalling bias of GLP-1(9-36)amide at the GLP-1 receptor	Kerry Barkan	686	Molecular Pharmacology of GPCRs
687	Inhibition of human neuronal Cav2.3 channels via μ-, δ- and κ-opioid receptoractivation	Geza Berecki	687	Molecular Pharmacology of GPCRs
688	Functional analysis of the N-terminal residues of human amylin	Rebekah Bower	688	Molecular Pharmacology of GPCRs
689	The third intracellular loop of the human L-amino acid receptor GPRC6A controls cell surface expression and receptor function	Hans Bräuner-Osborne	689	Molecular Pharmacology of GPCRs
690	Allosteric coupling in the calcium-sensing receptor	Alice Brown	690	Molecular Pharmacology of GPCRs
691	Deciphering the complex mode of relaxin receptor (RXFP1) activation	Shoni Bruell	691	Molecular Pharmacology of GPCRs
692	Characterising the conformational diversity of the neurotensin receptor 1	Fabian Bumbak	692	Molecular Pharmacology of GPCRs
693	Identifying novel sites of ligand interaction on the α1 adrenoceptors	Adrian Campbell	693	Molecular Pharmacology of GPCRs
694	Signalling of indole-2-carboxamides as allosteric modulators of the cannabinoid receptors	Erin Cawston	694	Molecular Pharmacology of GPCRs
695	GPCR MAS modulates purinergic P2Y2 and GLUT1 activities via interacting with MBP7-like motif	Wing-Tai Cheung	695	Molecular Pharmacology of GPCRs
696	Architecture and function of signalling complexes assembled by ß2-adrenergicand M_3 muscarinic receptors	Srgjan Civciristov	696	Molecular Pharmacology of GPCRs
697	GPR37L1: an orphan G protein-coupled receptor with constitutive activitymodulated by novel metalloprotease-mediated N-terminal cleavage	James Coleman	401	Molecular Pharmacology of GPCRs
698	Multi-pathway analysis of allosteric modulation of the calcium-sensing receptor by calcilytics	Anna Cook	698	Molecular Pharmacology of GPCRs
699	Improving G protein-coupled receptor virtual screening outcomes: Method for binding pocket refinement	Thomas Coudrat	699	Molecular Pharmacology of GPCRs
700	Discovery of novel selective C5a2 ligands that modulate IL-6 release from macrophages	Daniel Croker	700	Molecular Pharmacology of GPCRs
701	Functional expression of modified calcitonin receptor in nanodics forbiophysical measurements of family B GPCR conformational changes	Emma Dal Maso	701	Molecular Pharmacology of GPCRs
702	Comparative pharmacology of structurally distinct calcimimetics acting at the calcium-sensing receptor	Natalie Diepenhorst	702	Molecular Pharmacology of GPCRs
703	Identification of the mechanisms which govern allosterism at the dopamine D_2 receptor	Christopher Draper-Joyce	402	Molecular Pharmacology of GPCRs
704	Roles of the calcium-sensing receptor intraloops -2 and -3 and the carboxy-terminus in signalling pathway selection	Kimberly Edwards	704	Molecular Pharmacology of GPCRs
705	*In silico* analysis and cardiac gene expression data provide novel insights into human and mouse taste receptor gene regulation	Simon Foster	705	Molecular Pharmacology of GPCRs
706	Understanding the nature of efficacy at a model family B GPCR	Sebastian Furness	706	Molecular Pharmacology of GPCRs
707	Hypotonic stress indirectly activates TRPV4 via intracellular signalling	Megan Grace	707	Molecular Pharmacology of GPCRs
708	Clickable photoaffinity labels for metabotropic glutamate receptor 5 based on allosteric modulator scaffolds	Karen Gregory	708	Molecular Pharmacology of GPCRs
709	Species-dependence of Cannabinoid Receptor 2 functional selectivity	Natasha Grimsey	709	Molecular Pharmacology of GPCRs
710	Do relaxin receptors function as obligatory dimers?	Bradley Hoare	710	Molecular Pharmacology of GPCRs
711	Biphasic Ca^{2+}-dependent control of 25-hydroxyvitamin D-1α-hydroxylase expression mediated by the calcium-sensing receptor	Alice Huang	711	Molecular Pharmacology of GPCRs
712	Identification of cAMP signalling phenotypes in subpopulations of cells using a flow cytometer	Morag Rose Hunter	712	Molecular Pharmacology of GPCRs
713	Signalling pathway-selective consequences of the common µ-opioid receptor variants A6V and N40D	Marina Junqueira Santiago	713	Molecular Pharmacology of GPCRs
714	Signalling profile of CRIP1a: G-protein activation and signal transduction	Nilushi Karunaratne	714	Molecular Pharmacology of GPCRs
715	Novel agonist engagement and signalling bias by bitopic ligands at the M_1 muscarinic ACh receptor	Peter Keov	715	Molecular Pharmacology of GPCRs
716	The modulatory effect of morphine on matrix metalloproteinase-9 production in alternatively polarized macrophages	Samira Khabbazi	716	Molecular Pharmacology of GPCRs
717	Ligand-biased signalling and ligand-biased allosteric modulation at CB1 cannabinoid receptors	Elham Khajehali	717	Molecular Pharmacology of GPCRs
718	Biased agonism in the 4th dimension: the contribution of binding kinetics to thebias profile of antipsychotic drugs at the dopamine D2 receptor	Carmen Klein Herenbrink	312	Molecular Pharmacology of GPCRs
719	Genetic encoding of unnatural amino acids in the Glucagon-like peptide-1 receptor (GLP-1R)	Cassandra Koole	719	Molecular Pharmacology of GPCRs
720	Disruption of GLP-1 receptor dimersation results in liraglutide sensitivity	Graham Ladds	720	Molecular Pharmacology of GPCRs
721	Determining the role of RAMP2 in modulating glucagon receptor G protein coupling	Graham Ladds	721	Molecular Pharmacology of GPCRs
722	Gαq-mediated signalling bias by adrenomedullin2 at the calcitonin gene-related peptide receptor	Graham Ladds	722	Molecular Pharmacology of GPCRs
723	Calcilytics and calcimimetics modulate calcium sensing receptor (CaSR) activity via overlapping but distinct allosteric binding sites	Katie Leach	723	Molecular Pharmacology of GPCRs
724	Structurally distinct calcimimetics engender biased signalling from the human calcium sensing receptor (CaSR)	Katie Leach	724	Molecular Pharmacology of GPCRs
725	Desensitization of α_{1A}-adrenoceptors by oxymetazoline	Linzi Lim	725	Molecular Pharmacology of GPCRs
726	Allosteric modulation of the mu opioid receptor	Kathryn Livingston	404	Molecular Pharmacology of GPCRs
727	Structural basis for G protein-mediated high-affinity agonist binding to GPCRs	Jacob Mahoney	403	Molecular Pharmacology of GPCRs
728	Lipid conjugation for targeting endosomal signalling	Quynh Mai	728	Molecular Pharmacology of GPCRs
729	Enhanced ß$_3$-adrenoceptor expression and function in rosiglitazone-treated inguinal white adipocytes	Jon Merlin	729	Molecular Pharmacology of GPCRs

Posterboard number	Speakers Paper Title	Presenter	Paper reference	Speakers Theme Description
730	Binding pocket residue analysis of orphan G protein-coupled receptor, GPR37L1, a potential mediator of cardiovascular homeostasis: Insights into ligand structural determinants	Tony Ngo	730	Molecular Pharmacology of GPCRs
731	Structural Elucidation of The Venus Fly Trap Module In The Calcium-Sensing Receptor	Maria Oh	731	Molecular Pharmacology of GPCRs
732	Characterizing novel aspects of signaling bias from GLP-1 receptor using RNA-seq	Kavita Pabreja	732	Molecular Pharmacology of GPCRs
733	Determination of signaling signature of 40 SNPs found in melatonin type 2 receptor: From ligand-biased signaling to mutation-bias signaling	Bianca Plouffe	733	Molecular Pharmacology of GPCRs
734	Determination of receptor binding kinetics of M_1 muscarinic acetylcholine receptor (M_1 mAChR) antagonists using whole cell binding	Darren Riddy	734	Molecular Pharmacology of GPCRs
735	Use of hemi-equilibrium to determine the dissociation kinetics of histamine H_3 receptor antagonists	Darren Riddy	735	Molecular Pharmacology of GPCRs
736	GPRC6a mediates cellular responses to L-amino acids, but not osteocalcin variants	Patricia Rueda	736	Molecular Pharmacology of GPCRs
737	Stimulation of glucose uptake by α_{1A}-adrenoeptors involves mTORC2, AMPK, and Rac1	Masaaki Sato	737	Molecular Pharmacology of GPCRs
738	Functional Selectivity of GPR43	Zoe Schofield	738	Molecular Pharmacology of GPCRs
739	Stimulus bias of allosteric modulators at the metabotropic glutamate receptor subtype 5.	Kathy Sengmany	739	Molecular Pharmacology of GPCRs
740	μ-Opioid receptor signaling mechanisms: Quantifying bias and kinetics	Setareh Sianati	740	Molecular Pharmacology of GPCRs
741	Does RXFP1 form heteromers with GPCRs relevant in heart failure?	Christopher Siwek	741	Molecular Pharmacology of GPCRs
742	Investigating a novel mechanism of activation of the relaxin family peptide 2 receptor (RXFP2)	Nicholas Smith	742	Molecular Pharmacology of GPCRs
743	Interaction of agonists and antagonists with the transmembrane region of Protease Activated Receptor 2	Jacky Suen	743	Molecular Pharmacology of GPCRs
744	Comprehensive analysis of factors influencing quantification of biased signalling at the mu-opioid receptor	Georgina Thompson	744	Molecular Pharmacology of GPCRs
745	Small molecule inhibitors of invertebrate dopamine receptors as leads for new mode-of-action insecticides	Catherine Hill	745	Molecular Pharmacology of GPCRs
746	The impact of internalization on compartmentalized trafficking and signalling of the delta-opioid receptor	Lih En Tiah	746	Molecular Pharmacology of GPCRs
747	Pharmacological characterisation of synthetic and putative endogenous allosteric modulators of the M_2 muscarinic acetylcholine receptor (mAChR)	Celine Valant	747	Molecular Pharmacology of GPCRs
748	A switched on receptor - a role for adenosine A2B receptor constitutive activityin cancer cell proliferation	Elizabeth Vecchio	313	Molecular Pharmacology of GPCRs
749	New fluorescent antagonists for the histamine H_1-receptor	Andrea Vernall	749	Molecular Pharmacology of GPCRs
750	The Calcitonin gene-related peptide family of receptors exhibit G-protein coupling bias	Harriet Watkins	750	Molecular Pharmacology of GPCRs
751	Probing agonist and antagonist interactions at the relaxin-3 receptor RXFP3	Lilian Wong	751	Molecular Pharmacology of GPCRs
752	Heterodimerization of the calcium-sensing receptor with T1 taste receptors	Kim Woollett	752	Molecular Pharmacology of GPCRs
753	Harnessing unnatural selection: Engineering thermostabilised α_1-adrenoceptors for drug discovery	Kelvin Yong	753	Molecular Pharmacology of GPCRs
754	The role of phosphorylation sites in rapid desensitization of the μ-opioid receptor	Arsalan Yousuf	754	Molecular Pharmacology of GPCRs

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NSAIDS: How clarifying a question of toxicity added to the vocabulary of pharmacology (A memoir).

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Background: Sixty years ago, synthetic corticosteroids (SCS) were being (over)-prescribed for arthritis and other inflammatory disorders. They came at enormous cost, notably to a patient's connective tissues (CT), fluid and fat regulation. But how were physicians and their patients to be weaned from their deceptive euphoric effects?
Procedures: a) Show how SCS were developed from toxicity assays in rats eg thymus involution, reducing experimental granuloma, etc.; b) identify chemical mechanisms of SCS side effects eg muscle wasting, impaired CT anabolism/regeneration; c) 'flag' alternative treatments providing analgesia and reducing inflammation without the metabolic havoc of SCS: d) use the descriptor 'Non-steroid' to clearly direct attention to other less noxious anti-inflammatories (1). What happened: Jonas Buer, Norway, recently reported the term NSAID had been used in over 4 million citations (2), tracing its first use to published abstracts in 1960 and subsequent publication in *Nature* (1961) in which Jay Lash (U.Pennsylvania) and I reported a chemical basis for the toxic effects of SCS on embryonic cartilage development *in vitro* (3). [This was my first serious venture into biochemical toxicology.]
Conclusion: The need to define a biochemical/toxicological distinction between SCS and all other anti-inflammatories has served its original purpose. This initial report showed how objective markers of toxic drug effects on tissue development and turnover could be quantified *ex vivo*. Extensive adoption of the NSAID acronym by most reputable sources of drug information was surprising but gratifying.
Disclaimer: I may not have been the first to colloquially use the term 'non-steroid(al)' as a prefix but merely note Buer's findings that I probably authored its first appearance in the English medical literature.
References: 1. Whitehouse MW (1964) Biochemical and pharmacological properties of anti-inflammatory drugs. *Progr Drug Res.* 8:321-429.
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3. Whitehouse MW, Lash JW (1961) Effect of cortisone on the biogenesis of cartilage. *Nature*. 109:37-39.

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Activation of AMPK prevents drug induced mitochondrial and hepatocellular damage

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Introduction. Drug induced liver injury (DILI) is responsible for many cases of acute liver failure; and it is the most common reason for withdrawing new agents during drug development or after approval. To date, few strategies have focused on mitochondrial damage for treatment of DILI. Understanding the cellular basis of mitochondrial damage during DILI would point to new approaches for treatment.
Aims. Using collagen sandwich culture of hepatocytes, our study investigates the mechanism of how various hepatotoxic drugs affect mitochondrial function, fusion/fission morphology and cell viability, and examines the preventative effect of AMP-activated kinase (AMPK) on mitochondrial and cellular damage in DILI.
Methods. Hepatocytes and mitochondrial morphology were examined by immunofluorescence and confocal microscopy. Mitochondrial potential and hepatocyte viability were measured using confocal microscopy. Protein expressions were detected by Western blot.
Results. Hepatotoxic drugs, paracetamol (10 mM) and diclofenac (250 microM), caused mitochondrial fragmentation; and significantly decreased mitochondrial membrane potential as well as the cellular ATP levels. These drugs either decreased expression of mitochondrial fusion proteins or increased mitochondrial fission protein activity, which were associated with mitochondrial fragmentation. Both drugs decreased hepatocyte viability and caused depolarization, confirming their hepatotoxicity. Pharmacological activation of AMPK restored mitochondrial function (mitochondrial potential and cellular ATP), morphology, hepatocyte polarization and cell viability; demonstrating that AMPK activation can prevent drug induced mitochondrial and hepatocellular damage.
Discussion. The current study reveals that hepatotoxic drugs paracetamol and diclofenac cause mitochondrial fragmentation and dysfunction, which result in hepatocellular injury. Through its effects on mitochondrial biogenesis/function and mitophagy, activation of AMPK prevents mitochondrial and hepatocellular damage, therefore, targeting AMPK can be a potential novel strategy for treatment of DILI.

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Isoniazid does not activate the intrinsic death pathway *in vivo* in young and old Fischer 344 rats

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Background: Isoniazid is the first-line medication for tuberculosis however it causes hepatotoxicity in 10-20% of patients. The mechanism of isoniazid induced hepatotoxicity has not been well defined. *In vitro,* toxic doses of isoniazid activate the intrinsic death pathway and stimulate apoptosis however this has not been validated *in vivo*.
Aims: To investigate the effect of a toxic isoniazid regimen on the expression of proteins involved in the intrinsic death pathway and apoptosis in young and old Fischer 344 rats.
Methods: Saline or a toxic regimen of isoniazid (4 doses/day: 100, 75, 75, 75mg/kg ip every 3 hours over 2 days) was administered to young (6 months) and old (24 months) male Fischer 344 rats (isoniazid: young n=7, old n=5; saline: young n=7, old n=8). Fifteen hours post last injection animals were euthanized by ip injection of ketamine (75mg/kg)/ xylazine (10mg/kg) and sera was collected for biochemical analysis and livers for histology and immunoblotting of target proteins.
Results: Isoniazid treatment significantly increased serum hepatotoxicity markers (aspartate transaminase, glutamate dehydrogenase and sorbitol dehydrogenase; $p<0.05$) and non-significantly increased the prevalence of necrosis ($p<0.08$) in young treated animals but not in old animals. Compared to saline treated animals, a toxic regimen of Isoniazid did not alter apoptosis or hepatic mitochondria expression of pro- (BAX, BAK, VDAC-1 and Bid) and anti-apoptotic (Bcl-2 and Bcl-Xl) proteins in either young or old animals.
Conclusion: Our preliminary results show that a toxic regimen of isoniazid does not activate the intrinsic death pathway or increase apoptosis *in vivo* and suggest no critical role of this pathway in the mechanism of toxicity in young or old Fischer 344 rats.

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Rapid therapeutic monitoring of atorvastatin and three metabolites in human plasma by LC/MS

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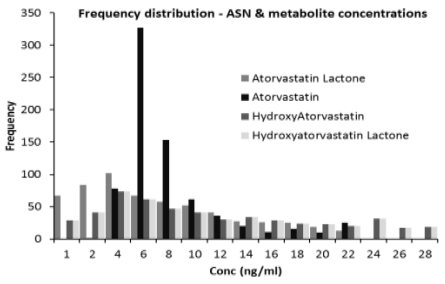
Introduction: Atorvastatin (ASN) is widely used for the treatment of dyslipidemia and the prevention of cardiovascular disease. It has two active metabolites, ortho- and the para hydroxy atorvastatin as well as inactive lactone metabolites. Both active metabolites are known to be equipotent to the parent drug. Quantification of ASN and its metabolites in human plasma by LC/MS is well established but requires involved sample preparation.
Aim: The aim was to develop and validate a simple and rapid method for the simultaneous quantitation of ASN and three of its metabolites for monitoring ASN therapy and in critically ill patients with sepsis.
Method: Online sample clean-up was achieved on a Security Guard column (C_{18} 4x3 mm, 5 µm). The chromatographic separation was performed on a Luna analytical column (C_{18}(2) 50 × 2.0mm, 5 µm). An isocratic 5:95 mixture of 0.1% aqueous formic acid and acetonitrile elutes the analytes with good chromatographic separation. The LLOQ was 0.5 ng/mL on an ABSciex API2000 MS/MS system.
Results. The assay was validated based on FDA guidelines for repeatability, matrix effect and stability. The precision and accuracy for all analytes were acceptable (<15%).



Discussion: The chromatographic resolution between both pairs of acid and lactone forms is adequate to determine that there was no significant in-source interconversion between the two forms. The method was successfully applied to nearly 2000 clinical samples from ICU patients with sepsis on prior atorvastatin therapy (see Figure). The on-line clean-up method allows rapid and reliable determination with relatively basic equipment.

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Detecting adulteration and contamination in traditional Chinese medicine (TCM): combined DNA and toxicological audits

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Adulteration and contamination of herbal and complementary medicines continues to be a significant problem worldwide. Herbal medicines may contain conventional medications, heavy metals and undeclared plant or animal material. One of the challenges of assessing herbal medicines for the presence of adulterants and contaminants is the sheer complexity of herbal preparations themselves, and the range of potential contaminants. Identifying herbal materials is often confounded by the lack of biomarkers and that processing removes all morphological markers that may identify herbs. Next generation DNA sequencing (NGS) can be used to rapidly evaluate complex mixtures unable to be tested effectively by previous techniques. In this study we used a combination of NGS and toxicological screening to evaluate contamination and adulteration in Traditional Chinese Medicines. Twenty-six pre-packaged TCM samples (capsules, tablets, and herbal teas) were purchased from retail stores and TCM practitioners in Adelaide, South Australia. 17 (65%) had undeclared content found by DNA and/or toxicological analyses. In some herbals DNA detection correlated with toxicological data (eg Ephedrine and *Ephedra* species). This study shows that combined DNA and toxicological screening enables more effective evaluation of TCMS

Coghlan, M. L. et al. (2012) PLoS Genetics 8, e1002657
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Structure-function relationships of human dimethylarginine dimethylaminohydrolase (DDAH) isoforms, DDAH-1 & DDAH-2: an approach to rational drug design

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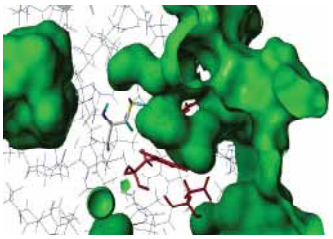

Introduction: The DDAH enzymes represents a potential target to mediate nitric oxide (NO) production, particularly in conditions characterized by excessive NO release, e.g. neurodegenerative diseases and septic shock. The isoform DDAH-1 is well characterised due to its role in cardiovascular homeostasis. However, little is known regarding DDAH-2, its tissue distribution in the body, or known substrates.
Aims: To identify the structure-function relationships of DDAH-1 and DDAH-2 and to elucidate the catalytic mechanism(s) that underpin substrate metabolism.
Methods: DNA cloning and site-directed mutagenesis was undertaken to generate a number of DDAH mutants. Recombinant protein was generated in HEK293T cells and their catalytic activity determined by mass spectrometry. The crystal template of DDAH-1 and an *in silico* model generated of DDAH-2, were utilized in automated docking experiments using the prototypic DDAH-1 substrate, asymmetric dimethylarginine (ADMA).
Results: Preliminary kinetic data with wild-type DDAH-1 and DDAH-1 mutants D73L, F76L, R145W and C274A revealed significant increases in Vmax relative to the WT enzyme. These results were not anticipated. DDAH-2 mutants L71D, L74F, W92G, T127G, W143R, and S274C were designed to investigate whether ADMA can be used as substrate in this enzyme. However no data is currently available. The catalytic mechanism of ADMA metabolism is currently unknown, however my structural investigations of DDAH-1 and DDAH-2 reveal a putative mechanism whereby the central core of each enzyme opens to accept the incoming substrate molecule.
Discussion: Computational docking experiments combined with enzyme kinetic data have provided insight into the physicochemical properties required for energetically favourable ADMA binding in DDAH-1. These data will be used to further design and develop DDAH inhibitors for use in neurodegenerative conditions, such as Alzheimer's Disease and Parkinson's Disease.

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Tamoxifen and its active metabolite 4-hydroxytamoxifen up-regulate UDP-glucuronosyltransferases (UGT) 2B15 and 2B17 in breast cancer cells: a novel mechanism of tamoxifen antiestrogenic activity

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Introduction. Testosterone is converted into estrogen in breast cancer cells. Hence, removal of testosterone by glucuronidation has a potential impact on estrogen-driven breast carcinogenesis and cancer progression. As the testosterone-glucuronidating UDP glucuronosyltransferases 2B15 and 2B17 are expressed in breast cells, factors that control their expression may have an important role in modulating these pro-carcinogenic estrogen effects.
Aim. To study the potential regulation of *UGT2B15* and *2B17* by tamoxifen and its active metabolite in breast cancer cells.
Methods & Results. *UGT2B15* and *2B17* mRNA levels and testosterone-glucuronidating activity were significantly increased in breast cancer MCF-7 cells treated with tamoxifen and 4-hydroxytamoxifen (4-OH-tamoxifen). This increase was abrogated by either knockdown of estrogen receptor α (ERα) by siRNA or the ER antagonist, ICI 182,780. Furthermore, tamoxifen and 4-OH-tamoxifen stimulated the activity of the *UGT2B15* promoter. This stimulation was enhanced by overexpression of ERα but significantly reduced by mutation of the previously reported functional estrogen response unit (ERU) at the *UGT2B15* proximal promoter. Chromatin immunoprecipitation (ChIP) assay showed enrichment of ERα binding at the ERU of the *UGT2B15* promoter upon 4-OH-tamoxifen exposure.
Discussion. Tamoxifen and its active metabolite up-regulate *UGT2B15* and *2B17* expression via ERα in breast cancer cells. This tamoxifen-induced *UGT2B15* and *2B17* enzymatic activity may facilitate the removal of testosterone from tumour cells. This is a novel mechanism that may contribute to the antiestrogenic effects of tamoxifen which is used in the treatment of estrogen receptor positive breast cancers.

Hu DG et al (2009) Mol Pharmacol 76:425-439

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A mouse model to determine the effects of polypharmacy on adverse geriatric outcomes

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Introduction. Polypharmacy (use of ≥5 different medicines) is common in older Australians. In observational studies, it is associated with an increased risk of adverse geriatric outcomes such as falls and frailty.
Aims. (1) To establish a mouse model of polypharmacy; (2) To establish whether short term polypharmacy causes adverse geriatric outcomes in young and old age.
Methods. Male young (3 months) and old (23 months) C57BL/6 mice were administered control or polypharmacy diet for 4 weeks: simvastatin (20 mg/kg/day), metoprolol (350 mg/kg/day), omeprazole (10 mg/kg/day), paracetamol (100 mg/kg/day) and citalopram (10 mg/kg/day). Mouse food intake, weight, blood pressure, rotarod latency (balance and coordination), front paw hang (holding impulse, grip strength) and clinical frailty index were assessed.
Results. The polypharmacy regimen was well tolerated, with no change in oral intake or weight observed in young or old mice. Polypharmacy did not significantly affect outcomes in young mice. In old mice, polypharmacy lowered blood pressure and impaired physical function but did not significantly increase frailty.

Mean (±SEM) change after 4 weeks of diet	**Young**		**Old**	
	Control (n=5)	**Polypharmacy (n=5)**	**Control (n=8)**	**Polypharmacy (n=13)**
Systolic BP (mmHg)	↑ 3± 6	↑ 9 ± 7	↑ 0.2 ± 3	↓ 11 ± 4*
Rotarod latency (s)	↑ 43 ± 38	↑ 34 ± 11	↑ 59 ± 11	↑ 2 ± 17*
Front paw hang (N sec)	↑ 1078 ± 664	↓ 529 ± 233	↑ 250 ± 104	↓ 203 ± 122*
Frailty Index	N/A	N/A	↑ 0.03 ± 0.01	↑ 0.05 ± 0.01

**p < 0.05 for change seen in control diet mice vs polypharmacy diet mice within each age group; BP, blood pressure; N, newton*

Discussion. This model showed that short term polypharmacy impairs balance, co-ordination and grip strength in old but not young mice. The model can be used to determine the effects of long term polypharmacy and deprescribing (withdrawing medicines) on geriatric outcomes in old age.

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A validated mouse frailty index to assess longevity interventions: Impact of calorie restriction and resveratrol

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Introduction. Frailty, a state of increased vulnerability, predicts adverse outcomes in old age. A frailty index based on deficit accumulation was recently validated in ageing mice and is comparable to the frailty index developed for use in humans (Whitehead *et al.*, 2014).
Aims. We aimed to assess the effect of calorie restriction and the calorie restriction-mimetic, resveratrol (interventions known to improve lifespan and healthspan in mice), on the mouse frailty index.
Methods. Frailty index scores were measured on male C57BL/6 mice aged 18 months that were ad-libitum fed (n=9) or calorie restricted from age 6 months (n=9). Frailty index scores were also measured on male C57BL/6 mice aged 24 months that were ad-libitum fed with (n=9) or without (n=16) resveratrol (100mg/kg/day) in their diet from age 19 months. Mean frailty index scores of 2 raters were compared between the treatment groups using t-tests and inter-rater reliability was assessed with the intraclass correlation coefficient (ICC).
Results. Calorie restriction reduced mean frailty index in aged C57BL/6 mice (FI=0.13±0.03 calorie restriction, 0.20±0.08 ad-libitum, p=0.04). Furthermore aged mice with chronic resveratrol treatment had a lower frailty index than age-matched ad-libitum fed mice without treatment (FI=0.18±0.01 resveratrol, 0.22±0.01 ad-libitum, p=0.01). The 2 raters had excellent inter-rater reliability (ICC = 0.88, 95% CI [0.80, 0.92]).
Discussion. Both interventions were shown to decrease the frailty index in male C57BL/6 mice, compared to age matched controls. This study further validates the mouse frailty index developed by Whitehead *et al.* (2014) in a different mouse colony with dietary and pharmaceutical interventions. The mouse frailty index scale is an important tool that could be used to assess the effect of any intervention on the important clinical outcome of frailty in an animal model.

Whitehead JC et al (2014) J Gerontol A Biol Sci Med Sci.69(6):621-32

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Detection of Testosterone dosing in racing male Greyhounds

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Introduction. Anabolic androgenic steroids that are derivatives of testosterone are used to increase performance in racing greyhounds. The analytical detection of these steroids in male dogs is complicated by the presence of endogenous hormone. As exogenous testosterone is administered as esters, which do not naturally occur, detection of the esters in biological samples would provide unequivocal evidence of illicit administration of drug.
Aims. The aim of this study was to develop and validate a confirmatory analytical method for detection in hair of testosterone-esters from greyhound dogs, and to identify the duration for which detection is possible.
Methods. A liquid chromatographic-tandem mass spectrometric method (LC-MS/MS) was developed and validated for the detection in hair of testosterone-esters; propionate, phenyl propionate, isocaproate, decanoate and enanthate. Drug administration studies were carried out by injection of testosterone-ester mix in five Greyhound dogs, and hair samples were collected on alternate days during the first week, followed by weekly for one month, and thereafter fortnightly until 3 months post dosing. These samples were analyzed using the above LC-MS/MS method.
Results. Preliminary results show that each testosterone ester was detectable from 2 hours and up to nine weeks post administration, with the mean maximum hair concentration occurring at 48 h for all esters; propionate 2.09±0.8 (ng/g), phenyl propionate 8.75±4.62 (ng/g), isocaproate 7.09±3.45 (ng/g), and decanoate 8.38±3.56 (ng/g). Propionate was readily detectable at nine weeks in all five dogs, compared to the other esters, which were detected in only some of the dogs. In addition, the concentration of the longer chain decanoate, was low or undetectable at nine weeks, which suggests that lipophilicity is not a major factor in the incorporation of anabolic steroids into hair.
Discussion. A sensitive LCMSMS method has been developed which provides an unequivocal confirmation of administration of testosterone-esters to greyhound dogs and bitches, and this new method should find useful application in racing regulation.

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Study of a novel regulatory mechanism that controls UGT1A8 synergistic activation by Cdx2 (Caudal-related Homeodomain protein-2) and HNF4a (Hepatocyte Nuclear Factor-4 alpha)

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Introduction. UDP-glucuronosyltransferase (UGT) 1A8 is a UGT1A family enzyme exclusively expressed in extrahepatic tissues. It is a major mediator of first pass drug metabolism in the gastrointestinal system. We recently found that expression of this gene is controlled by the intestinal master transcription factor Cdx2 as well as HNF4a through a novel regulatory element that we have defined in the proximal UGT1A8 promoter. In the present study we further characterize the mechanism of Cdx2 and HNF4a synergistic action.
Aim. To determine the mechanism of UGT1A8 transcriptional activation by Cdx2 and HNF4a
Methods. All experiments were performed in Caco-2 cells. Functional analysis of regulatory elements used promoter-luciferase reporter assays, site directed mutagenesis and co-transfection assays. Endogenous UGT1A8 expression was assessed by qRTPCR. Electrophoretic mobility shift assays (EMSA), and chromatin immunoprecipitation (ChIP) were performed to assesspromoter binding by Cdx2 and HNF4a.
Results and Discussion. We have defined a novel functional regulatory element in the proximal promoter of UGT1A8 (nt-44), which contains a consensus HNF4a binding motif. Mutagenesis assays show this HNF4a element to be essential for synergistic activation of the promoter by Cdx2 and HNF4a. Surprisingly however, a previously characterized Cdx2 element located upstream at nt -77 was not required for synergy. EMSA assays show that the -44 HNF4a element binds both HNF4a and Cdx2. Sequence analysis shows that the element is a composite of a consensus HNF4 motif and a cryptic Cdx2 recognition motif. Mutagenesis analysis shows that both motifs within this composite element are required for synergy of Cdx2+HNF4a, suggesting that the factors may interact at the composite element. This idea is supported by results from both EMSA and ChIP analysis. Moreover, mechanism analysis using dominant-negative Cdx2 mutants suggests that HNF4a may play the major role by recruiting Cdx2, which is in contrast to recent reports that in adult intestine Cdx2 induces chromatin remodeling to facilitate HNF4a binding. Ongoing analysis using clustered regularly interspaced short palindromic repeats (CRISPR) to remove this novel element from the genome will allow characterization of Cdx2+HNF4a functional synergy in vivo.

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Xenobiotic metabolism in Australian marsupial koala, *Phascolarctos cinereus*: Molecular and biochemical perspectives

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Introduction. Unlike eutherian mammals, marsupial herbivores ingest large amounts of eucalyptus leaves containing dietary toxins. It is hypothesised that eucalyptus herbivores, such as the koala (*Phascolarctos cinereus*) utilise highly efficient enzyme systems in order to metabolise and excrete these toxins as non-toxic metabolites. Enzymes that have been identified to date as those that carry out this biotransformation are those of the Cytochrome P450 subfamilies (CYPs). Whilst CYP3A is considered the most abundant enzyme involved in mammalian xenobiotic metabolism, biochemical analysis has determined that koalas may express higher CYP2C activity. These studies indicate the possibility that CYP2 and CYP3 activity may play an important role in xenobiotic metabolism. Recent studies have highlighted that dietary terpenes act as peroxisome proliferators, activating an array of CYP genes upon binding to peroxisome proliferators activated receptor-alpha (PPAR-α). Higher expression of hepatic PPAR-α mRNA was detected in the koala in comparison with non-eucalyptus feeders, suggesting that dietary terpenes upon binding to PPAR-α possess the ability to up-regulate CYP subfamilies.
Aims. The aim of the current study was to provide an update on molecular and biochemical aspects of biotransformation enzymes in Australian marsupials and their possible roles in dietary toxin detoxification.
Methods. The study focuses on molecular and biochemical findings as well as approaches employed over the last 2 decades to investigate Phase I and II drug metabolising enzymes and processes involved in marsupial metabolism.
Discussion. 1,8-cineole, the main terpenoid in Eucalyptus has been shown to have much lower LD50 values in mammalian species in comparison to that of marsupials. Possums and koalas have been found to tolerate a much higher daily intake of cineole. Administration of terpenes has been reported to induce certain CYPs, with studies of several mammalian species showing increased hepatic enzyme activity upon administration. Marsupials show elevated levels of CYP2C6 and CYP2C11 upon terpene administration, and also increased NAD activity. CYP4A increases hepatic NAD activity, thus elevating metabolic reactions. This elevation is an important finding in determining the efficiency of metabolic processes. The koala has been shown to have significantly higher NAD levels, suggesting that they have increased CYP activity and highly efficient hepatic metabolism. Moreover, koalas and possums have shown to rapidly produce and eliminate oxidised metabolites. The koala excretes 95% of administered cineole as carboxylic acid metabolites with complete absence of precursor metabolites in the urine. This indicates that oxidative reactions occur more rapidly in the koala than mammals. In summary, the unique

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Xenobiotic metabolism in Australian marsupial koala, *Phascolarctos cinereus*: Comparative PKs/PDs and drug disposition.

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Introduction. The koala, *Phascolarctos cinereus* is a biologically unique and evolutionarily distinct Australian marsupial that often requires drug treatment for a variety of conditions, including infectious diseases, pain and inflammatory condition resulted from road injury, relocation after fire, etc. However, little is known about the differences in the metabolism, disposition and pharmacokinetics/pharmacokinetics of drugs used in koalas compared to humans and other species.

Aims. The aim of the current study was to provide an overview on what is known about the comparative pharmacokinetics, efficacy and also toxicity of drugs used in koalas and to identify the possible contributing factors to PK/PD differences.

Methods. The study focuses on PK/PD profiles of koalas' commonly used drugs over the last 2 decades.

Results. The identified agents include antibiotics fluoroquinolones, fluconazole, chloramphenicol and non-steroidal anti-inflammatory drugs (NSAIDs), in particular meloxicam. In general, most studies found that the currently accepted/suggested dose rates are inadequate for koalas, attributed mainly to poor absorption and oral bioavailability. For example, the bioavailability of most drugs and drug classes, including the above antibiotics and NSAID, administered orally to koalas has been found to be extremely poor ($F<0.1$ or negligible) in comparison to humans and other species. Toxicity has also been reported with antibiotics administered orally to koalas, resulted from reduced gut micro-flora population. Current literature also indicates that the unique physiology of the koala involves rapid hepatic metabolism, especially the metabolic role of cytochrome P450 CYP3As and high plasma binding. As a result, approaches such as allometric scaling would be ineffective at predicting pharmacokinetics and dose rates of drugs in koalas.

Discussion. In summary, common dosing regimens for drug treatment in koalas at present are inadequate. Oral administration of many drugs in koalas exhibits poor bioavailability that was mainly due to poor absorption. Thus, the efficacy of drugs administered to koalas via oral route is not optimal; other routes of administration would be more effective for NSAIDs and antibiotics.

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Black LA et al (2013) J Vet Pharmacol Ther 36:478-85

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Shape of the ciprofloxacin and tobramycin concentration-time profile is critical for resistance prevention in *Pseudomonas aeruginosa*

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Introduction. The area under the free concentration time curve divided by the minimum inhibitory concentration (*f*AUC/MIC) best predicts bacterial killing at 24 h and clinical success of quinolones and aminoglycosides.
Aim. To determine whether delivering the same *f*AUC over 4 or 10 h vs. 24 h provides better killing and minimizes resistance.
Methods. *P. aeruginosa* ATCC 27853 was evaluated in 24 h *in vitro* static time-kills with ciprofloxacin (CIP; MIC=0.25 mg/L) at *f*AUC/MIC of 44 and 132 and tobramycin (TOB; MIC=0.5 mg/L) at *f*AUC/MIC of 36, 72 and 168, in duplicate. Log_{10} initial inocula (CFUo) were 4, 5 and 6 (95 profiles in total). Antibiotic was added at 0 h and removed at 1, 4, 10, 16 or 24 h by spinning and re-suspending bacteria 2- or 3-times in antibiotic-free broth. Drug concentrations were chosen to achieve the targeted *f*AUC/MIC for each exposure and serial viable counts were determined. Log_{10} mutation frequencies (MF, at 3x MIC, baseline: -6.6 ± 0.3 for CIP and -7.0 ± 0.3 for TOB) and MICs were assessed from the drug-free bacterial suspensions at 0 and 24 h.
Results. High CIP concentrations of the 4 h duration of exposure yielded extensive killing at 6 h and eradication at 24 h for CFUo=4, but were followed by regrowth for CFUo=6 (Table). For the 24 h duration of exposure, less regrowth occurred, but the CIP-resistant population almost completely replaced the susceptible population by 24 h and MICs increased 4 to 8-fold for CFUo=6 (Table). No resistance emerged for the 4 h (Table) and 10 h (not shown) duration of CIP exposure. For TOB *f*AUC/MIC of 36, the MF at 24 h was -6.4 ± 0.5 (mean±SD) for 1 and 4 h duration of exposure, but was higher (-0.53 ± 0.40; $p<0.05$) for 24 h exposure; TOB MICs at 24 h were 0.5 mg/L for 4 h duration of exposure and 4 mg/L for 24 h exposure.

Endpoint	CIP	Inoculum (CFU/mL) / duration of exposure (h)			
	*f*AUC/MIC	10^6 / 24 h	10^6 / 4 h	10^4 / 24 h	10^4 / 4 h
Log_{10} CFU/mL at 6 h vs 0 h	132	-4.5 ± 0.02	-6 *	-3.4 ± 1.1	-4 *
Log_{10} CFU/mL at 24 h vs 0 h	132	-3.0 ± 0.1	1.9 ± 0.3	-1.9 ± 0.4	-4 *
MF at 24 h	44	-0.8	-6.2 ± 0.4	< -3.1 *	-5.9 ± 0.1
MF at 24 h	132	-1.0 ± 0.8	-6.4 ± 0.4	< -1.7 *	*
MIC (mg/L) at 24 h	44 & 132	1 - 2	0.25	0.125-0.25	0.25

*: not detectable

Discussion. At the same *f*AUC/MIC, short 1, 4 [and 10] h durations of antibiotic exposure yielded extensive initial killing without resistance, whereas the 24 h duration of exposure caused less initial killing and substantial resistance emergence due to pre-existing resistant mutants at a CFUo of 6. High dose, short-course exposure of rapidly killing antibiotics seems promising for innovative combination therapies.

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Older peoples' attitudes and beliefs about statin use and their willingness to have statins deprescribed.

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Introduction. In older people, the evidence for benefit of statins in primary prevention in cardiovascular disease is limited, and the risks of non-cardiovascular statin side effects increase. Individualisation of statin therapy in older people through regular assessment of potential benefits and risks is required.
Aims. The aim of this study was to capture older patients' attitudes towards statin use and their willingness to have statins deprescribed (withdrawn).
Methods. A cross-sectional study of patients aged ≥ 65 years and taking a statin admitted to a Sydney teaching hospital was conducted. Data on socio-demographics, comorbidities, and medication use were collected from medical records. Participant views to cessation of statins were explored through the validated 10-item Patients' Attitudes Towards Deprescribing (PATD) questionnaire, modified with 7 additional statin specific items.
Results. A total of 116 participants have been recruited to date, with a median age of 79 (IQR= 15). Approximately 30% of participants were unsure whether they should continue taking the statin, 28% were concerned about the potential side effects of statin use and over 90% trusted their doctor to inform them if their statin should be ceased. Over 35% of participants had heard negative information regarding statins, either from media (93%), family members or friends (5%) or healthcare professionals (2%). Compared to patients prescribed statins for primary prevention (n= 33), those taking statins for secondary prevention (n= 83) were more likely to be unsure whether they needed to continue taking it (gamma= 0.33, p= 0.04).
Discussion. Underlying concerns and uncertainties regarding statin therapy may influence older people's willingness to have the statin deprescribed. Future interventions aimed at optimising the use of statins in older adults should include patient education at a population level and through their consultations with health care practitioners.

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Comparing utilisation of mycophenolate, tacrolimus, cyclosporin, sirolimus and everolimus in Australia and Northern Europe

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Introduction. Mycophenolate, tacrolimus, cyclosporin, sirolimus and everolimus, used in management of transplant rejection, receive restricted public subsidy in Australia and Northern Europe.
Aims. To examine the utilisation and expenditure of each immunosuppressant in Australia between 2007-2013 and to identify specific patterns of immunosuppressant utilisation in Denmark, Sweden, Norway and the Netherlands.
Methods. Data were gathered from the Pharmaceutical Benefits Scheme (PBS) database, Medicare Australia; the Danish Health and Medicines Agency; the Norwegian Institute of Public Health; the Swedish Board of Health and Welfare; and the Netherlands Healthcare Insurance Board. Drug utilisation was expressed as WHO Anatomical Therapeutic Chemical Defined Daily Dose (2013)/1000population/day (DDD/1000/day).
Results. In Australia in 2013, mycophenolate PBS usage was 0.28 DDD/1000/day, up 6.5-fold from 2007 with AUD$24.2million spent. Tacrolimus PBS utilisation was 0.23 DDD/1000/day, up 9.3-fold from 2007 with AUD$32.3million spent. Cyclosporin PBS utilisation was 0.13 DDD/1000pop/day, up 1.9-fold from 2007 with AUD$15million spent. Sirolimus PBS utilisation was 0.02 DDD/1000pop/day, up 4.6-fold from 2007 with AUD$3million spent and everolimus PBS usage was 0.04 DDD/1000/day, up 26-fold from 2007 with AUD$8.2million spent. Total Australian PBS expenditure across all immunosuppressants increased 4.2-fold between 2007 and 2013. In comparison to Northern Europe, 2012 Australian PBS immunosuppressant utilisation was approximately 50% lower but growing 2.8-fold faster, anticipating future convergence with the higher European usage. Transplantation rates have historically been higher in these Northern European countries.
Discussion. Immunosuppressant usage and subsequent expenditure are steadily rising with increased numbers of Australians living with a transplant. With transplant patients living longer, the potential for new subsidised indications and the observed growth potential predicted from Northern European data immunosuppressants can be predicted to continue consuming an increasing share of Australian PBS expenditures.

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Analgesic use, pain and daytime sedation in people with and without dementia in aged care facilities

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Introduction. Managing pain in residents of aged care facilities is challenging, especially for people with dementia. Clinicians must weigh the benefits of analgesic use against the potential for adverse effects such as sedation.
Aims. To investigate the prevalence and correlates of analgesic use, pain and daytime sedation in people with and without dementia in Australian aged care facilities.
Methods. A cross-sectional study of 349 residents with and without dementia from five aged care facilities in South Australia was conducted. Trained study nurses administered validated and dementia-specific assessments of pain, sedation and other clinical outcomes. Medicine use data were extracted directly from each resident's medication administration chart. Logistic regression was used to compute odds ratio (ORs) and confidence intervals (CIs) for factors associated with analgesic use.
Results. Analgesics were charted for 95.4% of residents. There was no difference in the administration of regular analgesics in the previous 24 hours between residents with and without dementia (72.2% vs 69.0%, p=0.21). Residents with dementia received fewer when-required analgesics in the previous 24-hours compared to residents without dementia (1.3% vs 5.5%, p=0.04). The prevalence of clinician-observed pain was similar in residents with and without dementia (25.2% vs 30.8%, p=0.25). Residents with dementia experienced more daytime sedation than residents without dementia (18.5% vs 7.0%, p<0.01). Analgesic use within the previous 24 hours was associated with both morning drowsiness (OR2.12, 95%CI 1.26 – 3.55) and napping frequency (OR2.01, 95%CI 1.20 – 3.40) when adjusting for age, sex, dementia diagnosis and pain.
Discussion. Encouragingly, there was a higher prevalence of regular analgesic use and lower prevalence of pain than reported in previous research, which did not differ in residents with and without dementia. These findings may indicate adequate pain control from analgesia. The presence of daytime sedation highlights the importance of ongoing assessment of the need for analgesia, especially in people with dementia.

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Prevalence of statin-drug interactions in older people: a systematic review

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Introduction. Statins are amongst the most frequently prescribed medications internationally. Older people are commonly prescribed multiple medications, and are at an increased risk of drug-drug interactions, including statin-drug interactions.
Aim. To conduct a systematic review of the current evidence on the prevalence of statin-drug interactions in older people.
Methods. A systematic search of Embase, Medline and PubMed was conducted and augmented with a manual search of the reference list of identified articles. Articles were included if they were published in English in the past 14 years (July 2000–July 2014) and reported on the prevalence of any statin-drug interactions in people over 65 years of age. Two reviewers independently assessed the articles for eligibility and extracted the data.
Results. The search returned 1555 records. A total of 19 articles met the inclusion criteria. In studies that focused on statin-users only (n=7), the prevalence of potential statin-drug interactions defined using different international sources, ranged from 0.19% to 33.0%. Of these seven studies, only one reported specifically on clinically relevant statin-drug interactions and found a prevalence of 4%, with amiodarone and atorvastatin or simvastatin being the most common statin-drug interaction. In studies that examined drug interactions across a population of both statin-users and non-users (n=12), the prevalence of potential statin-drug interactions ranged from less than 0.1% to 7.1%, and the prevalence of clinically relevant statin-drug interactions ranged from 1.5% to 4% (n=4).
Discussion. Current published evidence suggests substantial variations in the prevalence of statin-drug interactions. This may be due to lack of consensus in defining statin-drug interactions across different study populations. Further studies are necessary to provide a better understanding of the prevalence and clinical impact of clinically relevant statin-drug interactions, and the medications most frequently contributing to statin-drug interactions in older people.

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Polypharmacy in older people with cancer: how many medicines are too many?

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Introduction. Polypharmacy is often defined as use of five or more medicines. However, it is unclear if there is an optimal polypharmacy cut-off for predicting clinically important adverse events.
Aims. To determine the sensitivity and specificity of a range of common polypharmacy cut-offs for predicting frailty, physical function and falls in older people with cancer.
Methods. Between January 2009 and July 2010 385 patients aged ≥70 years presented to the medical oncology outpatient clinic at the Royal Adelaide Hospital and completed a structured data collection instrument including medicine use and falls. Frailty was determined using weight loss (patient self-reported over previous 6 months), exhaustion (CES-D), physical function (SF-36), instrumental activities of daily living and Karnofsky Performance Scale. Receiver operating characteristic (ROC) curves were derived to examine the sensitivities and specificities for varying definitions of polypharmacy in relation to frailty, physical function and falls (≥2 in previous 6 months).
Results. Patients had a mean age of 76.7 years and used a median of 5 medicines. Using Youden's index, the optimal polypharmacy cut-point was 6.5 medicines for frailty (sensitivity 70.0%, specificity 67.0%,), 6.5 for physical function (49.3%, 80.2%) and 5.5 for falls (73.0%, 59.2%). For polypharmacy defined as 'five or more medicines,' the sensitivity and specificity were acceptable for frailty (77.5%,, 44.9%), physical function (68.6%, 58.0%) and falls (75.7%, 44.5%).
Discussion. Our results suggest no one polypharmacy cut-off is optimal for predicting all adverse events in older people with cancer. The value of a specific polypharmacy cut-off is likely to be dependent on the clinical and medicine use characteristics of the population to which it is applied. However, the common polypharmacy cut-off of 'five or more medicines' appears reasonable to use as a prompt for referral for a comprehensive medication review in the patient population.

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Identifying the impact of medication complexity on chronic disease

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Introduction. Medication complexity refers to the overall difficulty associated with following a medication regimen. Greater responsibility has been placed on patients to manage their chronic disease on a daily basis in the community. They face many challenges that result from polypharmacy due to multiple co-morbidities, medication alterations and food and drug interactions. This contributes to a complex medication regimen that without proper knowledge and management can impact on a patient's outcome.
Aims. We aimed to review the literature to determine the different facets of medication complexity and its impact on patient management of their chronic disease
Methods. A narrative review was conducted using the search engines of MEDLINE and EMBASE for articles related to medication complexity, and the Medication Regimen Complexity Index (MRCI). This was limited to the elderly in EMBASE to further narrow results. The PRISMA statement was used to provide a structure in which the articles were assessed.
Results. In total 23 studies met the inclusion criteria, comprising of 10 cross-sectional, 4 retrospective cohort, 4 validation, 2 prospective cohort, and 3 separate studies of quasi-randomised, before/after, and correlational design. All studies were based on improving patient adherence by altering medication complexity. Various factors were identified as affecting medication complexity including age, knowledge, comorbidities, hospitalisation, mental health, cognitive function and medication related factors such as dosage form, frequency and additional directions.
Discussion. Reducing medication complexity produced better adherence in the majority of studies. However, in order to address medication complexity, it needs to first be quantified through use of the MRCI. This enables objective assessment of potential challenges and allows a targeted approach towards the various factors that could negatively affect patient medication regimen adherence. The older population are particularly susceptible as they have increased comorbidity and are more vulnerable to adverse events. Medication complexity needs to be addressed in order to improve adherence and benefit patient outcomes in the chronic setting.

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Medication Regimen Complexity Index: An indicator of bleeding risk

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Introduction. Medication complexity refers to the overall difficulty associated with adhering to a therapy and impacts negatively on patient outcomes. Anticoagulants are being increasingly used and are a large cause of medication related adverse events.
Aims. To determine if the Medication Regimen Complexity Index can be used as an indicator of patients at risk of bleeding.
Methods. A case control study of 487 participants on warfarin therapy was conducted. Factors impacting on warfarin management and patient outcomes were determined. The MRCI was used to quantify complexity and odds ratios (OR) with 95% confidence intervals calculated to determine factors impacting on bleeding risk.
Results. The calculated MRCI was right skewed with a mean of 19.12 (3-65.5) and median of 17. 33.33% of cases were found to have a high MRCI compared to 18.77% of controls. After adjusting for patient and pyscho-social factors, participants having a high MRCI were found to be 2.24 time more likely to be at significant risk of bleeding (95% CI 1.32-3.00).
Discussion. Anticoagulants are associated with serious adverse events and given their rise in use, there needs to be an improvement in identifying patients at risk of bleeding. The MRCI is a validated tool. Having a high MRCI was associated with a greater likelihood of bleeding risk. The MRCI has been found to be a good indicator of bleeding risk and has the potential to be used in the clinical setting to direct warfarin management.

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Medication-administration problems in older people in Sweden: a population-based case-control study

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Introduction. In older people, functional ability such as cognitive status often declines. Medication administration problems (MAPs) such as difficulties to remember, handle or administer medication can be the result.
Aims. To investigate factors associated with MAPs in older people.
Methods. Data were collected as part of the Swedish National Study of Aging and Care Kungsholmen (SNAC-K) study, a prospective population-based cohort study including people aged ≥60 years. MAPs were self-reported by participants during structured physician interviews. Using a case-control design, people with MAPs were individually matched 1:1 (age, sex and number of medications) with those without MAPs. The cognitive status was assessed using the Mini-Mental State Examination (MMSE). Medication regimen complexity was calculated using the 65-item validated Medication Regimen Complexity Index (MRCI). Mann-Whitney U tests were used to compare clinical, demographic and medication-related factors in people with and without MAPs.
Results. Of 3348 participants, 1891 participants had documentation regarding the presence or absence of MAPs. Overall, 10.7% (n=202) of participants self-reported one or more MAPs. The median age of people without MAPs was 73.0 years (range 60.0-105.0) versus 81.0 years (range 60-104.0) in people with MAPs. 1149/1689 (68%) of those who did not reporting MAPs and 161/202 (79.7%) of those who reported MAPs were women. The median number of medications in people without MAPs was four (range 1-23) versus seven (range 1-19) in people reporting MAPs. In the matched group, people with MAPs were more likely to have lower MMSE (U=14938, $p<0.001$, two-tailed). Mean MRCI for people with and without MAPs was 20.2 and 18.7, respectively. There was no significant difference in MRCI between people who did and did not self-report MAPs (U=21637, $p=0.29$, two-tailed).
Discussion. People with MAPs were more likely to have lower MMSE. In people with and without MAPs medication regimen complexity was not significantly different.

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Inhibition of platelet aggregation by vanilloid-like agents is not mediated by transient receptor potential vanilloid-1 (TRPV1) channels or cannabinoid (CB1, CB2) receptors

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Introduction. Vanilloid-like agents, including capsaicin (CAP), N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA), inhibit platelet aggregation (Almaghrabi et al 2014), and hence may protect against CVD.
Aim. To determine whether inhibition of platelet aggregation by vanilloid-like agents is mediated by TRPV1 channels and/or cannabinoid receptors.
Methods. Platelet-rich and -poor plasma were obtained from venous blood of healthy subjects. The effects of 50 μM CAP and NADA on aggregation induced by arachidonic acid (AA, 300 μg/mL) and of 50 μM OLDA and NADA induced by collagen (4 μg/ml) were determined in the absence and presence of TRPV1 (SB452553), CB1 (AM251) and CB2 (AM630) antagonists (10, 50 μM). Aggregation data (% maximum; mean±SEM; all n=4) were compared using ordinal logistic regression. Platelets were counted pre- and post-incubation.
Results. CAP and NADA significantly inhibited AA-induced aggregation (0 vs 50 μmol/L, 67.4±10.6% vs 29.6±14.5%, $p<0.05$, 66.8±9.9% vs 49.6±12.7%, $p<0.05$, CAP and NADA, respectively). SB452553, AM251 and AM630 did not affect CAP- or NADA-induced inhibition of aggregation. OLDA and NADA significantly inhibited aggregation induced by collagen, with the effect of OLDA enhanced significantly ($p<0.05$) by SB452553 (50 μM) (84.6±3.1% vs 32±17%) compared to OLDA alone (65.3±10%). The effect of NADA (86.9±2.8% vs 43.7±16.5%) was enhanced by 50 μM AM630 (13.4±2.8%, $p<0.005$). Finally, no vanilloid/endovanilloid or antagonist had an effect on platelet count.
Discussion. The inhibitory effects of capsaicin, OLDA and NADA on platelets are not mediated through TRPV1, CB1 or CB2 receptors. However, blocking TRPV1 and/or CB2 receptors may contribute to the action of vanilloids.

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Evidence against a role for pannexin-1 channels in α_1-adrenoceptor-mediated vasoconstriction in resistance arteries

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Introduction. Recent reports have provided evidence for a new concept that in small resistance arteries α_{1D}-adrenoceptor-mediated contraction is intimately linked to pannexin-1 hemichannels that open to allow the release of ATP, from the smooth muscle effector cell, that acts back on P2Y purinoceptors to cause contraction (Billaud et al, 2011). This concept relied largely on the use of mefloquine 10-20 μM acting as a selective pannexin-1 blocking agent to completely inhibit the contraction to phenylephrine, but not K^+ 40 mM. Lower concentrations of mefloquine had no effect.
Aims. The aim of the present study was to explore the specificity of mefloquine at 10 μM for pannexin-1 channels and the role of these channels in small artery contraction.
Methods. In mouse and rat isolated small resistance arteries, either pressurised or set up for wire myography, the effects of mefloquine on contractions to K^+, phenylephrine and a range of vasoconstrictor agents were assessed and compared with the pannexin-1 inhibitor carbenoxolone.
Results. Mefloquine had a wide range of inhibitory actions at 10-20 μM, some 200-fold above the concentrations previously shown to inhibit expressed pannexin-1 channel activity. Mefloquine 3-10 μM inhibited phenylephrine, U46619, vasopressin, endothelin-1, sympathetic nerve stimulation and K^+ 40 mM-mediated contractions in rat and mouse small mesenteric, and mouse thoracodorsal, arteries. Carbenoxolone 1-100 μM did not inhibit the contractile responses to these vasoconstrictor agents in small resistance arteries.
Discussion. The present study demonstrates that in small resistance arteries there is no evidence that pannexin-1 channels releasing ATP have any role in the constrictor actions of α_1-adrenoceptor activation and cautions the use of mefloquine at high concentrations that appear to elicit non-specific inhibition of various cellular mechanisms.

Billaud M et al (2011) Circ Res 109:80-85

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Glucagon-like peptide-1 receptor as a target in treatment of cardiovascular disease.
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Introduction. Glucagon-like peptide-1 (GLP-1) based therapies are used to treat type II diabetes via increasing insulin secretion and inhibiting glucagon production (Mundil et al., 2012). Recent evidence suggests that activating the GLP-1 receptor may also mediate direct vaso-protective effects (Gaspari et al., 2011).

Aim. To determine whether GLP-1R stimulation conferred cardio- and vaso-protection in a non-diabetic setting using the angiotensin (Ang) II infusion model of hypertension and cardiovascular dysfunction.

Method. Male C57Bl/6J mice (4-6 months) were assigned to one of the following 4 week treatment protocols: 1) vehicle (saline), 2) Ang II (800ng/kg/day), 3) Ang II + liraglutide (30µg/kg/day), 4) Ang II + liraglutide (300µg/kg/day). All treatments were administered via osmotic mini-pumps (s.c). After 4 weeks the effect of liraglutide treatment on blood pressure, vascular function and cardiac remodelling was examined.

Results. Liraglutide (both doses) attenuated Ang II-induced increase in systolic blood pressure without affecting blood glucose levels. Vascular function studies demonstrated a vaso-protective effect of chronic liraglutide treatment with prevention of Ang II-induced endothelial dysfunction. In the heart, liraglutide prevented Ang II-induced cardiomyocyte hypertrophy ($n=7\text{-}10$; $p<0.05$) and reduced collagen deposition ($n=7\text{-}9$; $p<0.01$). This anti-fibrotic effect was attributed to reduced fibroblast/myofibroblast expression ($n=7\text{-}10$; $p<0.001$) as well as decreased inflammation with a significant reduction in superoxide production using high dose of liraglutide ($n=5\text{-}7$; $p<0.01$).

Discussion. Overall, stimulation of GLP-1R in a non-diabetic setting protected against Ang II-mediated cardiac hypertrophy, cardiac fibrosis and vascular dysfunction, indicating potential for use of GLP-1 based therapies in treatment of cardiovascular disease independent of diabetes.

Gaspari et al (2011) Diabetes Vasc Dis Res 8 : 117-124
Mundil et al (2012) Diabetes Vasc Dis Res 9: 95-108

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Long chain omega-3 polyunsaturated fatty acids improve aortic relaxation in a mouse model of abdominal aortic aneurysm
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Introduction. Abdominal aortic aneurysm (AAA) is associated with vascular inflammation, localised dilation and increased risk of aortic rupture. Impaired aortic relaxation to Ach has been reported in an angiotensin II-infused, apolipoprotein E-deficient ($ApoE^{-/-}$) mouse model of AAA (Seto *et al*., 2013). Long chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) have BP-lowering and anti-inflammatory effects, making them a potential treatment option for AAA.

Aim. To investigate whether dietary supplementation of $ApoE^{-/-}$ mice with LC n-3 PUFAs can preserve vascular reactivity after 2-week infusion with angiotensin II.

Methods. $ApoE^{-/-}$ mice were fed a low (n=12) or high (n=13) LC n-3 PUFA diet for 10 weeks, with infusion of saline or angiotensin II (1000 ng/kg/min) for the last 2 weeks. BP was measured using a non-invasive tail-cuff method. Wire myography was performed on isolated thoracic aorta.

Results. Mean arterial pressure (MAP) was higher in low diet/angiotensin II-infused mice (93.7±4.3 mmHg) compared to saline-infused mice (86.9±6.8 mmHg, $P<0.05$). MAP was not different between high diet/angiotensin II-infused (85.4±6.4 mmHg) or saline-infused (85.4±2.9 mmHg) mice. Ach stimulated a relaxant response in low diet/saline-infused mice (28.9±6.0%; EC_{50}, 0.22±0.05 µM). Relaxation was negligible in low diet/angiotensin II-infused mice (6.7±8.0%). A similar relaxant response was observed in high diet/saline-infused (13.2±2.1%, EC_{50}, 0.49±0.24 µM), and angiotensin II-infused mice (19.3±3.1%, EC_{50}, 0.92±0.23 µM).

Discussion. A high LC n-3 PUFA diet was protective against increased BP and preserved Ach-mediated aortic relaxation in angiotensin II-infused $ApoE^{-/-}$ mice.

Seto SW et al (2013) PLoS ONE 8: e58481

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VCP746: A cardioprotective adenosine receptor agonist with minimal haemodynamic effects

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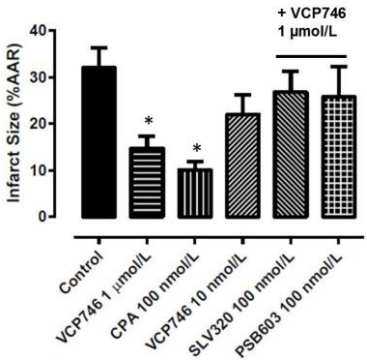
Introduction. It is well accepted that adenosine receptor (AR) activation provides powerful cardioprotection. However, AR activation is also associated with undesirable side effects in the form of profound heart rate (HR) and blood pressure (BP) reduction. This has hindered the transition of AR agonists into the clinic.

Aims. The aim of this study was to determine the effect of VCP746, an A_1/A_{2B} AR agonist, on infarct size, HR and BP.

Methods. Animals were anaesthetised with sodium pentobarbital (60 mg/kg ip). To determine infarct size, a Langendorff-perfused isolated rat heart model was used where hearts were subjected to 30 mins of ischaemia followed by 60 minutes of reperfusion. Post-reperfusion, hearts were frozen, sliced and incubated in 1% 2,3,5-triphenyl-tetrazolium chloride solution to distinguish the infarcted tissue. An anaesthetised rat model was used to measure HR and BP. The jugular vein and carotid artery were cannulated for compound administration and HR/BP measurements respectively.



Results. Infarct size was found to be significantly reduced with VCP746 treatment compared to the vehicle-treated ischaemia/reperfusion control group (Figure inset;14.7 ± 2.6% vs 32.1 ± 4.2% of area at risk, respectively; n = 5, $P<0.05$). Co-treatment with SLV320 and PSB603 (A_1AR and A_{2B}AR antagonist respectively) attenuated the infarct-sparing effect of VCP746. VCP746 also had minimal effects on HR and BP (maximum HR reduction: 6.3 ± 6.7 beats per min, BP reduction: 3.8 ± 1.6 mmHg; n = 5) as opposed to the profound bradycardia and hypotension mediated by N6-cyclopentyladenosine (CPA), a prototypical A_1AR agonist (maximum HR reduction: 325.3 ± 64.4 beats per min, BP reduction: 103.0 ± 11.1 mmHg; n = 5).

Discussion. The results suggest that VCP746 is able to reduce infarct size and requires both the A_1AR and A_{2B}AR to mediate its cardioprotective effect. VCP746 also has minimal effects on HR/BP in vivo at doses that are known to result in cardioprotective plasma concentrations, unlike CPA.

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Major role of endothelial nitric oxide release in the responses to noradrenaline in arteries isolated from different regions of the rat cerebral circulation

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Introduction. Cerebral arteries receive an abundant sympathetic innervation originating mainly from the superior cervical ganglia. However, the precise role of the sympathetic nervous system in regulation of cerebral blood flow is unclear. For example, studies in rat cerebral arteries have not been conclusive regarding the effects of adrenoceptor activation.

Aims. To analyse the effects of adrenoceptor stimulation in arteries isolated from different regions of the rat cerebral circulation and to test the involvement of endothelial nitric oxide (NO) in modifying the responses.

Methods. Rat isolated anterior cerebral (ACA), middle cerebral (MCA), posterior communicating (PCA) and basilar (BA) arteries were mounted for wire myography and the effects of noradrenaline analysed.

Results. Noradrenaline caused a weak contraction in ACA and MCA (~17% of maximum K^+-induced contraction) that was significantly enhanced (~70%) by inhibiting the production of NO with L-NAME (N_ω-nitro-L-arginine methyl ester) or in the presence of β-adrenoceptor antagonists, propranolol, atenolol (β_1) or ICI 11855 (β_2). Methoxamine (α_1-agonist) also caused contraction in ACA and MCA, but not in PCA or BA. In all cerebral arteries pre-contracted with vasopressin, noradrenaline induced relaxation (60-80%) while isoprenaline (β-agonist) caused maximum relaxation. Endothelium removal or pre-treatment with L-NAME abolished relaxation to noradrenaline and isoprenaline. The relaxation to noradrenaline was also antagonised by all β-adrenoceptor antagonists.

Discussion. This study demonstrated that noradrenaline causes both contraction and relaxation of the arteries of the anterior cerebral circulation (ACA and MCA). The contraction is related to the α_1-adrenoceptors which may be limited to the anterior parts of the rat cerebral circulation. The relaxation to noradrenaline is mediated by both β_1- and β_2-adrenoceptor activation and is dependent on the release of endothelial NO. There was no evidence of β-adrenoceptors located on rat cerebral artery smooth muscle mediating the relaxation induced by noradrenaline and isoprenaline. Therefore, the response to sympathetic stimulation in the cerebral circulation may be directly modified by endothelial dysfunction.

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Genetic deletion of the complement receptor C5aR1 has no effect on angiotensin II-induced hypertension and vascular remodelling

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Introduction. Inflammation and immune system activation are now recognised as important contributors to the pathogenesis of hypertension and vascular damage. The complement system is a critical component of the innate immune system, and the complement peptide C5a is a potent pro-inflammatory mediator involved in both innate immune responses and adaptive immunity.
Aims. To determine whether genetic deletion of the primary receptor for C5a (C5aR1) affects the hypertension and microvascular remodelling caused by angiotensin (Ang) II.
Methods. Adult male C5aR1 knockout (KO) and wild type (WT) mice were anaesthetized with inhaled isoflurane and radiotelemetry probes implanted for the direct measurement of blood pressure. Osmotic pumps were implanted (sc) to administer either saline or Ang II (1000 ng/kg/min) continuously for 14 days. Pressurized myography was used to examine contractility, endothelial function, structure and mechanics in mesenteric resistance arteries.
Results. Basal 24 h mean arterial pressures (MAP) were similar in WT and C5aR1 KO mice (99.9±1.7 and 97.5±1.9 mmHg respectively, n=9). Infusion of Ang II for 14 days increased MAP by a similar amount in both WT and C5aR1 KO mice (to 147.2±11.2 and 136.2±4.4 mmHg respectively, n=3-4, *P<0.05 vs saline). Pressurized myography showed no differences between groups in microvascular contractility, endothelium-dependent or endothelium-independent relaxation (as measured by responses to phenylephrine, acetylcholine and sodium nitroprusside). There were no differences in microvascular structure (lumen diameter, media:lumen ratio, medial cross-sectional area) between saline-treated WT and C5aR1 KO mice (n=4-6). Ang II infusion caused inward vascular remodelling, and significantly reduced lumen diameters and increased media:lumen ratios in both WT and C5aR KO mice (n=4-6). Stress-strain curves were used to assess vascular stiffness, and were similar in all groups.
Discussion. These results demonstrate that a lack of complement C5a-C5aR1 signalling does not affect Ang II-induced hypertension and vascular remodelling in the mouse, suggesting complement-mediated inflammation does not contribute to Ang II-induced vascular pathology.

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A flavonoid rich extract from *Carpobrotus rossii* improves glucose tolerance but not lipid profile

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Introduction. We have previously shown that a crude extract of the native, plant *Carpobrotus rossii* (CR), lowers lipids in healthy rats (Pirie et al., 2014). Hyperlipidaemia, glucose intolerance and insulin resistance are features of type II diabetes and/or metabolic syndrome.
Aims. To determine whether consumption of CR extracts improves glucose tolerance and lipid profile in insulin resistant mice and hyperlipidaemic rats, respectively.
Methods. Glucose tolerance was assessed in 6 week old male C57/BL6 mice fed either a normal (Norm, 9% fat w/w) or high fat (HFD 22% fat w/w) diet, with or without crude (HFD+Crude) or flavonoid-rich extract (HFD+FLAV) for 28 days. Glucose responses to i.p. injection of glucose (2 g/kg bw) were measured at regular intervals over 2 h. Plasma lipids were measured in male Sprague-Dawley rats (~150g) on a high fat (10% fat, 2% cholesterol w/w) (HFR) or a standard rat chow diet (5% fat w/w) (NDR) for 28 days. Additional HFR animals were supplemented with crude CR (HFR+Crude), CR flavonoids (HFR+FLAV) or simvastatin at 2mg/kg body weight (HFR+S). Supplementation (70 mg/kg/day crude equivalent) was via drinking water. Responses were compared using two way ANOVA followed by Fisher's post-hoc test.
Results. HFD+FLAV supplemented mice had significantly lower blood glucose 45 to 90 min post glucose challenge (all P<0.05), compared with HFD controls. None of the treatments improved the lipid profile in HFR rats.
Discussion. CR flavonoids, but not the crude extract, improved glucose tolerance in insulin-resistant mice. Investigations to determine the effects of the CR flavonoids on other aspects of metabolic syndrome are recommended.

Pirie AD et al (2014) Food Chem Toxicol 66:134-139

Endothelium-dependent cAMP and cGMP responses to serelaxin in human smooth muscle cells: role of nitric oxide and prostanoids

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Introduction. The phase III clinical trial, RELAX-AHF, showed that 48 hour infusion of serelaxin (recombinant H2 relaxin), caused marked vasodilation in patients with acute heart failure (Ponikowski et al. 2013). However, the precise cellular mechanism(s) associated with its vascular effects in humans are poorly understood.

Aims. This study examined the effects of serelaxin in co-cultures of human primary endothelial cells (ECs) and smooth muscle cells (SMCs) on cAMP and cGMP signaling, markers of vascular function.

Methods. A co-culture model was established using cell culture inserts (Thincerts) to examine endothelium-dependent signalling. AlphaScreen cAMP and cGMP accumulation assays were conducted to examine serelaxin signalling.

Results. Serelaxin stimulation of human umbilical artery endothelial cells (HUAECs), cells that do not express the cognate serelaxin receptor RXFP1, had no effect on cAMP and cGMP accumulation in HUAECs. Importantly, serelaxin-stimulated HUAECs had no effect on cAMP or cGMP accumulation in HUASMCs and HUVSMCs. However, stimulation of human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) with serelaxin concentration-dependently increased cGMP accumulation in both HUASMCs and HUVSMCs. Pre-incubation of HUVECs and HCAECs with a nitric oxide synthase inhibitor, L-NOARG (30μM, 30min), significantly inhibited serelaxin-mediated (30nM) cGMP accumulation in HUVECs and HCAECs, but also significantly inhibited cGMP accumulation in HUASMCs and HUVSMCs. Additionally in HCAECs but not HUVECs, pre-incubation with a cyclooxygenase inhibitor (indomethacin: 30μM, 30min), significantly inhibited cGMP accumulation in HUASMCs and HUVSMCs. Surprisingly, serelaxin stimulation of HCAECs but not HUVECs also increased cAMP accumulation concentration-depedently in HUASMCs and HUVSMCs. Pre-incubation of HCAECs with indomethacin (30μM, 30min) but not L-NOARG (30μM, 30min) abolished this cAMP accumulation in vascular SMCs.

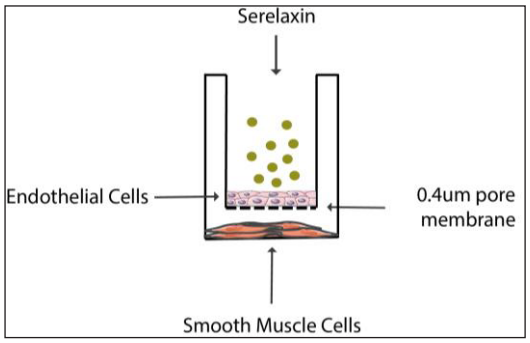

Figure 1 Cell co-culture model. Endothelial cells (EC) cultured on Thincerts (0.4μm, Greiner Bio-One, Australia) were stimulated with serelaxin. cAMP and cGMP accumulation were measured in EC and smooth muscle cells.

Discussion. Serelaxin caused EC-dependent cGMP accumulation in vascular SMCs but also when added to HCAECs caused EC-dependent cAMP accumulation. The responses involved nitric oxide and GC activation in vascular SMCs but also in HCAECs, EC-derived prostanoid production. Thus serelaxin utilizes several mechanisms to modulate vascular tone in different vascular beds.

Ponikowski P *et al*. (2013) *European Heart Journal* 35:431–441

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Endogenous hydrogen sulfide (H_2S) production in resistance arteries of the rat.

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Introduction. H_2S has been shown to be produced endogenously by several different biochemical pathways (Kimura 2014). It acts as a vasorelaxant gasotransmitter produced via cystathionine-γ-lyase (CSE) (Al-Magableh 2011), however it is not known whether other reported endogenous pathways for H_2S production play a role in vascular H_2S generation and vasoactivity.

Aims. To determine if the reported enzymatic pathways for H_2S production, CSE, cysteine aminotransferase (CAT) or D-amino acid oxidase (DAO) in concert with 3-mercaptosulfurtranferase (MST) or cystathionine-β-synthase (CBS) are present in the resistance vasculature and if so, what their functional role is in H_2S mediated vasoregulation.

Methods. Small mesenteric arteries were collected from male Sprague-Dawley rats (10-14 weeks old). CSE, CAT, DAO, MST and CBS protein levels were assessed via western blotting and cellular location via immunohistochemistry. Vasorelaxation responses elicited by the H_2S precursors, L-cysteine or D-cysteine, in the presence and absence of inhibitors of CSE, CAT, DAO, MST and CBS were examined using myography.

Results. CSE, CAT, DAO and MST, but not CBS, were present in mesenteric arteries. Vasorelaxation responses to L-cysteine were significantly inhibited by the CSE inhibitor propargylglycine (20mM, $P<0.05$), the CAT inhibitor L-Aspartic Acid (10mM, $P<0.05$), the MST inhibitor, sodium phenylpyruvate (1mM, $P<0.05$), but not the CBS inhibitor hydroxylamine (10mM). D-cysteine-induced vasorelaxation was not affected by the DAO inhibitor 3-methylpyrazole-5-carboxylic acid (10μM).

Discussion. These data show that CSE-derived but not CBS nor DAO-MST derived H_2S contribute to vasoregulation in resistance arteries. Further, this is the first demonstration that CAT-MST-derived H_2S has vasoactive effects.

Kimura (2014) Nitric Oxide 41C:4-10.
Al-Magableh (2011) Naunyn Schmiedebergs Arch Pharmacol.383(4):403-13.

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Chronic NaHS treatment protects vascular function in streptozotocin-induced diabetes in mice.

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Introduction. Hydrogen sulfide (H_2S) is endogenously produced in vascular tissue (Al-Magableh 2011). H_2S is an antioxidant (Al-Magableh 2014) and may be a useful therapeutic agent under conditions of increased oxidative stress.

Aim. The aim was to investigate whether chronic treatment with H_2S via NaHS could elicit a vasoprotective effect in diabetes, where there is known to be increased oxidative stress (Shen 2010).

Methods. Diabetes was induced in male C57 mice with streptozotocin (60mg/kg daily, ip for 2 weeks) and confirmed by elevated blood glucose and HbA_{1C} levels. Following a further 2 weeks, mice were then treated with NaHS (100μmol/kg/day) for 4 weeks, then tissues collected. Myography was employed to examine vascular reactivity, NO and H_2S bioavailability, and vasorelaxation elicited by the H_2S precursor L-Cysteine, a substrate for the H_2S producing enzyme cysthathionine-γ-lyase (CSE).

Results. ACh-mediated, endothelium-dependent vasorelaxation was significantly inhibited in diabetic aortae ($P<0.05$), but NaHS treatment restored the relaxation response to ACh. Vascular NO bioavailability was reduced ($P<0.01$) and H_2S bioavailability was increased ($P<0.01$) in diabetes. L-cysteine induced vasorelaxation was inhibited by the CSE inhibitor PPG in control aorta ($P<0.05$), but not in diabetic aorta, however none of these parameters were affected by chronic NaHS treatment.

Discussion. These data show that chronic NaHS treatment protects endothelial function *in vivo* in this model of vascular disease, however it does not appear to protect NO bioavailability. Vascular H_2S bioavailability is enhanced in diabetes, but this does not improve H_2S-induced vascular relaxation in diabetic aorta and is not affected by chronic NaHS treatment.

Al-Magableh (2011) Naunyn Schmiedeberg's Arch Pharmacol 383(4):403-13.
Al-Magableh (2014) Naunyn Schmiedeberg's Arch Pharmacol 387(1):67-74.
Shen G (2010) Can J Physiol Pharmacol 88(3):241-8.

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Th2-promoting cytokine treatment limits brain injury after cerebral ischemia in Th1-dominant mice.

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Introduction. Brain inflammation is a major contributor to secondary injury and infarction following ischemic stroke. Previous evidence indicates that T-helper type-1 (Th1) immunity is associated with a worse outcome in Th1-dominant versus Th2-dominant mouse strains after stroke. However, it is unknown whether brain injury and functional deficits can be limited by acute therapy to promote Th2-type immunity.

Aims. We therefore aim to test if Th2-promoting cytokines are able to switch the immune response in Th1-dominant C57BL/6 mice to a Th2-dominant phenotype, leading to reduced brain inflammation and improved functional outcome.

Methods. Male mice were treated with vehicle, IL-4 or IL-33 (1% Bovine serum albumin, 5 µg or 2 µg, respectively, i.p.) 24 h before and 1 h after cerebral ischemia. Mice were anesthetised with ketamine (80 mg/kg) and xylazine (10 mg/kg) i.p. prior to middle cerebral artery occlusion (1 h). Neurological and hanging wire assessments were performed 24 h after stroke. Brains were removed and frozen brain sections (30 µm) were stained with thionin for infarct analysis. Antibiotics (ampicillin and gentamycin, 300 mg/kg and 12 mg/kg, respectively, s.c.) were administered to some mice (n = 6) in combination with IL-33.

Results. Brain infarction was reduced by ~35% by IL-33 or IL-4 treatment, as compared to vehicle (26.09 ± 3.136 mm^3, 29.10 ± 3.657 mm^3 and 43.77 ± 4.589 mm^3, respectively, n = 13-16, both $P < 0.05$). However, mortality and neurological deficit were exacerbated by IL-33 and IL-4 ($n \geq 18$; both $P < 0.01$). Flow cytometric analysis indicated that IL-33 reduced pro-inflammatory $Ly6C^{hi}$ monocytes in ischemic brains, as compared to vehicle (166.6 ± 94.68 versus 782.2 ± 279.1 cells, respectively, n = 5-9; $P < 0.05$). Ongoing studies suggest that combined IL-33 and antibiotic therapy improves functional recovery compared with IL-33 alone.

Discussion. These data indicate that acute administration of Th2-promoting cytokines limits brain injury but exacerbates functional deficit after stroke, possibly due to increased bacterial infections. Post-stroke cytokine therapy may be feasible together with antibiotics.

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Improving warfarin education in community patients

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Introduction. Patient education has been identified as an important factor associated with the safe use of warfarin.

Aims. To assess the warfarin education received by a Melbourne population of older, community-based warfarin patients and to examine the relationship between warfarin education and bleeding risk.

Methods. A case control study was conducted from March 2008 - June 2009 in which participants completed a 19-item warfarin education test (WET). Questions focused on patient's warfarin knowledge, skills and education received. Cases were at risk of bleeding whilst controls were stable on warfarin. A univariate analysis followed by a multivariate analysis of patient factors associated with bleeding risk was performed.

Results.A total of 486 participants, 158 cases and 328 controls, completed the WET with a mean age of 76 years. Adequate warfarin education was not associated with a decreased bleeding risk (OR=1.080, p-value $\leq$ 0.776), however, 11 of the 19 questions in the WET displayed a significant relationship between an incorrect response and increased bleeding risk. Specifically, patients that did not understand what warfarin is and how it works showed a 3.126 increased bleeding risk ($p \leq 0.000$) and patients that did not inform their other healthcare professionals about their warfarin treatment displayed a 5.940 increased risk in bleeding ($p \leq 0.000$). After adjusting for patient factors (employment status, duration on warfarin, functional health literacy, depression, internet usage and vision), adequate warfarin education resulted in a 0.487 decreased risk of bleeding (0.487, <0.05).

Discussion. Adequate patient education was not associated with a decreased risk of bleeding in older warfarin patients. Conversely, doing poorly on specific WET questions was associated with increased risk. Patients that display poor health literacy, possible depression or are visually impaired may require more personalised education. Thus, future research should aim to improve the warfarin education delivered to patients in order to reduce warfarin related adverse events.

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Improving student outcomes and perceptions by enhancing engagement

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Introduction. This paper reports the results of a case study on the use of reflective practice to enhance student engagement and student success in a first year biosciences course at Griffith University.
Aims. The study was undertaken to evaluate different teaching methodologies, learning activities and assessment strategies and determine those that gave positive outcomes, with the aim of incorporating them into the course to enhance student engagement and success. Specifically, the study examined the development and modification of a course aimed at engaging student interest in contemporary issues and current research in the biosciences.
Methods. Through the course of the study, the curriculum was developed and adapted to maximise student engagement and a suite of teaching modalities and philosophies were implemented and trialled. All teaching methods and activities trialled have received recent interest and all are purported to enhance student engagement. Each modification was critically examined in terms of its effect on student outcomes and on student perceptions (as determined via anonymous student questionaires).
Results. The course Topics in Biosciences (1003 BPS) at Griffith University, Australia was developed with the aim of engaging first year university students in the biosciences and thereby aid in increasing student retention rates and the transition of students to the second year of their studies. The course incorporated learning activities that have previously been shown to have positive effects on student engagement including collaborative group work, writing to learn activities as well as oral and written presentations. Incorporation of other teaching practices which have been established to positively influence student engagement and success such as clear and rapid feedback on assessment, directed approaches to group assignment and in lecture activities to engage student participation were all included. The result was a well-rounded course that achieved good student engagement and success rates and that was positively received by the student cohort.
Discussion. Results from this study strongly indicate a positive influence for incorporating teaching activities that encourage active learning and engagement (such as in-lecture quizzes, collaborative group presentations, writing-to-learn activities) into the course structure. A clear correlation between incorporating these teaching practices with both student outcomes and student perceptions with the course was noted.

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Development of a smartphone 'app' for converting brand names of drugs to approved drug names to assist trainee doctors in taking drug histories and in prescribing

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Introduction. The World Health Organisation's Approved names of drugs are used exclusively in the teaching of pharmacology to medical students. Likewise, refereed medical literature use Approved names and not brand names of drugs primarily because brand names can vary between countries depending on copyright. Unfortunately, most patients only know the drugs that they are taking by the brand name, shown on the label of drugs from their community pharmacy. With the increase in widely used drugs coming off patent, there has been a proliferation in brand names with some widely used drugs having 6 or more brand names in Australia. This makes it difficult for medical interns to take drug histories, to assess both clinical needs for these drugs and potential drug interactions.
Aim. The latest generation of smart phones with touch screen input, large storage and computing capacity makes them ideal as portable computers to assist trainees in prescribing (Haffey et al 2013). We have written an application ('app'), specific for use in Australia, which will assist trainees deal with brand names that they do not know.
Methods. Approved drug names (n = 840) and corresponding brand names (n = 1762) were taken primarily from the Pharmaceutical Benefits Scheme website (www.pbs.gov.au). This data was compiled into an 'app' that runs on any modern browser and is agnostic to the operating system (e.g. iOS, Android, Windows). Users must access the 'app' once with an internet connection and the dataset is then stored locally on their device and can be accessed offline until the device is turned off.
Results and Discussion. When a brand name is entered into the phone screen, it quickly goes to a new screen showing the Approved name in uppercase and below are listed the appropriate brand names in alphabetical order in lower case. Additional brief information to prompt the memory of interns is the clinical classification and uses of the Approved drug (e.g. antidepressant, broad spectrum antibacterial, proton pump inhibitor etc) followed by the accepted pharmacological Mode of Action of that drug.

Haffey F et al (2013) Br J Clin Pharmacol 77: 31-38

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Pannexin-2 expression in the human intestine – possible roles in gastrointestinal motility and secretion

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Introduction. Pannexin-2 (Panx2) is a member of the novel group of membrane spanning protein channels, pannexins, and is primarily found in the CNS (Bond et al, 2014). Definitive Panx2 function has yet to be elucidated, and since no studies have examined Panx2 in the intestine, it may have important physiological roles in the enteric nervous system (ENS) which contains chemically coded neuron populations similar to those found in the CNS.
Aims. The present study characterised Panx2 expression and localisation in the human colon in health and disease.
Methods. Immunofluorescence was conducted on cross-sections of intact human colon tissues to determine Panx2 localisation and co-localisation with neuronal markers for intrinsic and extrinsic neurons. Colon specimens were separated into mucosa and muscularis layers for determining Panx2 gene and protein expression using quantitative real-time PCR and Western blot, respectively, in control, ulcerative colitis (UC) and Crohn's disease (CD) colon.
Results. Intense Panx2 immunoreactive staining was found in myenteric and submucosal plexuses, where Panx2 was localised to neuronal cell bodies, as well as nerve fibres. Double labelling immunohistochemistry demonstrated co-localisation of Panx2 with Class III β-tubulin, neuronal nitric oxide synthase, substance P, vesicular acetylcholine transporter, and calcitonin gene-related peptide, indicating widespread Panx2 expression in neurons such as motorneurons, and neurons involved in pain sensation. Molecular studies revealed a 3.4-fold higher level of Panx2 mRNA in ascending compared to sigmoid muscularis (**$P < 0.05$), despite similar protein levels. Similarly, UC muscularis showed a ~35-fold up-regulation in Panx2 mRNA (**$P < 0.05$), but not in protein.
Discussion. Dense and broad Panx2 expression in enteric neurons suggests important roles in intercellular signalling, especially in the modulation of ENS mediated sensation, motility and secretion. The dense expression of Panx2 in the ENS implies that Panx2 may be important in regulating neuronal function, and may contribute to the dysmotility symptoms seen in UC.

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Calcium homeostasis modulator 1 as a potential ATP release conduit in human colonic epithelial cells

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Introduction. ATP released from intestinal epithelial cells behaves as a sensory mediator that excites subepithelial sensory nerve terminals to regulate enteric motor reflexes. The mechanisms of ATP release in the human colon remain totally unexplored, but may involve calcium homeostasis modulator 1 (CALHM1), an ion channel recently shown to behave as an ATP-release conduit in primate taste bud cells (Taruno *et al.*, 2013).
Aims. This study was aimed to localise CALHM1 expression in the human colon, and to determine whether this ion channel mediates colonic ATP release during Ca^{2+} depletion and mechanical stimulation.
Methods. Immunohistochemistry was conducted to localise CALHM1 in human colon specimens. CRL1790 cells (human colonic gestational epithelial cell line) were incubated in either Ca^{2+}-free or hypotonic solution to investigate the effect of Ca^{2+} depletion and mechanical stretch, respectively, on ATP release. Parallel studies were performed in the presence of the selective CALHM1 blocker, ruthenium red (RuR, 20 μM, 1 hour of preincubation).
Results. Immunofluorescence staining showed CALHM1 expression on many cell types of the human colon, including smooth muscle cells, blood vessels, and enteric ganglia, as well as very dense staining on the membranes of epithelial cells. Both Ca^{2+} depletion and stretch significantly stimulated ATP release from CRL1790 cells, with levels peaking at 2 min and 10 min, respectively. Ca^{2+} -free solution produced a 1.5-fold increase in ATP release ($P<0.001$, one-way ANOVA) which was reduced to baseline levels by incubation with RuR. Stretch produced a 3.5-fold increase in ATP release ($P<0.01$), which was significantly enhanced by RuR.
Discussion. CALHM1 is widely expressed in the human colon. The different time courses suggest that ATP release in response to Ca^{2+} depletion and stretch may occur via different mechanisms/pathways. CALHM1 appeared to be a primary mechanism of ATP release in the Ca^{2+}-free condition, whereas enhancement of ATP release by RuR implies that other pathways participate in stretch-mediate ATP release. These pathways may involve a compensatory mechanism that overrides the pharmacological blockage of CALHM1. Alternatively, there may be a negative autoregulatory mechanism associated with CALHM1 channels, which was impaired by blockage of CALHM1.

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Gemcitabine enhances release of ATP from bladder urothelial cells but is selectively cytotoxic to bladder cancer cell lines

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Introduction. Intravesical chemotherapy for bladder cancer limits systemic absorption of cytotoxic drugs, but significant local urological side effects including dysuria and urgency still occur. No studies have investigated the potency of cytotoxic therapies on normal urothelial cells, which changes to may be responsible for the reported side effects. During bladder filling the normal urothelium releases mediators (ATP, acetylcholine, PGE_2) which activate sensory nerves, but intravesical treatment with cytotoxic drugs may possibly disrupt this process.
Aims. This study compared the toxicity of gemcitabine in cultured bladder urothelial cell lines (cancer versus non-cancer cells), and investigated drug induced changes in basal and stretch-induced release of urothelial mediators.
Methods. The human urothelial bladder cancer cell lines RT4 and T24, and non-cancer UROtsa urothelial cells were treated with gemcitabine. Cell viability was measured 72 hours post-treatment using resazurin reduction assay. Basal and stretch-induced release (using hypotonic solution) of mediators was measured from urothelial cells post-gemcitabine treatment.
Results. A concentration-dependent reduction in cell survival was seen 72 hours post-gemcitabine treatment. The potency of gemcitabine on cancer cells was approximately 100,000 fold greater than its potency on non-cancer urothelial cells ($p<0.001$). In urothelial cells treated with 4mg/mL gemcitabine, basal ATP release was significantly increased compared to control (16.4±2.5nM to 6.0±0.7nM, n=8-13, $p<0.05$). Levels of basal and stimulated acetylcholine and prostaglandin E_2 release from urothelial cells were unchanged after incubation with gemcitabine.
Discussion. Gemcitabine was selectively toxic to malignant urothelial cells with comparison to the normal urothelial cells, although the mechanism of this currently unknown. The increased release of urothelial ATP after incubation with gemcitabine may contribute to the urinary urgency and pain experienced by patients after intravesical treatment with this cytotoxic drug.

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The α_1-adrenoceptor antagonist prazosin enhances sensitivity of hypoxic prostate cancer cells to irradiation

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Introduction. Tumour hypoxia is an important determinant in the outcome of many malignancies and is associated with increased resistance to radiotherapy in solid human prostate tumours and biochemical relapse. *In vitro*, α_1-adrenoceptor antagonists are suggested to have cytotoxic effects on prostate cancer cells but little is known about their effects in hypoxic conditions and in the presence of irradiation.
Aims. The aim of this study was to investigate the potential radiosensitising actions of prazosin in prostate cancer cells irradiation under normoxic and hypoxic conditions.
Methods. Human androgen receptor negative prostate cancer PC-3 cells were treated acutely (2 h) with prazosin (0-100 μM) and irradiated (6MV, 5Gy) under normoxic or hypoxic conditions. Hypoxia (<0.2% oxygen concentration in culture medium) was generated by purging controlled-atmosphere chambers containing cells with oxygen free nitrogen for 38 min. Following acute prazosin treatment, cells were washed and incubated in drug-free medium for 5 days. Cell survival was determined by resazurin reduction.
Results. Following acute treatment and 5 day recovery, prazosin toxicity appeared to be selective for hypoxic PC-3 cells (n=5, $P<0.05$). Under normoxic conditions, irradiation resulted in approximately 75% reduction in PC-3 survival and the presence of prazosin (10-100 μM) during irradiation did not affect cell survival. Hypoxia protected PC-3 cells from irradiation-induced cytotoxicity, with a 3-fold increase in cell survival compared to nomoxic cells (n=5, $P<0.001$). Prazosin increased sensitivity of hypoxic PC-3 cells to irradiation in a concentration-dependent manner, with 30 and 100 μM prazosin reducing survival to that of normoxic cells (n=5, $P>0.05$).
Discussion. Prazosin has cytotoxic actions, which are enhanced in the presence of acute hypoxia. Furthermore, prazosin abolished hypoxia-mediated resistance to irradiation in PC-3 cells. These findings suggest prazosin, or other α_1-adrenoceptor antagonists, may be useful in combination with radiation therapy to overcome radioresistance of hypoxic solid prostate tumours.

The differing effects of doxorubicin treatment on neurogenic detrusor responses of young and old porcine bladders

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Introduction. Intravesical chemotherapeutic agent doxorubicin is commonly used for the treatment of superficial bladder cancer. It causes significant urological adverse effects including dysuria, increased urinary frequency, and urgency. Despite the large number of patients receiving this agent intravesically and the high percentage suffering urological adverse effects, there have been no investigations of the actions of this agent on the non-cancerous tissues of the bladder.

Aims. This study investigates the effects of chemotherapeutic agent, doxorubicin, on neurogenic detrusor response of both young and old porcine bladders when exposed to electrical field stimulations. The results derived from the young and old bladders were then compared to determine whether the effects of the drug on neurogenic detrusor responses differ between the two age groups.

Methods. As a model of intravesical doxorubicin administration in patients, doxorubicin (1mg/mL) was applied to the luminal surface of young (4 to 6 months) and old (1 to 2 years) porcine bladders for 60min. Following treatment, neurogenic responses to electrical field stimulation (20v, 0.5ms pulse-width, applied for 5s every 100s) were investigated.

Results. The responses of young porcine detrusor muscle strips to electrical field stimulation were significantly enhanced in the doxorubicin pre-treated tissues compared to control tissues. With increasing stimulation frequency, responses increased, but at every frequency examined (1, 5, 10 & 20Hz), responses were significantly greater ($P<0.05$) in tissues that had received the doxorubicin pre-treatment. In contrast, the responses of old porcine detrusor muscle strips to electrical field stimulation were depressed in doxorubicin pre-treated tissues compared to control tissues with significant inhibition observed at 10 and 20Hz ($P<0.05$).

Discussion. The results suggest that doxorubicin, when administered intravesically for bladder cancer, may alter the release of neurotransmitters within the detrusor smooth muscle depending on the age of the bladder. Hence, the effects of the drug on older porcine bladders may be more relevant to real life patients as the majority of those affected by bladder cancer are within the senior age range.

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Alterations in bladder urothelial acetylcholine, ATP, prostaglandin E2 and inflammatory cytokines by the chemotherapeutic agent epirubicin

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Introduction. Epirubicin is a cytotoxic agent administered intravesically for the treatment of superficial bladder cancer. During treatment, the drug comes into close contact with the urothelium, and after the treatment, patients may suffer urinary adverse effects.

Aims. The aim of this study was to assess the effects of epirubicin on the release of urothelial mediators and inflammatory cytokines to determine if they may play a role in the adverse effects associated with intravesical epirubicin treatment.

Methods. Immediately and 24 hours following a 1 hour treatment of cultured UROtsa human urothelial cells with epirubicin, samples of incubation medium were prepared for analysis of basal and stretch-induced (using hypotonic solution) mediator release. Incubation medium was collected 24 hr after epirubicin pretreatment for analysis of inflammatory cytokines.

Results. Immediately following epirubicin treatment, basal Ach release was significantly increased (2-fold, $P<0.01$) at 1mg/ml compared to the untreated controls and the Ach response to hypotonic stimulation was abolished ($P<0.01$). Both basal release and stretch-induced ATP release were significantly increased (2-fold, $P<0.01$) after 0.1mg/ml epirubicin. In addition, basal PGE2 release was significantly increased (2-fold, $P<0.01$), but stretch-induced release was abolished ($P<0.05$) after 1mg/ml epirubicin. Twenty four hours after pretreatment, basal Ach release was significantly increased ($P<0.05$) and stretch-induced Ach release was reduced by 70% ($P<0.01$) by 0.01mg/ml epirubicin. Both basal and stretch-induced ATP release were significantly increased (5-fold, $P<0.01$ and 4-fold, $P<0.01$ respectively) by 0.01mg/ml epirubicin. In contrast, PGE2 release was unaffected by epirubicin at any concentration. In addition, interleukin-6 and -8 were significantly enhanced (66-fold, $P<0.01$ and 5-fold, $P<0.01$ respectively) 24 hours after epirubicin pretreatment (0.01mg/ml).

Discussion. These findings indicate that inflammatory cytokines interleukin-6 and interleukin-8 are induced and urothelial mediator release is affected by treatment with epirubicin at clinically relevant concentrations and durations of treatment. These changes may play a role in the adverse effects observed in patients following intravesical epirubicin treatment.

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KCl potentiates subsequent responses to noradrenaline of the porcine urethra by enhancing ROCK and Ca^{2+} release.

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Introduction. Potassium chloride (KCl) has long been used in pharmacology to induce depolarisation and produce a non-receptor mediated contraction of smooth muscle to test tissue viability and also allow normalization of responses. However, recently it has been shown that KCl may also cause calcium sensitisation by activating the Rho kinase (ROCK) pathway in some smooth muscle preparations (Borysova *et al*., 2011). The effect of KCl on smooth muscle sensitization and contraction in the urethra has not been investigated.
Aim. To investigate the effects of KCl-pretreatement on subsequent noradrenaline-induced contractions of the porcine urethra.
Methods. Strips of urethra were mounted in organ baths at 37^0C in Krebs-bicarbonate solution. Test tissues were pre-contracted with KCl (60mM) for 5mins (control tissues were not pre-contracted). After 5mins, the tissues were washed for 30mins and then contracted to noradrenaline (100µM) in the presence or absence of the rho-kinase inhibitor fasudil (10µM), the PKC inhibitor calphostin C (1µM), and the inhibitor of intracellular calcium release cyclopiazonic acid (10µM).
Result. Pre-incubation of tissues with KCl resulted in significant potentiation of subsequent noradrenaline-induced contractions by (83.6±43.48%, Mean±SEM, n=5-10; $p<0.05$). This potentiation was still observed when PKC was inhibited. However, inhibition of ROCK or intracellular Ca^{2+} release abolished the potentiation of responses by KCl and reduced noradrenaline contractions by 54.6±8.4% and 48.4±11.0% respectively. The potentiating effects of KCl were long lasting and were even greater 3 hours after pre-incubation with KCl (90.34±14.292%, $P<0.05$).
Discussion/Conclusion. Prior exposure of urethral tissues to KCl causes potentiation of subsequent noradrenaline induced contractions of the urethra, which may be associated with both Ca^{2+} sensitization and enhanced Ca^{2+} release from the sarcoplasmic reticulum.

Borysova *et al*. (2011). Cell Calcium, 50 (4):393–405

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The role of Rho-kinase in phenylephrine and 5-HT-induced contractile activity of the porcine ureter

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Introduction. Ureteral calculus is frequently accompanied by ureteral colic, which is understood to be caused by constriction of the ureteral smooth muscle (Canda *et al*., 2007). In a previous study, we showed that porcine ureters from older animals generated greater contractions to α-adrenoceptor stimulation, and smaller contractions to 5-HT (Lim *et al*., 2013). Recently, calcium sensitisation via the Rho-kinase pathway has been proposed to play a significant role in smooth muscle contraction (de Godoy & Rattan, 2011),
Aims. To investigate the role of Rho-kinase in phenylephrine and 5-HT induced contractile activity in isolated ureters from old and young pigs.
Methods. Contractile responses of isolated smooth muscle to EC_{50} (low) and maximal (high) doses of phenylephrine and 5-HT were examined in distal ureteral tissues from young (20 weeks) and older (56 weeks) pigs, in the absence and presence of Rho-kinase inhibitors, Y-27632 (10µM) or fasudil (30µM). Tissues developed spontaneous contractile activity and responses were expressed as AUC as a percentage of the maximal contraction.
Results. Fasudil significantly attenuated ($P<0.0001$ vs control, n=6) ureteral contractions to low and high doses of phenylephrine by 86.81±4.18% and 81.96±0.97% in younger animals. Fasudil also reduced contractions to low and high doses of 5-HT by 83.69±2.97% and 77.67±3.16 ($P<0.0001$, n=6). In older animals, similar effects were observed, with contractions to phenylephrine being reduced by 82.01±1.71% and 78.69±3.01%, and to 5-HT, by 80.16±3.43% and 79.31±3.24% at low and high doses respectively ($P<0.0001$, n=6). The Rho-kinase inhibitor Y-27632 produced similar effects with both agonists in tissues from young and old animals.
Discussion. These results show that Rho-kinase mediates contractile responses to phenylephrine and 5-HT in isolated ureteral tissues and contribution to contraction is similar in ureters from both young and old animals.

Canda AE *et al.* (2007) Urol Int 56:23-33
de Godoy MA and Rattan S (2007) Trends Pharmacol Sci 32:384-393
Lim I *et al.* (2013) Proceedings of ASCEPT ASM 2013 437:94

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Comparative evaluation of different polyphenols of green tea and to study the most effective polyphenol in nanotechnology based drug delivery system alone and in combination with sulfasalazine in experimental inflammatory bowel disease in rats

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Background: Inflammatory bowel disease (IBD) is associated with activation of nuclear factor kappa B (NF-κB) involved in regulating the expression of proinflammatory cytokine in serum and decreased levels of antioxidants along with morphological and histopathological changes. Green tea and its polyphenols acts by inhibiting NF-κB along with antioxidant activity.

Methodology: The in vivo efficacy of different polyphenols of green tea was assessed in rat with trinitrobenzene sulfonic acid (TNBS)-induced colitis. The rats were randomized into eleven groups (n =6) and were treated with vehicle (ethanol), TNBS, Epicatchin (10 mg/kg, p.o.), Epigallocatchin (10 mg/kg, p.o.) Epicatchin gallate (10 mg/kg, p.o.), Epigallocatchin gallate(10 mg/kg, p.o.), sulfasalazine(360 mg/kg, p.o.), green tea(70 mg/kg, p.o.), Epigallocatchin gallate(10 mg/kg, p.o.) in combination with sulfasalazine(360 mg/kg, p.o.), nano Epigallocatchin gallate and its combination with sulfaslazine for 14 days. Myeloperoxidase, tissue antioxidant levels were assessed with using biochemical methods. Serum inflammatory cytokines and expression of NF-κB assessed with ELISA-kit. Morphological and histopathological changes assessed by pathologist.

Results: Among different polyphenols of green tea treated groups Epigallocatchin gallate(10 mg/kg, p.o.) reduced colonic inflammation, myeloperoxidase, biochemical parameters, NF-κB and serum anti-inflammatory cytokines were significantly reduced compared with other polyphenols ($p < 0.01$). Epigallocatchin gallate(10 mg/kg, p.o.) was more effective than green tea by significantly reducing above mentioned parameters ($p < 0.01$). Comparison Epigallocatchin gallate and its nanoformulation there was no difference in efficacy. Comparison of Epigallocatchin gallate(10 mg/kg, p.o.) in combination with sulfasalazine(360 mg/kg, p.o.), nano Epigallocatchin gallate and its combination with sulfaslazine, in later group was more efficacious by significantly reducing above inflammatory parameters ($p < 0.01$).

Conclusions: The present study indicates that EGCG is most efficacious polyphenol among different polyphenols of green tea. Nano Epigallocatchin gallate is effective as it has additive effect in experimental induced colitis, however these results require further confirmation in human studies.

Loss of fenestrations in liver sinusoidal endothelial cells impairs hepatic insulin signaling and glucose homeostasis

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Introduction. The liver plays a major role in the regulation of glucose homeostasis, which was tightly regulated by insulin. Prior to hepatic metabolism, substrates travel through fenestrations in the liver sinusoidal endothelial cells to gain access to hepatocytes. We propose loss of fenestrations (defenestration), such as seen in ageing, will impair the transfer of insulin and glucose across the hepatic sinusoidal endothelium, thus contributing to hepatic insulin resistance.

Aims. To investigate changes in hepatic insulin signaling and glucose homeostasis in normal and defenestrated livers.

Methods. Defenestration is induced in F344 rats with a single i.p. injection of P407 24h prior to experimentation (1g/kg). Livers were snap frozen and subjected to qrtPCR using RT2 PCR array and immunoblotting. Radiolabelled glucose tolerance test was performed, where rats were fasted and injected with glucose (2g/kg i.v.) spiked with 10 µCi ^{14}C-glucose for assessment of insulin action and 10 µCi ^{3}H-2-deoxyglucose for assessment of glucose uptake. Blood glucose level were read at 0, 15, 30, 45, 60 and 90m using glucometer and insulin level were determined at the beginning and end of experiment for calculation of Homeostatic Model Assessment (HOMA) index. Liver, white adipose tissue (WAT) and muscle were collected for radioactivity analysis.

Results. Animals treated with p407 showed a significant increase in triglyceride and cholesterol levels compared to control ($p<0.001$), together with a marked defenestration in the liver sinusoidal endothelial cells. Hepatic volume of distribution as a fraction of the extracellular space for both insulin and glucose were significantly decreased in the hyperlipidemic rats, indicating impeded substrate transfer as a result of defenestration (glucose: 1.54±0.06 control vs 1.10±0.10 P407; insulin: 1.08±0.08 control vs 0.81±0.05 P407, $p<0.001$). There was a significant up-regulation of Insulin Receptor Substrate-2 mRNA (Control 1.3^{03} vs P407 2.2^{03}, 1.76 fold change, $p=0.02$), with a trend for down-regulation of the rate limiting gluconeogenic enzyme Glucose-6-phosphatase mRNA. Limited access of insulin to hepatocellular membrane is shown by a decreased phosphorylation of Insulin Receptor Substrate-1 protein ($p=0.045$) involved in early insulin signalling pathway. Defenestration also induced hyperinsulinemia (T0: Control 0.61±0.07 vs P407 1.35±0.22, $p=0.005$; T90: Control 0.94±0.15 vs P407 2.85±0.73, $p=0.036$) and reduced insulin sensitivity (HOMA index; Control 5.43±0.68 vs P407 11.7±2.06, $p=0.014$). In addition, there was decreased incorporation of glucose into glycogen in the liver (Control 4.2±0.38 vs P407 2.8±0.48, $p=0.042$), but not in muscle and WAT in the P407 treated group.

Discussion. This finding indicates that defenestration reduced the volumes of distribution of insulin and glucose in the liver, leading to alteration of the insulin signaling pathway, and impaired glucose homeostasis particularly in the liver. It further affirms the important role of the liver ultrastructure in hepatic metabolic processes and highlighting it as an important potential therapeutic target for insulin resistance in conditions such as ageing and liver diseases.

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Effect of chemical enhancers on the in vitro percutaneous penetration of caffeine

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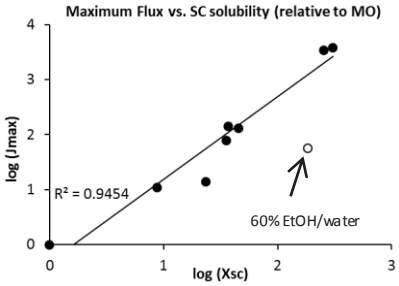
Introduction. The maximum penetration flux of topically applied solutes should be independent of the vehicle, unless the vehicle affects the skin (Zhang, 2009). In this study, we examined how skin penetration fluxes are affected by vehicles reported to interact with the skin barrier to different degrees. We compared *in vitro* maximum penetration fluxes (J_{max}) and mole fraction solubilities in the vehicles (X_v) and stratum corneum (X_{sc}) for caffeine from various solvent vehicles to evaluate vehicle effects on permeability parameters.
Methods. Solutions of caffeine were dissolved in a range of vehicles and applied to heat separated human epidermal membranes in Franz diffusion cells. Permeated solute concentrations were measured by HPLC and used to calculate permeation parameters. Caffeine solubilities in the vehicles and in stratum corneum were estimated after equilibration with saturated caffeine solutions for 24 hrs.
Results. J_{max} was independent of X_v for inert vehicles with no reported effects on the skin barrier, whereas enhanced caffeine flux was seen with vehicles containing the penetration enhancers oleic acid (OA) or eucalyptol (Eu). There was a linear relationship between J_{max} and X_{sc} for all vehicles, including those containing OA and Eu, while the 60% ethanol/water vehicle deviated from linearity with a reduced flux (see Figure).



Discussion. The results show that the maximum flux of solutes is related to their solubility in the stratum corneum, regardless of the vehicle used. Chemical penetration enhancers may increase stratum corneum solubility by mechanisms including reorganization of the ordered lipid packing. The reduced flux with 60% ethanol/water is likely to be due to dehydration of the skin, as demonstrated previously by this group.

Zhang, Q et al. (2009) Pharm Res 26:1974-85.

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Redefining normal variability of drug disposition

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Introduction. The pharmacokinetics of many drugs are said to be predictable. This is often used to imply the ease of dosing or dose-adjustments. However, predictability requires both accuracy (lack of bias) and precision (reproducibility). In the context of pharmacokinetics, precision refers to the ability to achieve a specified target concentration in different individuals. Precision is the inverse of the variance between-subjects, i.e. the greater the between subject variability (BSV) the less precise/predictable a parameter is across a patient population. BSV in PK parameters is quantified by the coefficient of variation (CV%). The current convention is that BSV in PK parameters is considered "low" (CV% ≤ 10%), "moderate" (CV% ~ 25%), or "high" (CV% > 40%). [1]
Aims. To explore the range of BSV values in PK parameters in patient populations of preselected drug classes.
Methods. A literature review of population PK studies from various data sources was conducted. Drug classes studied included psychotropics, immunosuppressants, cardiovascular drugs, and antibiotics. Estimates of clearance (CL) and volume of distribution (V) and their corresponding CV% were recorded.
Results. A total of 181 studies involving 95 drugs were found. The mean CV% in CL/F was 40.3% and in V/F was 51.3%. The mean CV% in CL/F in predominately renally cleared drugs was 31% (after accounting for renal function) and those predominately hepatically cleared drugs was 47.4%. Age, sex, weight, and renal function were among the most significant covariates reported across the drug classes.
Discussion. According to the current convention most drugs show "moderate" to "high" BSV. The current convention needs to be recalibrated to consider that a low BSV in CL is < 25%, 25 – 50% is normal, and > 50% is high. Clinically, this means that a normal level of variability in CL would result in a 4- to 5-fold variability in steady state average plasma concentrations and therefore for all drugs with a low therapeutic index, monitoring plasma concentration or response and dose-individualisation will be essential.

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Resolvin D2 has mitogenic activity in estrogen receptor positive breast cancer cell lines via activation of estrogen receptor

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Introduction: Inflammation has been implicated in tumour initiation, angiogenesis and metastasis and linked to the development of more aggressive, therapy-resistant estrogen receptor positive breast cancer (Baumgarten and Frasor, 2012). Resolvin D2 (RvD2) is a potent anti-inflammatory lipid mediator. RvD2 is present in plasma at bioactive concentrations and the enzymes involved in its synthesis are expressed by both tumour and stromal cells.
Aim: To investigate the impact of RvD2 on cell processes underlying breast tumour growth and spread.
Methods: Viable cells were enumerated by Trypan-blue exclusion. Transactivation of estrogen response element (ERE) was assessed by transient transfection with an ERE reporter. RT-qPCR was used to examine gene expression. Binding to the estrogen receptor (ER) was investigated by radioligand binding assays. Western blotting and immunofluorescence techniques were used to ascertain ER α nuclear localization.
Results: RvD2 (10-1000 nM) supported the proliferation of the ER-positive breast tumour, MCF-7, but not the ER-negative, MDA-MB-231 cells. RvD2 mitogenesis in MCF-7 cells was prevented by the estrogen receptor antagonist ICI 182,780 (100nM). Furthermore, RvD2 increased ERE transcriptional activity in MCF-7, T47D, BT474 and SKOV3 cells. RvD2 altered the expression of a subset of estrogen-responsive genes. Prior exposure of MCF-7 cells to RvD2 reduced the apparent cytosolic ER density. RvD2 did not directly compete with ^{3}[H]-17 β-estradiol (E2) for ER binding. Confocal immunofluorescence localisation and western blotting studies showed that RvD2 promoted nuclear localization of ERα with a corresponding decrease in cytosol ER density.
Discussion: RvD2 displays significant but indirect estrogenic activities and that it has the potential to play a role in estrogen-dependent breast cancer progression.

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Mas E et al (2012). *Clinical Chemistry* 58, 1476-1484.

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Safety and Pharmacokinetics of Metformin in Liver Disease

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Introduction. Metformin is the first-line treatment of type II diabetes mellitus. In the product information, metformin use is contraindicated in patients with hepatic impairment due to concerns of heightening the risk of precipitating lactic acidosis. However, this causal relation is contentious, and there is little research conducted in this area.
Aims. The primary objectives of this study was to evaluate the safety and PK parameters of metformin in subjects with liver fibrosis.
Methods. Liver disease patients currently taking metformin were recruited for this observational cohort study. Subjects were stratified according to degree of liver fibrosis; minimal fibrosis (n = 3) and cirrhosis (n=5). At least one blood sample was collected from each patient. Metformin concentrations in plasma were determined using HPLC. Biochemical parameters such as lactate, bicarbonate and anion gap were also assessed. PK parameters were determined by using TCIWorks (Version 1.0) and a published population PK model[1].
Results. The mean (± SD) metformin CL/F was 55 (±16) L/h and the apparent volume of distribution was 185 (±71) L. The mean ratio of metformin CL/F to creatinine CL was 13.5 (±3.4) which was very similar to values found in without significant liver disease, 12.6 (±4.3)[1]. The plasma concentrations of lactate and bicarbonate were 2.2 (± 0.7) mmol/L and 25.8 (± 3.9) mmol/L, respectively. The anion gap was 17.1 (±2.3) mmol/L. There was no correlation between metformin concentrations and lactate, bicarbonate or anion gap concentrations.
Discussion. Metformin clearance was similar to that seen in previous literature, with concentrations remaining within the therapeutic range. This study shows that liver disease has no significant impact on metformin PK and safety although further studies are required. Renal function and body weight are still the most important determinants of metformin PK.

Duong et al., (2013). Clin Pharmacokinet 52:373-84

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Domperidone, QT prolongation and sudden cardiac death – is there really a risk?

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Introduction. Domperidone (DOM) fills a prescribing niche for gastrointestinal motility and chemotherapy nausea and vomiting. The European Medicines Agency (EMA) recently concluded that it poses a significant risk of QT prolongation and sudden cardiac death (SCD) and subsequently restricted the use in Europe.
Aims. To review the risk of QT prolongation and cardiac adverse effects with DOM and provide information to allow prescribers to make informed decisions on usage.
Methods. A search of two bibliographic databases, the EMA website, Micromedex, Lexicomp and reference texts was undertaken for DOM related reports of QT prolongation, cardiac arrhythmias and/or SCD. The New Zealand Centre for Adverse Drugs Reaction Monitoring was also contacted for cardiac adverse event reports with DOM.
Results. Over 30 published papers, EMA documents and other information sources were collated including two studies that met thorough QT study (TQT) criteria (ICH-E14). The first TQT[1] was negative while the second[2] was marginally positive. Reports of QT prolongation, ventricular arrhythmias and SCD were located (predominantly high/very high-dose IV DOM). With oral DOM, a Dutch case controlled study[3] reported an adjusted odds ratio of SCD of 11.4 (95% CI 1.99-65.2), based on only 3 patients out of 1366 cases of SCD. A second nested case-controlled study[4] calculated an odds ratio of ventricular arrhythmia or SCD of 1.59 (1.28-1.98) vs. placebo.
Discussion. Based on the results of the two TQT (regulatory agency gold standard for assessment of QT prolongation) DOM does not appear to be strongly associated with QT prolongation at oral doses of 20 mg QID in healthy volunteers. Further, there are limited case reports supporting an association, and the frequently cited case-control studies have significant flaws. While there remains an ill-defined risk at higher systemic concentrations, especially in patients with a higher baseline risk of QT prolongation, our review does not support the view that DOM presents intolerable risk.

1. EMA Assessment Report 06/03/2014 (EMEA/H/A-31/1365)
2. BJCP 2012; 73 (3): 411-21
3. Drug Saf 2010; 33(11): 1003-14

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Microglia-Derived BDNF Modulates Dopamine Circuitry in Opioid Dependent States

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Opioid dependent states are associated with adaptations within the mesocorticolimbic dopaminergic system that contribute to the negative affective state when the drug is absent (withdrawal). Here, we analyze molecular adaptations in the ventral tegmental area (VTA) and their effect on drug reward.
C57Bl/6 mice were made opioid dependent with increasing injections of morphine (10-40mg/kg, i.p) twice daily for 4 days. Opioid dependent animals displayed significant microglial activation in the VTA and blocking microglial activation decreased BDNF expression. Chronic morphine exposure decreased expression of the potassium/chloride (Cl-) co-transporter, KCC2, within VTA GABAergic neurons, which resulted in a loss of Cl- extrusion as measured by fluorescent lifetime imaging. Interfering with BDNF signaling recovered Cl- extrusion in morphine-dependent GABAergic neurons.
Loss of Cl- extrusion is known to undermine GABAergic inhibitory potential, which translates into an increased inhibition in dopaminergic VTA neurons. In support of this, cocaine reinforcement (as measured using the conditioned place preference paradigm) was diminished in opioid dependent animals. Cocaine place preference was restored in opioid dependent animals by cotreatment with microglial inhibitors.
This study provides evidence for disrupted reward circuitry in opioid dependent animals that is driven by microglial activation within the VTA. Reactive microglia release BDNF that precipitates a shift in E_{GABA} within VTA GABAergic inhibitory interneurons leading to dysregulation of dopaminergic circuitry.

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Audit of clinical adherence to National Health Medical Research Council (NHMRC) venous thromboprophylaxis (VTE) guidelines in acutely ill older medical inpatients.

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Introduction: Clinical adherence to national and local VTE thromboprophylaxis guidelines is reported to be 40-60% for medical inpatients. Adherence to and applicability of these guidelines to older frail and robust medical inpatients is unknown.
Aims: Amongst frail and robust older medical inpatients, to evaluate the clinical adherence to NHMRC thromboprophylaxis guidelines, assess clinical prescribing behaviour and identify patient groups not described in the guidelines.
Methods: In this prospective cohort study, medical patients aged ≥65 years were recruited from the Emergency Department of Royal North Shore Hospital. The Reported Edmonton Frailty Scale was used to assess frailty. A predetermined protocol was used to assess adherence to the NHMRC guidelines for thromboprophylaxis and cases that did not fit this protocol were classified using expert consensus.
Results: To date, 90 patients with complete data were analysed: 46.7% female, median (range) age 79.5 (65-99). Concordance with the NHMRC guidelines was observed for all 59 participants who received VTE prophylaxis but for only three of the 31 participants who did not receive prophylaxis. This equates to 68.9% overall adherence. There was no correlation between non-adherence and frailty. Frail patients were not significantly more likely to receive heparin or enoxaparin dose reductions than robust patients (frail 34.5%, robust 25.9%, $p>0.18$). Eleven patients had pharmacologic dose reduction but only one was clearly appropriate due to renal impairment. Of those who received thromboprophylaxis, 55/59 patients received prophylaxis though their whole inpatient stay. There was variable prescribing for drug and dosage observed for groups not explicitly covered by the guidelines (n=4), such as palliative patients and those undergoing minor surgical procedures such as angiograms or pacemaker implantation.
Discussion: There is a need for more evidence to inform clearer thromboprophylaxis guidelines to guide clinical prescribing for older medical inpatients, particularly for those receiving palliative care and minor procedures.

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Ongoing poor management of medicines in the older-aged living independently in a rental retirement village

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Introduction. Previously, we have suggested that the older-aged living in a low socioeconomic, rental retirement village have a low adherence to medicines and a poor understanding of their illnesses (Doggrell & Kairuz, 2012). However, as the sample size was relatively small for the rental retirement villages it was not clear whether this is a consistent finding.
Aims. The aim was to determine the ongoing management of medicines by the older-aged living independently in a rental retirement village.
Methods. We returned to the rental retirement villages after one and two years, and reassessed the management of medicines by the older-aged living in the village, using semi-structured interviews.
Results. Although similar numbers participated in the study in 2011 (25 participants from 60 units), 2012 (n = 25) and 2013 (n = 23), the cohort changed. Thus, only 9 of the older-aged interviewed in 2011 were re-interviewed 2012, and similarly, only 9 interviewed in 2012 were re-interviewed in 2013. Nevertheless, the findings over the 3 years were very similar. The participants at the rental retirement village had a mean age ~75 years, each time we interviewed, and less than 50% were adherent at the time of the study and unlikely to have problems in the next 6-12 months. Only about 50% of the participants had a good knowledge of the illnesses, which they were being prescribed medicines for. Participants were taking about 7 medicines each, and cardiovascular/antithrombotic drugs were the most common medicines prescribed in the village, followed by psychotropic and gastrointestinal medicines.
Discussion. The management of medicines by the older-aged living in a low socioeconomic rental retirement villages is poor, and this finding is ongoing and consistent. This supports the need for extra assistance and resources for the older-aged living to manage their medicines in rental retirement villages.

Doggrell SA, Kairuz T. (2012) J Pharmac Pract Res 42:208-12.

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Metformin pharmacokinetics in haemodialysis

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Introduction. Metformin is eliminated largely if not entirely by renal excretion. Currently, it is contraindicated in dialysed patients because there is no data on PK during hemodialysis (HD) at therapeutic doses. Furthermore, excessive retention of metformin could lead to lactic acidosis, a major, although rare, adverse effect of the drug.
Aim. To investigate the PK and safety of metformin in patients on HD.
Methods. Two patients were dosed with 500 mg of metformin after each dialysis session (3 x 500 mg per week) for 4 weeks. Biochemical parameters were measured weekly. Blood was collected 4, 24 and 48 hours post dose after Week 2. HD CL was calculated on 3 occasions by collecting blood entering and exiting the dialyser at various time points for the entire dialysis session (Weeks 2-4). Metformin concentrations were measured by HPLC.
Results. Metformin concentrations did not exceed 5 mg/L and were generally constant between 4 and 48 hours. The dialyser CL of metformin from plasma was very high (191 ± 20 and 183 ± 11 mL/min for patient 1 and 2, respectively) and approached the plasma flow rate (approximately 200 mL/min). The CL of metformin from red blood cells was approximately 80% lower than CL from plasma. The patients tolerated metformin well with no adverse effects. Plasma lactate concentrations remained within the normal range (up to 2.2 mmol/L) and did not increase during dosage with metformin. The anion gap also did not increase but was slightly higher than the normal range (10 to 18 mmol/L) throughout.
Discussion. Metformin is readily dialyzable from plasma. The lower dialyser clearance from red blood cells is due to slow equilibration of metformin between red blood cells and plasma. Further studies are in progress.

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Utilisation and outcomes of pharmacological venous thromboembolism (VTE) prophylaxis in older frail and robust medical inpatients.

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Introduction. Older adults, particularly the frail have multiple co-morbidities and are under-represented in pharmacological clinical trials. The safety and efficacy of VTE prophylaxis is unknown in this population.
Aims. To evaluate the utilisation of pharmacological VTE prophylaxis in frail and robust older medical inpatients and investigate the proportion that would have been excluded from previous clinical trials. To describe the prevalence of haemorrhage and VTE in this population.
Methods. In this prospective, cohort study, medical patients (≥65years) were recruited from the Emergency Department of Royal North Shore Hospital. The Reported Edmonton Frailty Scale, IMPROVE bleeding risk assessment and Padua prediction score were utilised to assess frailty, bleeding risk and VTE risk respectively. Use of VTE prophylaxis and adverse events in hospital were obtained from medical notes and participant questionnaires.
Results. To date, 94 patients have been recruited, 46.8% female, median (range) age 79.5 (65-99) and 44.7% frail. Frail patients had more co-morbidities than robust (3.2±1.6 frail, 2.4±1.6 robust, $p<0.05$); higher risk of bleeding (31.0% frail, 11.5% robust, $p<0.05$) and higher risk of VTE (65.9% frail, 24.0% robust, $p<0.05$). Frail patients were not significantly more likely to receive VTE prophylaxis (73.8% frail, 55.8% robust, $p=0.07$). Of the patients on prophylaxis, 68.3% to 88.3% would have been excluded from past clinical trials. Minor bleeding occurred in two patients who both received VTE prophylaxis, one frail and one robust. One VTE event occurred in this study in a frail participant who did not receive VTE prophylaxis.
Discussion. Frail older medical inpatients have higher risk scores for VTE and bleeding than the robust. The majority of older medical inpatients would not have been represented in previous clinical trials for VTE prophylaxis, as such further research is warranted to explore the safety and efficacy of VTE prophylaxis in older adults.

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Barriers to care in gout: A qualitative study of patients' and prescribers' understanding and management

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Introduction. Gout is a painful inflammatory arthritis characterised by excessive uric acid in joints. Its prevalence is increasing worldwide. Despite good pathophysiological understanding of gout, current management practices are suboptimal. No qualitative research examining barriers to effective care in gout has been conducted in Australia.
Aims. To investigate general practitioners' (GPs) and patients' understanding and management of gout, and to identify barriers to optimal gout management practices in a local setting.
Methods. A snowball recruitment strategy was used, with GPs and eligible patients known to the research team invited to participate. Semi-structured interviews were conducted with GPs (n=15) and patients (n=22), focusing on experiences with gout, medication usage, lifestyle interventions and gout education. Interviews were transcribed verbatim and analysed by two independent reviewers to identify themes.
Results. Management of gout by GPs was often inconsistent with Therapeutic Guidelines, particularly management related to prophylaxis, up-titration and patient education. The main barriers to optimal management by doctors were identified to be poor awareness of guidelines, inadequate education, cultural factors and poor understanding of risk factors. There appeared to be strong disparities between GP and patient beliefs, especially regarding the impact of diet. Whilst the majority of patients only had a basic understanding of gout, they were interested in learning more. Most patients took allopurinol, but barriers to adherence were identified, including cost, lifestyle and comorbidities.
Discussion. Insufficient educational resources, poor patient adherence and non-concordance with guidelines were prominent factors hindering advancements in care. Overall, both patients and GPs lack a holistic understanding of gout and its management due to inadequate education and resources. Hence an intervention that targets these areas is necessary to ensure better management of gout by GPs, and for all patients.

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Factors associated with a high anticholinergic burden in elderly Australians with and without dementia

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Introduction. Elderly patients taking anticholinergic drugs have been shown to have an increased risk for cognitive decline and dementia and it is recommended that these drugs be prescribed cautiously (Carriere et al, 2009).
Aims. To determine the predictors of anticholinergic medication use in community-dwelling older primary care Australian patients with and without dementia. This will help identify patients most at risk.
Methods. The cognitive status of patients aged 75 years or older (n=1044) was determined using a subsection of the revised Cambridge Examination for Mental Disorders of the Elderly (CAMCOG-R; Roth et al, 1986). A CAMCOG-R score of 79 or less was used as a relatively sensitive indicator of dementia. Patient demographics and lists of their medications were collected. Anticholinergic load was determined using the Anticholinergic Drug Scale (Carnahan et al, 2006). Quality of life and depression were determined using an Australian version of the WHOQOL-BREF (Hawthorne, et al 2006) and a 15 item scale (Sheikh et al, 1986), respectively.
Results. The dementia group had a significantly higher anticholinergic load (1.5 *vs* 0.8; $P = 0.002$). Multivariate analysis identified several patient factors that were associated with a higher anticholinergic burden including polypharmacy (i.e. taking five or more medications) ($P < 0.001$), increasing age ($P = 0.018$), CAMCOG-R dementia ($P = 0.003$), depression ($P = 0.003$) and lower physical quality of life ($P < 0.001$).
Discussion. There is considerable scope for the improvement of prescribing practices in the elderly.

Carnahan RM et al (2006) J Clin Pharmacol 46:1481-1486
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Declining intra-lymphocyte concentrations of mycophenolic acid correlate with the incidence of graft rejection in renal transplant recipients: preliminary results of a prospective study

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Introduction: Mycophenolic acid (MPA), a key immunosuppressant used to prevent rejection following solid organ transplantation, exerts antiproliferative effects on lymphocytes. Routine therapeutic drug monitoring (TDM) of MPA concentrations in plasma is currently recommended to individualise MPA doses, but little is known regarding MPA concentrations at the site of action, within lymphocytes. Intra-lymphocyte MPA concentrations might be better predictors of graft rejection compared to plasma concentrations.
Aims: This study investigated the relationships between intra-lymphocyte and plasma MPA concentrations, and their association to graft rejection in renal transplant recipients.
Methods: Thirty-two renal transplant recipients receiving MPA were included and rejection was determined histologically from routine biopsies performed within 2 weeks post-transplantation. Lymphocytes were isolated from blood (9 mL) and intra-lymphocyte MPA concentrations were quantified using a validated LC-MS/MS method. Corresponding trough MPA plasma concentrations were collected from TDM records.
Results: The association between intra-lymphocyte MPA concentrations and trough MPA plasma concentrations was intra-lymphocyte MPA = 0.33 x plasma MPA + 0.27, r_s^2 = 0.34, P = 0.06. In 7 patients experiencing rejection (22%), intra-lymphocyte MPA concentrations were lower compared to those who did not reject, with median ± SD of 0.38 ± 0.48 and 1.1 ± 1.0 ng/10^7 cells (P = 0.05), respectively. There was no significant relationship between trough MPA plasma concentrations and graft rejection (P = 0.37).
Discussion: These observations suggest that, despite TDM to minimise inter-individual pharmacokinetic variability in systemic MPA concentrations, intra-lymphocyte MPA concentrations may represent an additional tool to further improve individualisation of MPA dose after transplantation with the purpose of either preventing graft rejection or minimising drug toxicities.

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Response to antiplatelet drugs in frail and non-frail older inpatients with atrial fibrillation

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Introduction. The utilisation of antiplatelet therapy in treatment for cardiovascular diseases is increasing, especially in older people. In frail older people it is unclear whether response to antiplatelet therapies is altered.
Aims. To study the platelet function of older inpatients with atrial fibrillation (AF) taking antiplatelet drugs and explore differences between frail and non-frail patients.
Methods. We recruited inpatients with AF aged ≥65years (mean 86±7) from Royal North Shore Hospital, Sydney. Frailty was determined using the Reported Edmonton Frail Scale. Platelet aggregation studies were performed using Whole Blood Impedance Aggregometry (Multiplate). The platelet agonists arachidonic acid and adenosine diphosphate were used to evaluate the response to aspirin and clopidogrel respectively. Cut-off for response to aspirin is an area under the curve (AUC) < 40 units (U). The target AUC in response to clopidogrel is comprised between 20U-42U.
Results. Amongst participants taking aspirin, mean±SD AUC was 15±13U (n=33) overall and did not differ with frailty (18±15U frail, n=20; 11±8U non-frail, n=13; p=0.1); 2 had AUC>40U (both frail). Amongst participants taking clopidogrel AUC was 31±15U overall (n=14), 30±16U in frail (n=8) and 33±15U in non-frail (n=6), p=0.8; 4 had AUC<20U and 3 had AUC>42U. Correlation of frailty and arachidonic acid-induced platelet aggregation in participants taking aspirin showed an increased variability in response to aspirin with increased frailty score.
Discussion. Nearly all participants responded to aspirin and half were outside the therapeutic range for clopidogrel. Platelet response did not differ between frail and non-frail older participants, although there was a trend towards increased variability in response to aspirin with increased frailty score.
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Capodanno D et al (2010) Journal of the American College of Cardiology 56(21):1683-1692

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Clinical Review and Documentation of Rationale for Potentially Inappropriate Medication Prescribing in Older Hospital In-Patients

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Introduction: While older persons are particularly vulnerable to polypharmacy, adverse drug reactions, and may benefit from deprescribing, 'potentially' inappropriate medications (PIMs) may warrant continuation when clinical judgement is exerted on a tailored case-by-case basis.
Aim: (1) Using the STOPP (Screening Tool of Older Persons' Potentially Inappropriate Prescriptions) criteria (Gallagher et al 2008), to investigate prevalence of PIMs at both admission and discharge, and (2) to explore the ongoing prescription of such PIMs had been reviewed and a clinical decision-making process for their continued use had (i) been documented in the hospital medical notes, and (ii) communicated to the General Practitioner (GP), who provide longterm community-based care.
Methods: 100 patients aged ≥65, admitted to our hospital January 2013, were randomly selected (computer-generated) for retrospective chart review. Hardcopy and electronic medical records and discharge letters (sent to GPs) were reviewed.
Results: In total, 80 PIMs, in 35 patients, were identified at admission, and 101 PIMs, in 47 patients, at discharge. The commonest PIM recorded was prolonged proton-pump inhibitor use (n=15 at admission, n=22 at discharge) (Table). Amongst the 101 PIMs identified at discharge, 82% (n=83) had a clinical reason for continued prescription documented. For 71% (59/83) of these, this rationale was documented in the GP discharge letter.
Discussion: While PIMs were common, the medication had been clinically reviewed in many cases, and a clinical reason for continuation documented. However, such decision-making was less optimally communicated to GPs. Prescribing for older patients requires an individually-tailored approach, and understanding of a complex interplay of comorbidities and therapeutic goals. Documentation and communication of reasoning for continued PIM use are important in ensuring best patient management. [Gallagher P, O'Mahony D (2008) Age Ageing 37:673–679]

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Patterns of Deprescribing Amongst Geriatricians: The Influence of Cognition, Dependency and Pill Burden

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Introduction. Deprescribing habits amongst physicians managing older, frailer, cognitively-impaired patients have not been well-investigated.
Aims. To analyse deprescribing habits amongst geriatricians.
Methods. Australian/New Zealand geriatricians (N=930) were presented with a series of case vignettes, including a list of patient medications, describing a similar patient with progressively more cognitive impairment and dependency (with ischaemic heart disease, hypertension, and constipation), and asked which medications they would change, and why.
Results. Amongst 134 respondents (14.4% response rate), 52.6% were male, 48.9% aged 35-50, 74.8% Caucasian, 84.1% specialists (15.9% advanced trainees [ATs]). With increasing dependency and cognitive impairment described, physicians were more likely to stop donepezil, aspirin, atorvastatin, ramipril, and amlodipine, or either antihypertensive (all $p<0.001$ for trend). On multivariate analysis, males (OR1.4, $p=0.05$) and ATs (OR 2.3, $p<0.001$) were more likely to stop antihypertensives, and males (OR 1.6, $p=0.05$) and older respondents ($p<0.001$) were more likely to discontinue senna. The commonest reason cited for discontinuing donepezil (49.6% of deprescriptions), aspirin (93.6%), atorvastatin (72.8%), ramipril (94.1%), and amlodipine (40.8%) was 'severity of dementia', with pill burden the next commonest cited reason, except in the case of paracetamol, where pill burden was the commonest reason for deprescribing (at 98.3%).
Discussion. On analyses of case-based scenarios, geriatricians are more likely to deprescribe multiple medications in the setting of advancing dependency and cognitive impairment, and report severity of dementia and pill burden as the main influencing factors. Physician characteristics are also associated with deprescribing habits. Further exploration of factors influencing deprescribing patterns, and whether these are associated with patient outcomes, is needed.

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Rates of Polypharmacy at Admission and Discharge: A Retrospective Hospital-Based Study

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Introduction. Polypharmacy is common, especially amongst older patients. Hospitalisation is associated with both medication initiation and cessation, but potentially affords an opportunity to rationalise medications.
Aims. To compare total numbers of medications at admission and discharge amongst older hospitalised patients.
Methods. Numbers of medications (regular, regular + PRN), at admission and discharge, were retrospectively calculated for all patients ≥65 years old admitted to our tertiary university hospital, January 2013.

Results. 306 patients were eligible for inclusion. Unknown medications led to exclusion of 111 from admission tallies, 53 from discharge calculations. At admission, the median number of regular medications was 7 (IQR 4-10), and of regular and/or PRN medications was 8 (IQR 5-11). 194 (63%) were on ≥5 regular medications, 68 (22%) on ≥10; 205 (67%) were on ≥5 regular and/or PRN medications, and 80 (26%) on ≥10. At discharge, the median number of regular medications was 5 (IQR 1-9), and of regular and/or PRN medications was 5.5 (IQR1-10). 144 (47%) were on ≥5 regular medications, 54 (18%) on ≥10; 153 (50%) were on ≥5 regular and/or PRN medications at discharge, and 68 (22%) on ≥10. The median difference in individual's total number of regular medications at admission and discharge was 0.

Discussion. Polypharmacy was common. Although hospitalisation offers a chance to rationalise medications, there was no change in the number of medications between admission and discharge in the patient population studied. Policies and structures to further promote reduction of unnecessary polypharmacy at opportunistic medical encounters are needed.

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Characterisation of the UDP-glucuronosyltransferase (UGT) enzyme inhibition selectivity of dapagliflozin: implications for drug-drug interactions.

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Introduction. Dapagliflozin (DPF) is a novel anti-diabetic drug that works via inhibition of renal sodium-glucose co-transporters. DPF is routinely used in combination with other anti-diabetic drugs (typically metformin) for the treatment of type-2 diabetes. DPF is primarily cleared via glucuronidation, a reaction that is catalysed by the enzyme superfamily UDP-glucuronosyltransferase (UGT). Despite the role of UGT in DPF clearance, the capacity of DPF to perpetrate metabolic drug interactions (DDIs) by inhibiting UGT is currently unknown. Given that DPF is frequently administered in combination with other anti-diabetic drugs to patients with multiple co-morbidities also requiring pharmacotherapy, the potential of DPF to perpetrate metabolic DDIs by inhibiting UGT requires urgent elucidation.
Aims. To assess the capacity of DPF to inhibit enzymes of the UGT1A and 2B sub-families, and where observed to characterise the mechanism and potency of inhibition.
Methods. The capacity of DPF (1, 10 and 100 μmol/L) to inhibit 4-methylumbelliferone (4MU) glucuronidation by recombinant UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B10, 2B15 and 2B17, and to inhibit lamotrigine glucuronidation by UGT1A4 and codeine glucuronidation by UGT2B4 was assessed using validated incubation conditions. Metabolite formation was quantified by HPLC with UV detection. Subsequent experiments were performed to characterise the potency and mechanism of DPF inhibition of human liver microsomal UGT1A9, using the selective substrate propofol, in the presence and absence of albumin (BSA; 1%).
Results. DPF caused modest inhibition of UGT2B enzymes (<40% at 100 μmol/L), but did inhibit multiple UGT1A enzymes; IC_{50} for UGT 1A1, 1A8, 1A9, and 1A10 were 66, 75, 39, and 55 μmol/L, respectively. DPF inhibited microsomal PRO glucuronidation in a non-competitive manner with an unbound K_i of 410 μmol/L, the presence of BSA (1%) in incubations resulted in an 6-fold reduction in K_i (to 67 μmol/L) but did not alter the mechanism of inhibition.
Discussion. DPF inhibited multiple UGT1A enzymes to a substantial extent. Inhibition was greatest with UGT1A9, which is a key enzyme in the metabolism of many drugs. As such further consideration of the clinical relevance of this inhibition is warranted.

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In vivo evaluation of felodipine as an inhibitor of cytochrome P450 enzymes and P-glycoprotein

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Introduction. Felodipine is an antihypertensive drug with low oral bioavailability that is sensitive to CYP3A and P-gp inhibition. *In vitro* studies indicate that inhibition of CYP3A4 by felodipine may also be a clinically important source of pharmacokinetic drug-drug interactions (PK-DDIs).
Aims. To evaluate felodipine as an *in vivo* inhibitor of drug metabolising CYP enzymes and P-gp.
Methods. Felodipine extended-release 10 mg was administered daily to 6 healthy subjects for 7 days (days 1 – 7). Subjects were administered a modified Inje cocktail comprising the selective probe substrates caffeine 100 mg (CYP1A2), losartan 25 mg (CYP2C9), omeprazole 20 mg (CYP2C19), dextromethorphan 30 mg (CYP2D6), midazolam 2 mg (CYP3A) and digoxin 250 µg (P-gp) on day 0 (prior to felodipine exposure) and day 7 (after felodipine exposure). Plasma samples were collected over 24 hours and drug concentrations measured by UPLC-MS/MS.
Results. The geometric means of the area under the plasma concentration-time curve ratios (probe AUC after felodipine exposure / probe AUC prior to felodipine exposure) and 95% confidence intervals for each probe were: caffeine 0.91 (0.64-1.30); losartan 1.05 (0.95-1.15); omeprazole 1.17 (0.78-1.76); dextromethorphan 1.46 (1.00-2.12); midazolam 1.23 (0.99-1.52) and digoxin 1.01 (0.89-1.15).
Discussion. Felodipine is a weak *in vivo* inhibitor of CYP3A and CYP2D6 but is unlikely to act as a clinically significant perpetrator of PK-DDIs.

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Emerging definition of deprescribing: implications for research and practice in older adults

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Introduction. The term ‘deprescribing’ first appeared in the English health literature on medication management in older people in 2003 with an exponential increase in use since then. Deprescribing however, has not been systematically defined and variations currently exist.
Aims. To conduct a systematic review of the definition of ‘deprescribing’.
Methods. Systematic literature searches were performed (earliest records to Feb 2014) in MEDLINE, EMBASE, CINAHL, Informit, Scopus and Google Scholar. The terms deprescrib* or de-prescrib* were employed as a keyword search in all fields. Articles were included if they were medication related and used the term in the title, abstract or body of the article. Two researchers independently reviewed each article for inclusion and then extracted and identified characteristics of the definition where available.
Results. Following removal of duplicates, 231 articles, conference abstracts and non-peer reviewed items (e.g. newsletters, blogs) were retrieved. Eighty-nine fulfilled the inclusion criteria, 37 of which included a definition. Eight characteristics of the definitions were identified: use of the term stop/withdraw/cease/discontinue (35 articles), aspect of prescribing included e.g. long term therapy/inappropriate medications (18), use of the term ‘process’ or ‘structured’ (13), withdrawal is planned/supervised/judicious (11), involving multiple steps (7), includes dose reduction/substitution (7), desired goals/outcomes described (5) and involves tapering (4).
Discussion. Our findings suggest there is lack of consensus on the definition of deprescribing. A consistent definition is necessary for future research into deprescribing (e.g. determining outcomes) and for clinical practice as it will inform guideline development and implementation. We propose the following definition: Deprescribing is the process of cessation, dose reduction or substitution of an inappropriate medication, which is supervised by a clinician with the goal of managing polypharmacy and improving outcomes.

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Patient autonomy in deprescibing: a qualitative study of the views, attitudes and beliefs of older adults and non-paid carers of older adults

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Introduction. The term deprescribing describes the complex process that is required for the safe and effective cessation of medications likely to cause more harm (maleficence) than benefit (beneficence). To conduct deprescribing ethically, there must also be respect for patient autonomy. However, little is known about how older adults and non-paid carers think and feel about deprescribing.
Aims. To explore attitudes, beliefs and views towards deprescribing in older adults and non-paid carers of older adults.
Methods. People were eligible to participate in focus groups if they or the person that they cared for were aged more than 65 years and taking at least one long term prescription medication. The focus groups were audiotaped and then transcribed in full. Results were analysed via line by line coding and grouped according to the themes identified in a systematic review of patient barriers and enablers of deprescribing (which included participants of all ages).
Results. Four focus groups were conducted: two with older adults and two with non-paid carers. The older adults (n=14) were 79.3 years old (±5.3) and were taking 3.8 (±2.5) regular medications. The non-paid carers (n=14) were caring for older adults aged 86.2 (±9.1) taking 5.6 (±4.5) regular medications. All five previously published themes arose in all four focus groups: appropriateness of cessation, process of withdrawal, influences, fear related to cessation and dislike of medications. Overall, participants initially reported dislike of medications and wanting to take as few as possible. However, when the discussion moved to actual cessation there was strong resistance expressed with the main verbalisation of 'why?'
Discussion. This study highlights that the initial discussion between the health care professional and the older patient/carer should revolve around why deprescribing is appropriate. Knowledge of these attitudes may empower medical practitioners to negotiate patient preferences while still respecting autonomy.

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Assay for Measurement of Etoposide (ETOP) and Selected Anti-Retrovirals (ART) in Dried Blood Spots (DBS)

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Introduction. DBS is an accepted method for sample collection in genetic, viral epidemiology, and pre-clinical animal studies. DBS use in pharmacokinetic (PK) studies is a more recent use of this approach and has not been used extensively in clinical cancer research.
Aims. To develop a DBS assay to quantify ETOP and ART to support a clinical study in a challenging environment.
Methods. Standard samples were prepared using spiked whole blood, which was then spotted onto Whatman 903 paper (GE Healthcare), filling the pre-printed ring. These were air dried for 2-3 hours, then placed in desiccator to complete dryness. A 6 mm punch was taken, rehydrated in water, precipitated with acetonitrile (ACN), transferred, dried, then dissolved in mobile phase (ACN:ammonium acetate). Using HPLC MS/MS (API4000, ABI Biosystems) with a Inertsil ODS-3 150X4.6 mm 5 micron column (GL Sciences) Q1/Q3 were determined for ETOP, lamivudine (LAM), lopinavir (LOP), indinavir (IND), zidovudine (ZID), nevirapine (NEV), stavudine (STAV), efavirenz (EFA) and tenofovir (TEN), using amprenavir (AMP) and teniposide (TENIP) as internal standard.
Results. All compounds could be quantified in a single HPLC run with lower limits of quantification as follows: 1 ng/mL – IND, LAM, LOP; 3 ng/mL – ZID; 10 ng/mL – ETOP, STAV, NEV; and 100 ng/mL – EFA, TEN. All compounds could be quantitated with a haematocrit (HCT) of 45% within acceptable error (<20%). Interestingly, with a HCT 30%, only ETOP and IND remained acceptable, and with HCT 60% only STAV remained acceptable. Filter paper developed for specifically microsampling (DMPKA,B and C, GE Healthcare) did not perform as well as Whatman 903 paper.
Discussion. An assay to quantify ETOP and ART was developed using DBS as a collection method. Use of DBS facilitated sample collection for a PK study in a challenging research environment (rural Western Kenya), allowing quantitation of ETOP and detection of ART. Further work into sample stability and the effect of HCT on sample quantitation needs to be explored.

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Oxycodone and other analgesics prescribing on discharge from Christchurch Hospital

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Introduction. There is a worldwide rise in prescription opioid use with oxycodone use reaching epidemic proportions in the USA[1]. A similar trend has been reported in New Zealand and Australia[2]. Concerns about the contribution of oxycodone prescribing at hospital discharge to the rising oxycodone use in the community have been raised. Establishing which analgesics are being prescribed on discharge from Hospital could contribute to understanding local factors affecting opioid use.
Aims. To describe the analgesics prescribed on discharge from Christchurch Hospital.
Methods. A retrospective cohort study of patients discharged from Christchurch Hospital was undertaken. The medical records of consecutive patients discharged over ten days in May 2014 were reviewed. Data collected included analgesic(s), dose, frequency prescribed and the documented duration/quantity of supply. The data were analysed using descriptive statistics
Results. During the study period the Emergency Department (ED) discharged 1473 patients, 250 of whom (17%) were prescribed one or more analgesics. None of these received a discharge prescription for oxycodone (95% CI 0-0.25%). The Orthopaedic Department discharged 533 patients, 295 of which (55%) were prescribed one or more analgesics. Seven of these received a discharge prescription for oxycodone 95% CI x-y%. The other analgesics prescribed were paracetamol (454), tramadol or codeine (355), non-steroidal anti-inflammatory drugs (244), morphine (7).
Discussion. Currently few patients are being discharged from ED with prescriptions for oxycodone. The Orthopaedic Department discharged approximately a patient a day on oxycodone and this should be explored further.
1.Manchikanti L et al (2010) Therapeutic use, abuse, and nonmedical use of opioids: A ten-year perspective. Pain Physician 13:401-435.2 Royal Australian College of Physicians. (April 2009) Prescription Opioid Policy: Improving the management of chronic non-malignant pain and prevention of problems associated with prescription opioid use.

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Anti-*Helicobacter* activities of Kakadu Plum and colloidal silver

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Background. The Kakadu Plum (KP) is wild-harvested in Northern Australia (NT.WA) and a revered Aboriginal remedy for many ailments. The fruit is rich in ascorbate, other anti-oxidants and contains potent anti-bacterials (1). Colloidal metallic silver (CMS) preparations have shown anti-*Helicobacter* activity (2).
Procedures. Gastric *Helicobacter pylori* (HP) infection was assessed with a MetAtron diagnostic monitor (IPP). Volunteers attending a naturopathic clinic took oral treatments with either dried KP extract (1 gm b.i.d.) or CMS (≤0.3 mg Ag b.i.d.) as Lunasol™ for five days only. Probability indices for HP infection were determined on days zero, 7, 14 and 28 to assess a) efficacy and b) duration of response post-treatment. [Protocols for clinical studies were approved by an appropriate Human Ethics Committee.]
Results: KP extract effectively reduced gastric HP burden >85% within one week. The KP was particularly valuable as second-line treatment in a few subjects (3/17) showing lesser responses (≤40%) to CMS alone.
Conclusion: At present, supply constraints and significant harvesting costs may limit the usefulness of KP as a phytotherapy to treat gastric inflammation/ulcers associated with HP. Nevertheless it is a valuable synergist for 'tandem therapy' with other traditional remedies for ulcer disease eg Bismuth, licorice (2).

1. Cock I, Mohanty S. (2011) Pharmacognosy J 3:72-79.
2. Whitehouse MW et al (2013) Proc ASCEPT Mtg Abstr

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The relationship between fractional urate clearance and renal transporter genotype in patients with gout.

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Introduction. The fractional clearance of urate (FCU) is the ratio of urate renal clearance and creatinine clearance. It has been proposed as a marker for altered urate renal handling and as a means of identifying urate 'under-excretors' (Indraratna et al). FCU is therefore expected to predict tubular urate transporter genotype in gout patients.
Aim. To explore the relationship between FCU and renal transporter genotype.
Methods. Plasma and urine concentrations of urate (P_u and U_u) and creatinine (P_{cr} and U_{cr}) from 116 gout patents were available for analysis. FCU was calculated using the formula; $U_u*P_{cr}/U_{Cr}*P_u$. Renal transporter variants associated with raised plasma urate concentrations included; URAT1 (rs3825018 G allele), OAT4 (rs17300741 A allele), GLUT9 (rs11942223 T allele), NPT1 (rs1183201 T allele), and, ABCG2 (rs2231142 G allele). The mean natural log of FCU (lnFCU) was compared across renal transporter genotypes and to the genetic risk score for hyperuricaemia.
Results. The mean lnFCU was significantly lower in carriers of the hyperuricaemia risk T allele for SLC2A9 compared to the CC genotype (1.39 vs 1.96, p=0.039). No differences were observed in mean lnFCU between carriers of the hyperuricaemia risk allele and the urate-lowering genotype for URAT1 (1.38 vs 1.47, p=0.19), OAT4 (1.37 vs 1.57, p=0.06), NPT1 (1.41 vs 1.38, p=0.79), and, ABCG2 (1.46 vs 1.35, p=0.16). No significant relationship was found between FCU and hyperuricaemia genetic risk score.
Discussion. FCU was found to predict SLC2A9 genotype, though there were only 2 individuals with the urate-lowering CC genotype. The result may therefore be due to statistical error. We found a trend towards reduced FCU in carriers of hyperuricaemia risk alleles for URAT1 and OAT4 but differences were not statistically significant. Overall, our results do not provide strong support for the use of FCU as a phenotypic marker of urate tubular handling in patients with gout.

Indraratna PL et al (2010) Arthritis Res Ther, 12:149-151.

Novel approach to optimising synergistic carbapenem plus aminoglycoside combinations to combat carbapenem-resistant *Acinetobacter baumannii*

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Introduction. The high rates of *A. baumannii* resistance highlight the urgent need for alternative treatment options such as rationally optimised combination dosage regimens.
Aim. To identify and rationally optimise β-lactam plus aminoglycoside combinations via novel mechanism-based modelling that synergistically kill and prevent resistance of carbapenem-resistant *A. baumannii*.
Methods. We studied combinations of ten β-lactams and three aminoglycosides against four *A. baumannii* strains, including two imipenem-intermediate (MIC_{IPM}: 4 mg/L) and one imipenem-resistant (MIC_{IPM}: 32 mg/L) clinical isolate, using high inoculum static concentration time-kill studies. We present the first application of mechanism-based modelling for bacterial killing and resistance with Monte Carlo simulations of human pharmacokinetics to rationally optimise combination dosage regimens for immune-compromised, critically-ill patients. Population pharmacodynamic modelling was performed in the S-ADAPT software and Monte Carlo simulations in Berkeley Madonna.
Results. All monotherapies achieved limited killing (≤ 2.3 log_{10}) of *A. baumannii* ATCC 19606 followed by extensive regrowth for aminoglycosides. Imipenem 8 mg/L plus tobramycin yielded synergistic killing (>5 log_{10}) and prevented regrowth against all four strains. Modelling demonstrated imipenem likely killed the aminoglycoside-resistant population and *vice versa* and aminoglycosides enhanced the target site penetration of imipenem. Against carbapenem-resistant *A. baumannii* (MIC_{IPM}: 32 mg/L), optimised combination regimens (imipenem 4 g/day as continuous infusion plus tobramycin 7 mg/kg every 24h) were predicted to achieve >5 log_{10} killing without regrowth in 98.2% of patients.

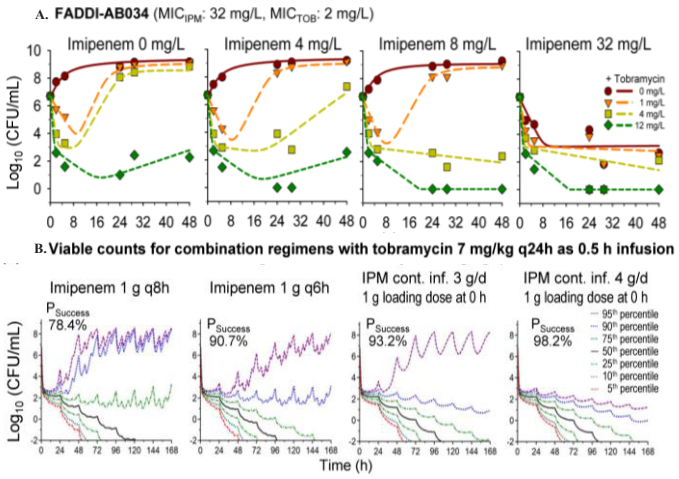

Figure: Observed and individually fitted viable count profiles from static time-kill studies (A) and Monte Carlo simulated viable counts for combination dosage regimens with 7 mg/kg tobramycin q24h in immune-compromised patients (B) against the imipenem-resistant *A. baumannii* isolate

Discussion. Bacterial killing by any β-lactam or aminoglycoside in monotherapy was limited against a high inoculum of wild-type *A. baumannii* ATCC 19606. Among all tested combinations, imipenem plus an aminoglycoside provided the most extensive killing without regrowth against high inocula of susceptible, carbapenem-intermediate and carbapenem-resistant strains. Mechanism-based modelling identified both subpopulation synergy and mechanistic synergy for imipenem plus aminoglycoside. Monte Carlo simulations predicted a 98.2% success rate for clinically relevant imipenem plus aminoglycoside combination dosage regimens against a carbapenem-resistant clinical *A. baumannii* isolate with an MIC of 32 mg/L. Imipenem plus aminoglycoside combination regimens are highly promising and warrant further evaluation.

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***In-vivo in-vitro* correlation (IVIVC) of topical salicylate esters**

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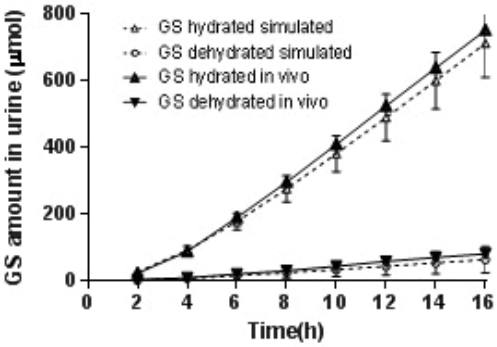
Introduction. In order to replace expensive, time-consuming and potentially harmful *in vivo* skin penetration trials with studies using excised skin, it is first necessary to demonstrate appropriate *in vitro-in vivo* correlation (IV-IVC).
Aims. Our goal was to examine the correlation between *in vitro* and *in vivo* skin penetration data and to assess the influence of the skin hydration state on *in vitro* topical delivery.
Methods. Salicylate esters were applied to human epidermal membranes in Franz diffusion cells for 24 hr., under either dehydrated or hydrated conditions. We then used a convolution approach to predict Wurster & Kramer's (1961) *in vivo* data from our *in vitro* data.



Results. Our convoluted data showed good agreement with previous *in vivo* results for glycol salicylate (GS) (Fig 1) and ethyl salicylate but not methyl salicylate, probably due to its vasodilatory effects *in vivo*. Salicylate penetration was strongly dependent on hydration state.
Discussion. Our results emphasise that excised human skin is an excellent model for *in vivo* conditions, provided the experimental conditions are replicated. The *in vitro* approach augmented with mathematical modelling to simulate urine and plasma levels of topically applied drugs saves time and effort; it can also help in accurate monitoring of therapeutic levels of drugs with narrow therapeutic windows.

Wurster, DE & Kramer, SF (1961) J Pharm Sci 50:288-293.

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Effect of Beta-endorphin fragments on interleukin-1beta release

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Introduction: Stimulation of opioid peptide-containing leukocytes by local factors results in release of beta-endorphin (BE 1-31) within the inflamed tissue. Released BE 1-31, although most well known for pain modulation, can also modulate immune cells through the modulation of pro-inflammatory cytokines such as interleukin1-beta (IL-1β). However, BE 1-31 is unstable in the inflamed environment and is transformed to several smaller fragments. It is possible that some of these BE 1-31 fragments may also play a role in the modulation of inflammatory cytokines such as IL-1β.
Aim: To investigate the modulatory effects of a selection of N-terminal BE 1-31 fragments on IL-1β release in differentiated THP-1 cells.
Method: Lipopolysaccharide (LPS) was applied to differentiated THP-1 cells, a cell line that displays a human tissue macrophage phenotype upon differentiation, to induce the production of IL-1β. BE 1-31 and selected fragments (BE 1-9, BE 1-11, BE 1-13, BE 1-17, BE 1-20) at concentrations of 10 pmol/L, 1 nmol/L, , and 0.1 µmol/L were incubated with differentiated THP-1 cells induced by LPS for 24 h to study their effect on IL-1β release. The modulatory effect on IL-1β release was also examined in the presence of naloxone (10 µmol/L), a non-selective antagonist of opioid receptors.
Results: A significant decrease in IL-1β release was observed for shorter fragments of BE 1-31 (i.e. BE 1-9, BE 1-11, BE 1-13) at 0.1 µmol/L. In contrast, at 0.1 µmol/L BE 1-17, BE 1-20, and BE 1-31 increased the release of IL-1β in differentiated THP-1 cells-induced by LPS. The modulatory effect of BE 1-31 fragments was not inhibited by naloxone characterising the effect as non-opioid activity for BE 1-31 fragments.
Discussion: BE 1-31 fragments can modulate the release of IL-1β in a concentration dependent manner. This may be potentially important, since BE 1-31 fragments are produced, *via* enzymatic degradation at different concentrations and at different times post-release of BE 1-31 into the inflammatory milieu. In conclusion, the examined fragments of BE 1-31 may modulate inflammatory processes differently as a result of enzymatic effects at the site of inflammation.

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Glucocorticoids induce skeletal muscle wasting in a mouse model of acute exacerbations of Chronic Obstructive Pulmonary Disease

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Introduction. Skeletal muscle wasting is a common comorbidity of Chronic Obstructive Pulmonary Disease (COPD), and is a strong predictor of mortality independent of declining lung function. In patients hospitalised for acute exacerbations of COPD (AECOPD) caused by respiratory infections, skeletal muscle wasting is worsened. These patients are usually given oral glucocorticoids, however there is evidence that systemic glucocorticoids can induce skeletal muscle wasting (McEvoy et al, 1997).
Aims. To investigate the effect of dexamethasone on skeletal muscle in a mouse model of AECOPD.
Methods. Balb/c mice were exposed to 3 cigarettes per day (CS) or air (sham) for 14 days (d). On d15, mice were intranasally inoculated with influenza virus (HKx31, $1x10^4$ plaque-forming units) or saline. Treatment with dexamethasone (Dex; 1mg/kg/d, ip) or saline occurred 1h prior to inoculation with flu, and for 4 d thereafter. Body weight and food intake were monitored daily. 5 d post infection, lung inflammation was assessed in bronchoalveolar lavage fluid (BALF) and by quantitative RT-PCR (qPCR) on lung tissue. Hind limb muscles were removed, weighed and gene expression changes were measured by qPCR.
Results. CS+flu mice had significantly more BALF inflammatory cells than all other groups (n=6-8 per group, $P<0.05$), and dex treatment did not change BALF inflammatory cell numbers. While CS+flu mice did not have significantly reduced bodyweight, dex treatment of CS+flu mice reduced body weight by 12% vs. CS alone (n=8 per group, $P<0.05$). Dex treatment of CS+flu mice significantly reduced gastrocnemius muscle weight by 9%, compared to CS mice ($P<0.05$).
Discussion. This model of AECOPD showed evidence of an exacerbation of lung inflammation. Treatment with dex did not reduce levels of lung inflammatory cells, however it did induce skeletal muscle wasting. These data could be used to elucidate mechanisms of glucocorticoid-induced wasting in patients hospitalised with AECOPD.

McEvoy CE, Niewoehner DE (1997) Chest 111: 732-743.

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Targeting oxidative stress reduces cigarette smoke-induced lung inflammation and wasting in mice

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Introduction. Oxidative stress and reactive oxygen species (ROS) have been implicated in chronic obstructive pulmonary disease (COPD) and the observed wasting associated with this debilitating disease. Therefore, targeting oxidative stress and ROS production may be a novel way to treat COPD and its comorbidities.
Aim. To determine whether inhibiting oxidative stress and ROS production reduces cigarette smoke (CS)-induced lung inflammation and wasting in mice.
Methods. Male Balb/C mice (7 weeks of age) were exposed to CS generated from 6 cigarettes/day, 5 days/week for 8 weeks. Sham mice were handled identically and exposed to room air. Mice were treated daily with apocynin (5mg/kg, 0.1% DMSO, i.p.) or vehicle (0.1% DMSO in PBS). Food intake was measured daily and body weights were recorded 3 times a week. On day 56, mice were culled and blood, bronchoalveolar lavage fluid (BALF), lungs, liver, kidneys, spleen, tibialis anterior, soleus, gastrocnemius, testicular and retroperitoneal white adipose tissue (WAT) were collected from the 4 groups of mice (n=8).
Results. CS exposure caused an increase in BALF total cells (911,405±67,231), macrophages (650,974±49,590) and neutrophils (256,397±25,457) compared to sham-exposed mice (248,406±24,472; 247,545±24616 and 860±506, respectively). However, CS-exposed mice treated with apocynin had markedly less total cells (653,052±46,205), macrophages (524,099±42,321) and neutrophils (128,391±8,991). Compared to sham-exposed mice, CS exposure caused a reduction in total body weight but mice treated with apocynin lost less weight. CS-exposed mice had a reduction in food intake which was unaffected by apocynin treatment. In addition, CS-exposed mice had increased lung mass and decreased tibialis anterior, soleus, testicular WAT and retroperitoneal WAT mass. Apocynin-treated CS-exposed mice lost less testicular WAT, retroperitoneal WAT and soleus muscle compared to vehicle-treated CS-exposed mice.
Discussion. These data indicate that targeting oxidative stress and ROS production with apocynin reduces CS-induced lung inflammation and wasting in mice and that this approach may be a novel means for treating COPD and its comorbidities.

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Biotransformed dynorphin A fragments attenuate lipopolysaccharide-stimulated activation of THP-1 cell-derived macrophages through inhibition of nuclear factor-kappa beta (NF-κB) translocation

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Introduction. Dynorphin A 1-17 (DYN A) is an endogenous opioid peptide that has been demonstrated previously to produce a variety of opioid and non-opioid fragments upon release in inflamed tissue. Prior studies have suggested that the nuclear factor-kappa beta (NF-κB) pro-inflammatory, transcription-mediated signalling pathway as one of the possible mechanisms involved in the immunomodulatory effects of opioids during inflammation.
Aims. To evaluate the effects of a selection of DYN A fragments on LPS-induced NF-κB nuclear translocation in the human THP-1 cell-derived macrophages.
Methods. Phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells were established as an *in vitro* model for macrophages in inflammation. The differentiated macrophages were stimulated with lipopolysaccharide (1 mg/L, 1 hour) followed by incubation with DYN A and a range of biotransformed DYN fragments discovered previously in inflamed tissue (DYN 1-6, 1-7, 1-9, 1-10, 1-11, 2-17, 6-12, 3-14, 7-17 and 8-17) (1 μmol/L and 10 nmol/L, for 1 hour). The nuclear localization of NF-κB p65 subunit in the treated macrophages was immunolabelled and nuclear translocation was assessed using the ImageXpress (Molecular Devices) high-content cellular imaging system.
Results. LPS markedly induced the translocation of NF-κB p65 subunits into the nuclei of the immunolabelled cells to that representing 89% of immunolabelled NF-κB, whilst minimal translocation was observed in unstimulated, THP-1 cells ($p < 0.05$). NF-κB p65 nuclear translocation was significantly attenuated following treatment with DYN A by 67.5 $\pm$ 7.0% at 10 nmol/L. All DYN A fragments reduced NF-κB translocation, with the greatest effect observed with DYN A (1-7), reducing translocation by 56.7 $\pm$ 7.0% at 10 nmol/L.
Discussion. These findings suggest that DYN A biotransformed fragments may be involved in the modulation of the cellular signal transduction pathway associated with inflammation by inhibiting the NF-κB signal and thereby limiting the exacerbation of the inflammatory response. Further investigation is required to elucidate the mechanisms involved in the inhibition of NF-κB by biotransformed DYN A fragments.

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How does early life viral-induced pneumococcal infection impact the adult mouse lung?

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Introduction. Severe respiratory viral infection in infancy has been linked to increased asthma risk in later life, long after pathogen is cleared. Bacterial carriage is also associated with wheezy episodes in young children. Influenza virus infection has been shown to facilitate pneumococcal disease and transmission in infant mice.
Aims. To assess whether early life viral and bacterial co-infection leads to persistent changes in lung function, structure and immunity into adulthood.
Methods. C57Bl/6 infant mice were infected intranasally at 5 days of age with *S. pneumonia* EF3030 (SP, $2X10^3$ CFU in saline, or saline vehicle) and at 12 days of age co-infected with influenza A virus (IAV, 20 PFU HKx31 in saline, or saline vehicle); 4 treatment groups in total. Outcomes were measured at 6 – 8 weeks of age (sodium pentobarbitone 0.2ml of 60mg/ml i.p.). Airway mechanics were assessed *in vivo* with SCIREQ Flexivent (ketamine/xylazine 10μg/200μg per g mouse i.p.) and *in vitro* with the lung slice technique in female mice. Whole lung immune profile was assessed using fluorescence-activated cell sorting, differential cell counts and H&E staining of lung sections in male mice.
Results. All groups gained weight similarly throughout the study, and displayed comparable baseline airway mechanics. Co-infected mice were hyper-responsive to methacholine challenge *in vivo*, but not *in vitro*. H&E stain revealed accumulation of immune cells in IAV mice only, that was reflective of inducible bronchus-associated lymphoid tissue (iBALT) formation, which are T and B cell aggregates that have been shown to be protective during IAV infection. In the bronchoalveolar lavage compartment, there were significantly more cells in SP mice, and an increase in lymphocytes in co-infected mice.
Discussion. Exposure to viral and/or bacterial infection in infancy had effects lasting into adulthood. The accumulation of iBALT-like immune structures was markedly prevented by transient colonisation with SP. Future studies will determine whether altered IAV-induced iBALT formation in co-infection models will prove to be beneficial or detrimental to development of an asthma-like phenotype following chronic allergen challenge.

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Extracts of Australian native stingless bee (*Tetragonula carbonaria*) cerumen modulate dermal fibroblast proliferation and myofibroblast differentiation *in vitro*

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Introduction. Cerumen is a resinous, plant-derived product of stingless bees. We previously identified extracts and fractions of Australian native stingless bee (*Tetragonula carbonaria*) cerumen with anti-oxidant and anti-inflammatory activities *in vitro*; however, their wound-healing potential are currently unknown.
Aims. To examine the effects of *T. carbonaria* cerumen extracts and active fractions on the proliferation of human fibroblasts obtained from normal dermis (NF) and chronic wounds (CWF), and on transforming growth factor (TGF)-β_1-mediated myofibroblast differentiation.
Methods. *T. carbonaria* cerumen collected from hives in South-East Queensland was partitioned into methanol and methanol-water extracts. Two active fractions (Fractions 1 and 9) were obtained from the methanol-water extract by preparative reversed-phase HPLC fractionation. Cultured NFs and CWFs were incubated in the absence or presence of cerumen extracts and fractions (0.3-20 µg/mL). MTT dye-reduction assays measured cellular proliferation over 120 h. The effects of extracts and fractions on myofibroblast differentiation were also investigated in TGF-β_1-stimulated NFs (10 ng/mL; 72 h), by examining α-smooth muscle actin (SMA) immunoreactivity.
Results. Increased proliferative activity was observed in NFs exposed to 5 µg/mL methanol extract (129.9±11.2%; 120 h), 5 µg/mL methanol-water extract (124.0±15.8%; 24 h), 1 µg/mL Fraction 1 (131.1±17.7%; 120 h) and 3 µg/mL Fraction 9 (214.6±26.4%; 120 h) ($P<0.05$). Proliferation was also enhanced in CWFs treated with 5 µg/mL methanol extract (134.6±10.0%; 120 h) and 3 µg/mL Fraction 9 (134.8±5.7%; 120 h) ($P<0.05$). Exposure of NFs to Fraction 9 (3 µg/mL; 72 h) inhibited TGF-β_1-mediated α-SMA expression and myofibroblast differentiation.
Discussion. Extracts and fractions of *T. carbonaria* cerumen possess proliferative and anti-scarring properties that may promote the healing of both acute and chronic wounds. Studies are underway to examine their effects on fibroblast migration; and to elucidate the active constituents of Fractions 1 and 9.

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Nanoparticles as potential novel mast cell modulating agents

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Introduction: Mast cells are well known for their pathophysiological role in allergic diseases such as allergic rhinitis. When activated, mast cells degranulate releasing pro-inflammatory mediators which produce allergic symptoms. A number of studies have shown mast cell modulation by nanoparticles (NPs). However, the use of acetylated-dextran nanoparticles (Ac-DEX NPs) in this setting has not been examined. Ac-DEX NPs offer a biologically compatible, tunable material that can be employed as a drug delivery vehicle whereby upon cellular uptake, drug payloads can be released in low pH environments such as that of mast cell granules.
Aims: To characterize the selective uptake, trafficking and actions of a range of Ac-DEX NPs in mast cells.
Methods: Fluorescently labelled Ac-DEX NPs were synthesised using established protocols and dynamic light scattering used to measure their size distribution. Immunofluorescence and confocal microscopy, alongside flow cytometry, was used to determine the binding, uptake trafficking behavior of Ac-DEX NPs in a range of cell types with a focus on the mast cell lines RBL and LAD2. Following nanoparticle treatment, β-hexosaminidase (β-Hex) release and resazurin-based assays were utilised to measure mast cell degranulation and assess cell viability respectively.
Results: Ac-DEX NPs of varying diameters were synthesised and were shown not to affect cell viability. Immunofluorescence and flow cytometric analysis using Ac-DEX NPs (average size 120 nm) demonstrated greater selectivity of interaction with mast cells than with a range other of relevant cells types. Higher resolution imaging using confocal microscopy with 'lysotracker' co-staining confirmed internalization and localization of NPs to acidic organelles. Ac-DEX NPs had no effect themselves on β-Hex release nor on IgE-dependent and IgE-independent degranulation in both RBL and LAD2 cell lines.
Conclusions: Ac-DEX NPs interact preferentially with mast cells and when internalized, localize to acidic organelles. This suggests that Ac-DEX NPs could be used to selectively deliver mast cell inhibitory compounds and act as novel anti-allergic therapies.

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Glucocorticoid gene regulation differs in airway epithelial cells compared to hepatocytes

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Introduction. Glucocorticoids are the most effective anti-inflammatory agents available to treat chronic inflammatory diseases, including asthma. However, severe and sometimes irreversible adverse effects (e.g. diabetes, peptic ulcer, osteoporosis, psychosis, glaucoma, Cushing's syndrome) present a major obstacle to effective and safe treatment. The generation of adverse effects has generally been attributed to induction of gene expression (in particular metabolic genes such as PDK4), whilst the anti-inflammatory effects are thought to be due to repression of pro-inflammatory transcription factors (e.g. NF-κB, AP-1). However, recent evidence suggests that in airway epithelium, induction of anti-inflammatory gene expression (e.g. the genes encoding MKP-1 and p57 Kip2) is an important anti-inflammatory mechanism (Clark & Belvisi 2012; Newton & Holden 2007).
Aims. In this study, we test the hypothesis that glucocorticoid-induction of gene expression differs in airway epithelial cells compared to hepatocytes.
Methods. Human bronchial epithelial cells (BEAS-2B) and hepatocellular carcinoma-derived epithelial cells (HepG2) were used in this study. Cells were serum-starved for 24 h – 48 h prior to stimulation with dexamethasone (Dex: 1 – 100 nM) after which total RNA was extracted and RNA expression was quantified by RT-PCR. Cy3- and Cy5-conjugated SmartFlare™ probes (Millipore) were used to detect RNA expression in live cells.
Results. Dex treatment of BEAS-2B cells induced a 19-fold upregulation of the gene encoding p57 Kip2, whereas this gene was undetectable in HepG2 cells. This difference was confirmed by SmartFlare™ live RNA detection. The gene encoding MKP-1 was expressed at a similar level in both cell types, but was induced to a greater extent by Dex in BEAS-2B cells. In contrast, the gene encoding PDK4 was induced to a greater extent in HepG2 cells.
Discussion. These results indicate that there is significant heterogeneity in glucocorticoid-induction of gene expression in cells derived from different tissue types. These findings have implications for optimisation strategies to improve the balance of efficacy and safety of glucocorticoid-like drugs.

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Relaxin and rosiglitazone exert differential inhibition of airway contraction to methacholine and endothelin-1

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Introduction. Relaxin (RLN) mediates anti-fibrotic actions in lung fibroblasts (Unemori et al., 1996) through multiple mechanisms including activation of the RXFP1 and PPARγ receptors and via generation of nitric oxide (Samuel et al., 2004; Chow et al., 2014). In the present study, we comparedbronchodilator effects of RLN, rosiglitazone (RGZ, a PPARγ agonist that relaxes airways) (Bourke et al, 2013) and isoprenaline (ISO, a β-adrenoceptor agonist).
Aims. To test whether pretreatment with RLN and RGZ inhibits the development of airway contraction.
Methods. Isolated mouse and guinea pig tracheal segments mounted in a wire myographwere contracted with KPSS or acetylcholine (30μM) to generate standard contractionsprior to treatment. Tissues were then preincubated with RLN (100nM), RGZ (10μM, 100μM), RLN/RGZ (100nM/10μM) orISO (10μM) prior to construction of full or partial concentration-response curves to methacholine (MCh, 10nM-100μM) or endothelin-1 (ET-1, 1-100nM).
Results. MCh-induced contractions in both species were significantly inhibited by RGZ, but not RLN, with reduced maximal responses but no loss of potency. The maximum MCh contraction (%ACh response in the same tissue) was 249±76% in untreated mouse trachea, and decreased to 57±18% and 17±7% in the presence of 10μM and 100μM RGZ respectively (n=4-6, P<0.05, 0.01). In contrast, ISO only reduced MCh potency in guinea pig trachea (pEC_{50} untreated 6.3±0.1, +ISO 5.5±0.2, n=3-4, P<0.05). All treatments reduced contraction to 100 nMET -1 (%KPSS: untreated 151±21%; +RLN 83±5%, +ISO 48±18%, n=3-4 mouse tracheal segments, P<0.05).
Discussion. Results from different species using different contractile agonists suggest that RLN and RGZ inhibitcontraction by alternative mechanisms to β-adrenoceptor agonists. Further investigation is required to define these mechanisms and thetherapeutic potential of RLN and RGZ toreduce the extent of airway narrowing in asthma.

Unemori E et al (1996) J Clin Invest 98: 2739-2745
Samuel CS et al (2004) Endocrinology 145: 4125-4133
Chow BS et al (2014) Kidney Int 86: 1-11
Bourke et al (2014) Am J Resp Cell MolBiol50:748-56

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Targeting ALX/FPR2 in models of bacterial and viral co-infection

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Introduction. COPD patients are susceptible to bacterial colonization and viral infections that drive acute exacerbations (AECOPD). The presence of both bacterial and viral pathogens is associated with higher levels of systemic inflammation, as determined by measuring Serum Amyloid A (SAA). SAA has been identified as a candid mediator of airway inflammation in COPD through its interaction with the GPCR, ALX/FPR2. In addition to interacting with pro-inflammatory SAA, ALX/FPR2 can interact with pro-resolving mediators such as Resolvin D1 (RvD1) to initiate resolution of inflammation.
Aims. To assess the effects of RvD1 compared to dexamethasone on airway inflammation and bacterial burden in a co-infection mouse model.
Methods. C57BL/6 mice were treated with *Streptococcus pneumonia* (Spn) (EF3030, 10^5 *CFU)* on day 1; followed by HKx31 influenza A virus (10^4 PFU) (IAV) on day 2. Mice were treated with dexamethasone (DEX, 0.5mg/kg, i.p.) or RvD1 (4μg/kg, i.p.) on day 4-6 with the control group receiving vehicle (0.5% ethanol in PBS). BALF inflammation and bacterial burden was measured through cell counts and CFU analysis.
Results. Mice inoculated with Spn alone efficiently cleared the bacteria by Day 7. In contrast, mice inoculated with both Spn and IAV displayed a 100-fold increase in pneumococcal load in the lungs. Increased pneumococcal load was associated with a decrease in neutrophilic inflammation at Day 2. By Day 7, there was a significant increase in inflammatory leukocytes that was predominately driven by IAV infection. Therapeutic administration of DEX resulted in a 2-fold increase in bacterial burden and neutrophilic inflammation. In contrast, RvD1 markedly suppressed inflammation and bacterial burden at Day7 relative to Vehicle treated mice.
Discussion. Glucocorticosteroids are routinely used to control inflammation in COPD and AECOPD. In our model, DEX failed to reduce bacterial burden and inflammation in the lungs, whereas RvD1 potently suppressed inflammation by over 50% and improved bacterial clearance relative to vehicle. Pro-resolving mediators that target ALX/FPR2 may offer a novel therapeutic strategy to combat co-infection in chronic lung diseases such as COPD.

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Morphine modulates IL-4- or breast cancer cell-induced pro-metastatic activation of macrophages

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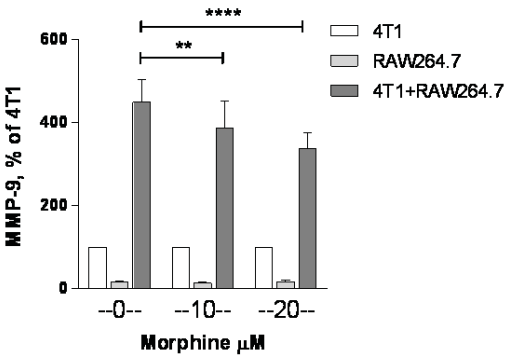
Introduction. Interactions between cancer cells and stromal cells in the tumour microenvironment play a key role in the control of invasiveness, metastasis and angiogenesis. Macrophages display a range of activation states in pathological contexts and alternatively activated (M2) macrophages can promote tumour aggressiveness. Opioids are able to modulate tumour growth and metastasis and are often administered to cancer patients.
Aims. We tested whether morphine can modulate the activation of RAW264.7 macrophages induced by (i) interleukin-4 (IL-4), the prototypical M2 polarization-inducing cytokine, or (ii) coculture with 4T1 breast cancer cells to induce a tumour-associated macrophage (TAM) phenotype *in vitro*.
Methods. The level of extra-cellular matrix (ECM) degrading enzymes, matrix metalloproteinase-9 (MMP-9) and -2 (MMP-2) was measured in cell-conditioned media using in-gel zymography. The expression of MMP-9 and M2 markers was measured at mRNA level using real time reverse transcriptase polymerase chain reaction (RT-PCR).
Results. We showed that IL-4 causes increased MMP-9 production and expression of the alternative activation markers arginase-1 and MRC-1. MMP-2 was unaffected. Morphine (10-20 μM) prevented the IL-4-induced increase in MMP-9 in a naloxone- and methylnaltrexone-reversible fashion. Morphine further prevented Il-4-elicited alternative activation of RAW264.7 macrophages. Expression of MMP-9 and arginase-1 were also increased when RAW264.7 macrophages were subjected to paracrine activation by breast cancer cells, and this effect was prevented by morphine.



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Poly I:C, a TLR3 ligand and a viral RNA mimic, and heat-inactivated respiratory syncytial virus (RSV) recapitulate the glucocorticoid insensitivity evoked by RSV infection

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Introduction. Although inhaled glucocorticoids are the cornerstone of asthma therapy, they are ineffective in virus-induced asthma exacerbations. Respiratory syncytial virus (RSV), the prime cause of severe paediatric bronchiolitis, induces glucocorticoid insensitivity in human airway epithelial cell line, BEAS-2B cells, by mechanisms still to be elucidated. Since airway epithelium is the major site for viral infection and replication, emergence of resistance may occur through epithelium activation by pathogen-associated molecular patterns (PAMPs) with release of cytokines, including transforming growth factor-β (TGF-β), a potent inducer of glucocorticoid resistance (Salem et al, 2012).
Aims. To ascertain whether PAMPs mediate the suppressive impact of the RSV infection on human bronchial epithelial cell responses to glucocorticoids via mobilization of TGF-β.
Methods. BEAS-2B cells were transiently transfected with a glucocorticoid response element (GRE)-controlled secretory alkaline phosphatase (SEAP) reporter construct and pGL3 as an internal control vector to assess GRE activity. The expression of TGF-β1 and plasminogen activator inhibitor-1 (PAI-1) was quantified by RT-qPCR. Smad 2/3 phosphorylation was analyzed by western blotting.
Results. Both poly I:C (10μg/mL) and heat-inactivated RSV at a multiplicity of infection 1 (MOI 1) caused a marked suppression of the GRE activation mediated by dexamethasone (30nM). The selective ALK5 inhibitor, SB431542 (1μM) fully prevented the attenuation of GRE activation, suggesting the mobilization of endogenous TGF-β. This observation was reinforced by the up regulation of the TGF-β1 and the TGF-β regulated PAI-l. Furthermore, smad 2/3 phosphorylation was detected after 24 hr of Poly I:C or HI-RSV exposure.
Discussion. Our data suggest Poly I:C and heat-inactivated RSV can potentially mimic RSV detrimental effects on glucocorticoid signalling in bronchial epithelium. This effect may be partly explained by mobilization of endogenous TGF-β.

Salem S et al. (2012) Br J Pharmacol 166: 2036–2048.

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Guanine Deaminase: Significance, Assay, Identification of Inhibitors, and Evidence for Redox Regulation

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Introduction. Mammalian guanine deaminase (GDA; EC 3.5.4.3) regulates both the intracellular guanine nucleotide pool and the production of xanthine, a substrate for the redox-sensitive immune regulator xanthine oxidase. GDA activity is vital for normal purine homeostasis, neural development and innate and acquired immunity. Oxidative stress stimulates GDA expression and detection of GDA activity in serum or cerebrospinal fluid indicates neoplasia or tissue trauma. Although it is an attractive target for drug development, studies of GDA have been hampered because of difficulties associated with its assay, notably the poor aqueous solubility of both substrate and product.
Aim. To develop a simple, versatile, robust and sensitive high-throughput GDA assay suitable for both research and routine diagnostic applications.
Methods. An ultrasensitive microtitre plate-based fluorimetric assay for GDA activity was devised by using guanosine as a pro-substrate and "nesting" the GDA-catalyzed reaction in a set of coupled enzymatic reactions that ultimately generate hydrogen peroxide, which was detected by oxidation of Amplex Red. The assay was used to establish a reference range for serum GDA activity and to monitor fluctuations in serum GDA activity sera obtained from 85 individuals during the course of interferon treatment for chronic hepatitis C infection. It was also used to screen some potential GDA inhibitors. Inhibitory activity was confirmed using an HPLC-base assay.
Results. Serum GDA activity in HCV infected individuals was elevated, increased during interferon treatment, then decreased to an extent that correlated with longer-term viral clearance. Besides those already reported by Fernandez *et al.* [1], 6-aza-thymine and 8-oxo-deoxyguaninosine (8-oxoGdR) were amongst the GDA inhibitors identified.
Discussion. Together with existing data, these observations provide evidence that GDA expression and activity are subject to redox regulation, being enhanced by pro-inflammatory cytokines and inhibited by 8-oxoGdR, a product of oxidative damage to DNA. They further emphasize the biological significance of GDA and broaden the scope for future investigation of its activities. [1] Fernández, J.R.*et al.* Bioorg Med Chem. 2010; 18:6748-55.

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Elastase activates PAR_2/TRPV4 signaling complex and causes inflammatory pain

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Introduction. Proteases such as mast cell tryptase cleave protease-activated receptor-2 (PAR_2) at R36/S37 and reveal a tethered ligand that binds to and activates the cleaved receptor. Activated PAR_2 then sensitize transient receptor potential (TRP) ion channels and thereby induce neurogenic inflammation and pain. Proteases that cleave the receptor at non-canonical sites may trigger different signalling cascades thereby act as biased agonists. Neutrophil elastase is activated during inflammation and is a biased agonist of PAR_2, However, whether elastase induces PAR- and TRP-dependnet neurogenic inflammatory pain is unknown.
Aims. To investigate whether elastase induces inflammatory pain via biased agonism of PAR_2 and TRPV4.
Methods. Elastase activation of PAR_2 was examined in HEK293 cells stably overexpressing human PAR_2 by measuring Ca^{2+} mobilization, cAMP accumulation, ERK1/2 activation and β-arrestin recruitment. Elastase signaling of dorsal root ganglion neurons from wild type, PAR_2 and TRPV4 knockout mice were determined using single cell Ca^{2+} imaging. mechanical hyperalgesia were evaluated after intraplantar injection of elastase to wild-type and PAR_2 knockout mice.
Results. In HEK-PAR2 cells, elastase stimulated ERK1/2 activation and cAMP formation, but not Ca^{2+} mobilization or β-arrestin recruitment. In primary neurons, elastase induced adenylyl cyclase-dependent Ca^{2+} influx via ion channels such as TRPV4. Intraplantar injection of elastase induced PAR_2-dependent mechanical hyperalgesia and inflammation.
Discussion. Our results identify a novel mechanism of elastase-induced activation of PAR_2, and expand the role of PAR2 as a mediator of protease-driven inflammation and pain.

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GHB activates a subset of $GABA_A$Rs expressed in *Xenopus* oocytes

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Introduction. γ-hydroxybutyrate (GHB) is a small molecule with complex pharmacology. Present in low concentrations in the mammalian brain, it acts as a neuromodulator. When taken exogenously, it is used to treat narcolepsy and to ameliorate the withdrawal effects of alcohol, and is used as a recreational drug at higher concentrations, sometimes used as a "date-rape" drug. GHB is known to activate the $GABA_B$ receptor at high concentrations, but ligand-binding studies identified "GHB receptors" that bind GHB with high affinity.
Aims. To determine the role of the interaction between GHB, NCS-382, THIP and $GABA_A$Rs in thermoregulation, and the differences in subtype selectivity that underlies these roles.
Methods. Thermoregulation was measured using radiotelemetry in wild-type and knockout mice injected intraperitoneally with GHB, NCS-382 and THIP. The activity of GHB, THIP and NCS-382 at $GABA_A$Rs was determined by injecting mRNA encoding the sequences of the α4, β1-3 and δ subunits of $GABA_A$Rs in various ratios and combinations into *Xenopus* oocytes and measuring currents by two-electrode voltage clamp..
Results. GHB, THIP and NCS-382 all induced hypothermia in wild-type mice, bur only THIP-mediated hypothermia was abolished in δ-knockout mice. We then investigated the pharmacology of NCS-382 at α4βδ $GABA_A$Rs by measuring concentration-response curves of THIP, GHB and NCS-382 on Xenopus oocytes injected with different combinations of α4β1-3 and α4β1-3δ RNA at different ratios. NCS-382 activated β3 homomeric receptors and these currents were inhibited with co-application of GHB. Furthermore, GHB activated α4β1 receptors injected with a 1:10 ratio significantly more potently than when injected with a 10:1 ratio.
Discussion. Taken together, these data demonstrate that THIP, but not GHB or NCS-382 induce hypothermia via the activation of δ-containing $GABA_A$Rs. It is likely that NCS-382 and GHB activates GABAA receptors that are expressed in Xenopus oocytes but not readily found on the extracellular surface of native neurons, and these receptors are most likely to contain a β-β interface. While NCS-382 has previously been reported as an antagonist of GHB receptors, the pharmacological profile of NCS-382 is considerably more complicated.

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Effect of positive allosteric modulators of M_1 muscarinic receptors on psychosis-like behaviours in mice

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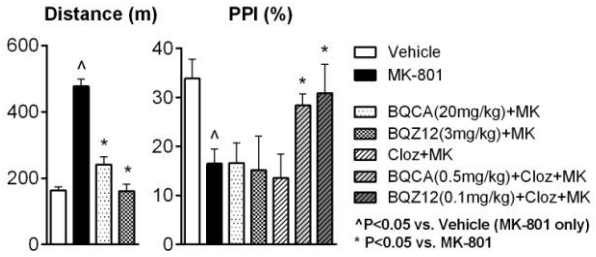
Introduction: Allosteric enhancement of M_1 muscarinic acetylcholine receptors (mAChR) has emerged as a potential therapeutic antipsychotic strategy, however its effect in animals presenting aspects of psychoses remain unclear.

Aim: To investigate the ability of the M_1 modulators, benzyl quinolone carboxylic acid (BQCA) and benzoquinazolinone-12 (BQZ12), to reverse locomotor hyperactivity and prepulse inhibition (PPI) deficits induced by the NMDA receptor antagonist MK-801.



Methods: Mice were treated with BQCA (0.5-20mg/kg) or BQZ12 (0.1-3mg/kg) prior to MK-801 (0.3mg/kg) and subjected to locomotor activity or PPI testing (n=8-15/group). Mice co-treated with a sub-effective dose of clozapine (2mg/kg) and the lowest doses of BQCA (0.5mg/kg) or BQZ12 (0.1mg/kg) were also included in PPI.

Results: BQCA (20mg/kg) and BQZ12 (3mg/kg) completely reversed MK-801-induced locomotor hyperactivity without affecting the baseline locomotor activity when administered without MK-801. Neither clozapine (2mg/kg), BQCA nor BQZ12 was capable of reversing MK-801 induced PPI disruptions. However, the combination of BQCA (0.5mg/kg) or BQZ12 (0.1mg/kg) and clozapine produced a reversal of MK-801-induced PPI disruptions.

Discussion: We provide an evidence of potential antipsychotic efficacy of the positive allosteric modulator of the M_1 mAChR, and also demonstrate its potential to broaden the therapeutic spectrum of current antipsychotics to improve the pharmacological treatment of the schizophrenic syndrome.

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Activity of illicit synthetic cannabinoids, UR-144, XLR-11 and 5-hydroxypentyl-UR-144 at human cannabinoid receptors and cannabinoid-sensitive ion channels

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Introduction: Illicit synthetic cannabinoids (SC) are of major concern in part because of apparently high toxicity associated with their use. Whether this arises through actions at CB receptors or other targets remains an open question, largely because almost nothing is known about their pharmacological activity.

Aim: Our aim was to compare the activity of 3 related SC, UR-144 (1-pentyl-1H-indol-3-yl)-(2,2,3,3-tetramethylcyclopropyl)methanone), its fluoropentyl analog (XLR-11) and its 5-hydroxypentyl metabolite (SB5035) at human CB1 and CB2 receptors and the cannabinoid-sensitive ion channel TRPA1.

Methods: CB1 and CB2 receptor mediated K channel activation was measured in AtT20 cells using a membrane potential sensitive dye, activity at hTRPA1 was measured in HEK 293 cells using a Ca-sensitive dye. Assays were performed using a Flexstation 3, with a minimum of 3 independent replicates.

Results: All 3 drugs were agonists at CB1 and CB2 receptors with pEC50s: UR-144, 6.4±0.05, 7.1±0.05; XLR-11, 7.0 ±0.5, 7.1 ± 0.15; SB5035, 5.7±0.1, 8.2±0.1 respectively. Maximum effects were similar to those of WIN55212, with exception of SB5035, which produced a maximum hyperpolarization approximately 50% larger than WIN55212 at CB1. Compared to the phytocannabinoid Δ9-THC, the SCs were less potent at CB1, but more potent and efficacious at CB2. UR-144 (30μM) activated TRPA1 to 38±4 % of the prototypic agonist cinnamaldehyde. XLR-11 and SB5035 (pEC_{50} 5.1±0.05 and 4.95±0.02 respectively) had similar efficacy to cinnamaldehyde.

Discussion: These SC are highly effective agonists at CB1 and CB2 receptors, with higher efficacy than Δ9-THC. These results are broadly consistent with previous studies of UR-144 and XLR-11 at CB receptors (Frost et al, 2010; Wiley *et al,* 2013). High CB1 and TRPA1 efficacy could contribute to the human toxicity of these compounds.

Frost JM et al (2010) J Med Chem 53, 295-315. Wiley JL et al (2013) Neuropharmacology 75, 145-154.

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Varenicline improves motor coordination and alters cytokine expression profiles in the YAC128 transgenic mouse model of Huntington's Disease

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Introduction. The nicotinic agonist varenicline improves motor, cognitive and affective symptoms in the YAC128 transgenic mouse model of Huntington's Disease (HD; D'Souza et al, unpublished). It remains unclear which mechanisms underlie this functional improvement. Increased pro-inflammatory cytokine expression in the brain, plasma and CSF of HD patients suggests that an altered innate immune response may play a role in HD pathology[1].
Aims. To investigate whether reduced brain inflammation contributes to functional recovery in varenicline-treated, late stage YAC128 mice.
Methods. Thirteen month old YAC128mice (n=10) and age-matched wild-type (WT) littermates (n=10) received varenicline (1mg/kg/day for 14 days) via subcutaneously implanted matrix-driven delivery pellet (IRA®). Mice were trained and tested in the accelerod test prior to and at 1, 2, 3, 4, 6, 8 and 10 weeks after varenicline treatment. Cytokine protein expression levels were assayed in striatum, hippocampus, cortex and cerebellum samples using a mouse cytokine/chemokine premixed 22plex Milliplex kit (Millipore).
Results. Varenicline significantly increased the fall latency of YAC128 mice in the accelerod test (pre-drug: 26.27± 4.77s v post-drug: 46.48±5.80s, $p<0.001$). Increased fall latency remained evident at week 8 (43.45s ± 6.28, $p= 0.012$) but not at week 10 (35.55s ± 6.12, $p=0.329$). Varenicline-treated YAC128 mice showed decreased expression of INFγ in all brain regions, and IL-9 in striatum and cerebellum relative to untreated animals. In contrast, MCP-1 and IL-1 expression levels were increased in the cortex and striatum and cortex and cerebellum respectively.
Discussion. Varenicline produced significant, long lasting improvements in motor function in late stage YAC128 transgenic HD mice. Complex changes in cytokine expression profiles within brain regions involved in motor coordination may underlie improvements in motor performance.

[1] Bjorkqvist M, Wild EJ *et al*. (2008) Journal of Experimental Medicine 205(8):1869-77

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Development of Novel Polymeric Microparticles for Sustained-Release Intrathecal Delivery of Analgesics

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Introduction. The management of moderate to severe persistent pain associated with advanced cancer is a great challenge to clinicians. At present, 10-30% of patients with cancer-related pain do not obtain benefit from existing analgesic drug treatments delivered by conventional dosing routes. Ketamine has been shown to enhance the analgesic effects of strong opioid analgesics, thereby enabling satisfactory pain relief to be achieved with an improved adverse event profile (1). Intrathecal drug administration enables logarithmic reductions in the doses of analgesic medications relative to those required for systemic dosing routes (2).
Aims. To develop a bioerodable, sustained-release, poly (lactic acid-co-glycolic acid) (PLGA) microparticle formulation of the strong opioid analgesic, hydromorphone, in combination with ketamine, for intrathecal delivery, as a means to produce prolonged periods of analgesia in patients with severe cancer-related pain.
Methods. Hydromorphone or ketamine loaded microparticles were prepared using a modified double emulsion solvent evaporation method. Drug loading of the microparticles and their *in vitro* release profiles were measured using UV-spectroscopy, GC-MS and HPLC with UV detection. The size and morphology of microspheres were analysed using a Mastersizer and scanning electron microscopy (SEM).
Results. To obtain a high yield (>90%) of microspheres with a desirable size and acceptable drug loading (1.3% to 6.1%), a *wow* method was used after the drugs of interest were converted to the free base form by adjusting the pH to 10 with subsequent dissolution in the oil phase.
Discussion. A modified double emulsion method has been used to develop microparticles containing the analgesic drugs ketamine and hydromorphone, with enhanced drug loading and an improved sustained release profile (>28 days, *in vitro*) for future assessment in rodent pain models.

Wasana C et al (2009) Current Drug Delivery 1: 69-75;
Yang Y et al (2001) *Biomaterials* 22: 231-241.

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Neurokinin 1 receptor signalling from endosomes: a key source of pain signalling.

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Introduction. G-protein coupled receptors (GPCRs) are the largest class of membrane bound receptors and are involved in the majority of pathophysiological signalling pathways. Upon activation, most GPCRs traffic to endosomes. Although GPCRs can continue to signal from endosomes, the mechanisms of endosomal signalling and its importance in complex pathophysiological processes are unknown. The substance P (SP) neurokinin 1 receptor (NK1R) is a mediator of pain and inflammation and is rapidly internalized to, and signals from, endosomes. However, the role of endosomal signalling in NK1R mediated pain is not understood.
Aims. Determine the importance of internalization and endosomal signalling of NK1R on pain and inflammation.
Methods. Dynamin-1 and clathrin inhibitors and siRNA were used to block NK1R endocytosis and trafficking to endosomes in cell lines and intact rats and mice. NK1R internalization and trafficking were quantified using BRET and immunofluorescence. Endosomally-mediated ERK, PKC and cAMP signalling were measured in cell lines using FRET biosensors and by immunofluorescence in rats. Capsaicin-evoked mechanical hyperalgesia was evaluated following intrathecal injection of dynamin, clathrin, NK1R, or MEK inhibitors in mice.
Results. In HEK293 cells, dynamin and clathrin disruption blocked SP stimulated NK1R endocytosis and inhibited SP-induced activation of nuclear pERK, cytoplasmic PKC, and cytoplasmic cAMP. Intrathecal injection of clathrin and dynamin inhibitors and siRNA blocked capsaicin-evoked endocytosis of the NK1R and activation of ERK in spinal neurons, and suppressed capsaicin-evoked mechanical hyperalgesia. Dynamin inhibitors also blocked SP-induced excitation of spinal neurons.
Discussion. Our results demonstrate a critical role for the endosomal signalling of the NK1R in pain transmission. These results also open new therapeutic targets for the treatment of NK1R mediated pain and inflammation.

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Type-1 interferon signalling regulates the glial-mediated neuroinflammatory response in models of Parkinson's disease

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Introduction.Neuroinflammation contributes to Parkinson's disease (PD) pathology. Previously, we have demonstrated that type-1 interferon (IFN) signalling drives the deleterious neuroinflammatory response in rotenone treated primary cultured neurons, however the role of type-1 IFNs in the glial cell response in PD is unclear.
Aims. To investigate the contribution of type-1 IFNs to the glial cell response in in-vitro and in-vivo models of PD.
Methods. Primary cultured wildtype and IFN-α receptor-1 knockout ($IFNAR1^{-/-}$) mixed glia were treated with 25nM rotenone (24-72h). Wildtype and $IFNAR1^{-/-}$ mice were administered MPTP (4x15mg/kg, 2h intervals) and brains collected at 1, 3 and 21 days post-MPTP for analysis.
Results. QPCR analysis confirmed $IFNAR1^{-/-}$ glia display reduced expression of IFNα and IFNβ following rotenone treatment compared to wildtype controls ($n=6$, $p<0.05$). $IFNAR1^{-/-}$ glia also exhibit diminished type-1 IFN signalling, with reduced STAT-3 phosphorylation identified by western blot. Significantly, levels of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 were reduced in $IFNAR1^{-/-}$ glia ($n=6$, $p<0.05$), as measured by QPCR and ELISA. In MPTP treated mice, reduced microglial activation ($Iba\text{-}1^{+}$) was identified in the substantia nigra (SN) of $IFNAR1^{-/-}$ mice 3 days post-MPTP. Additonally, $IFNAR1^{-/-}$ mice display reduced levels of M1 pro-inflammatory markers (CD11b, CD16, CD32, CD86) and increased M2 anti-inflammatory genes (YM1, IL-10) compared to wild type mice ($n=6$, $p<0.05$). This reduced neuroinflammatory response correlated with increased survival of SN neurons in $IFNAR1^{-/-}$ mice compared to wildtype mice (3907 ± 64.61 versus 3079 ± 70.59, $n=10$, $p<0.001$), identified by Tyrosine Hydroxylase quantification 21 days post-MPTP.
Discussion. These results confirm a role for type-1 IFN signalling in the neuroinflammatory glial cell response in neurotoxin models of PD. Targeting type-I IFN signalling may provide a novel therapy to reduce neuroinflammation via modulating the glial cell phenotype, and limiting cell death in PD.

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Varenicline improves motor, cognitive and psychiatric symptoms in the YAC128 mouse model of Huntington's Disease

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Introduction. Huntington's Disease (HD) is a fatal, inherited neurodegenerative disorder characterised by progressive movement, cognitive and psychiatric symptoms. Post mortem and genetic mouse model studies report a significant loss of acetylcholine and choline acetyl transferase activity in the HD brain, but no change in the number of nicotinic receptors[1]. This suggests impaired cholinergic neurotransmission may contribute to HD pathology.
Aims. To determine whether chronic treatment with the nicotinic agonist varenicline reduces motor, cognitive and affective symptoms in the YAC128 transgenic mouse model of HD.
Methods. The performance of 15 month old YAC128 mice (n=14) and age-matched wild type littermates (n=13) was assessed in the rotarod, T maze, novelty suppressed feeding (NSF) and forced swim test (FST) before and after treatment with varenicline for 4 weeks (5mg/kg/day). Thymidine analogues were used to assess progenitor cell proliferation and survival. DARPP32 immunohistochemistry was performed to visualise medium spiny neurons in the striatum, hippocampus and cortex.
Results. Chronic varenicline treatment significantly increased fall latency in the rotarod (14±3s vs 50 ±7s, p=0.004) and increased rewarded alternation in the T maze in YAC128 mice (65±2% vs 79±3%, p=0.045). Varenicline also, decreased latency to feed in the NSF test and reduced floating time in the FST in both YAC128 and wild type animals. Immunohistochemical analysis revealed increased progenitor cell proliferation and survival in addition to increased DARPP32 immunoreactivity in the striatum and cortex of varenicline treated animals.
Discussion. Chronic treatment with varenicline significantly improved motor coordination and spatial memory in late stage YAC128 mice. Varenicline also produced genotype-independent improvements in recognition memory, anxiety and depressive-like behaviour. Improved performance in YAC128 mice may be attributed to increased striatal and cortical neurogenesis.

1. Smith R, *et al* (2006) Hum. Mol. Genet. Vol. 15, No. 21, 3119–3131.

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Investigating the activity of opioid and non-opioid alkaloids at the Toll-like receptor 4

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Introduction. *In vitro* studies have implicated the Toll-like receptor 4 (TLR4) signaling pathway in the unwanted effects of opioids such as reward and dependence. This raises the possibility that the benzylisoquinoline and promorphinan opiate alkaloid backbones possess molecular patterns detectable by the TLR4 innate immune pattern recognition systems. Furthermore, a broader class of non-opiate benzylisoquinoline and promorphinan alkaloids may possess previously uncharacterised TLR4 signalling (agonist or antagonist) activity.
Aims.1: Determine the potential of benzylisoquinoline and promorphinan alkaloids to bind to the TLR4 chaperone MD2. 2. Characterise the action of naturally occurring opioid alkaloids at TLR4. 3: Interrogate and characterise the biological activity of naturally occurring non-opioid alkaloids at TLR4.
Methods. *In silico* docking simulations of ligands were conducted to the crystal structure of MD2. Additionally, the molecular signalling pathways activated by opioid alkaloid-dependent activation of TLR4 will be examined and detailed pharmacological profiles will be generated using HEK293 cell lines overexpressing TLR4 and MD2 utilising an inflammatory cytokine readout. The biological activity of non-opioid benzylisoquinoline and promorphinan alkaloids at TLR4 will be established by conducting functional assays on recombinant cell systems over-expressing TLR4 in agonist and antagonist screens.
Results. We have undertaken preliminary analyses conducting *in silico* MD2 docking simulations of benzylisoquinoline and promorphinan alkaloids. Data shown in the figure displays the preferred binding conformations of each alkaloid to MD2 (morphine binding is designated in the grey cloud). The docking of many of the alkaloids overlaps entirely or substantially with this established pharmacologically active site. *In vitro* assays are ongoing.

Discussion. Amongst these alkaloids may exist compounds that have beneficial TLR4 antagonist or detrimental agonist pharmacologies. Thus, understanding the TLR4 activity of these alkaloids, either its presence or its absence, will aid the development of novel potent drugs with reduced side effects owing to the greater understanding of the structure activity relationship at TLR4.

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Generic substitution of antiepileptic drugs: patient attitudes and perceptions

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Introduction. In recent years, the number of generic medicines of important prescription drugs has increased significantly. As generic medicines may be substantially lower in price than the innovator brands, they are perceived to provide major savings in healthcare cost, because they provide savings in terms of the purchase price of medications. Whether they produce other costs, such as increased doctor visits, serum monitoring, and hospitalizations, is in question. Moreover, generics are approved to be bioequivalent via comparison against the innovator, potentially there is greater disparity between two generic products than between any generic and the innovator.

Aims. We have previously found that patients in community pharmacies generally showed acceptable attitudes towards generic substitution. The aim of the current study was to understand and compare perceptions of patients with epilepsy about substitution of antiepileptic drugs and whether patients taking drugs for such critical conditions would have the same attitude as community patients who are not taking medications for such a critical condition.

Methods. A self-administered anonymous survey will be sent to patients with epilepsy in Australia by postal mail through Epilepsy Australia. This organization provides services to Australian patients with epilepsy. The survey is composed of multi-response and short-answer questions. The questionnaire is divided into two sections. Section 1 is designed to collect information on the patients' demographics, including age, gender, type of epilepsy, ethnicity, when did the last seizure occur, self-rated health status, and the patients' current medications. Section 2 collects information on the safety and efficacy of generic antiepileptic drugs and the patients' willingness to use generic medicines for the treatment of epilepsy. The questionnaire will be evaluated with a sample (n = 10-20) of patients with epilepsy before the study commences. Invitations and information packages will be sent to patients diagnosed with epilepsy as self-reported. About 500 invitations will be sent out to patients in each of the 6 states of Australia. Data collection will be 3 months after the invitations being posted. Approval for conducting the research and the study protocol will be obtained from The Human Research Ethics Committee of the Research Ethics and Compliance Unit, Research Branch, The University of Adelaide.

Discussion. Considerable concern exists among patients with epilepsy about generic substitution of their antiepileptic medications. This was based on our pilot study conducted previously with a sample (n= 47) of patients and from studies conducted overseas. More research on whether generic antiepileptic drugs are bioequivalent is therefore critically important. The findings will also help to address patient concerns.

Ngo SNT et al (2013) Epilepsy & Behavior 26(1):64-66
Cusack L et al (2013) Health Soc Care Com 21:373-80

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Mechanism of α4β2 nicotinic acetylcholine receptor modulation unraveled by x-ray crystallographic and functional studies

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(introduced by Thomas Balle, Faculty of Pharmacy, The University of Sydney, NSW, Australia)

Introduction. Allosteric modulators of nAChRs are interesting in a drug discovery perspective but the mechanisms underlying modulation are poorly understood. To aid this understanding we have explored the binding mode of the stoichiometry selective modulator NS9283. It modulates through a binding site in the α4α4 interface of $(\alpha4)_3(\beta2)_2$ receptor with a pharmacological profile similar to that of a benzodiazepine acting at $GABA_A$ receptors.

Aims. To unravel the mechanism of action of NS9283 as a representative for modulators with a benzodiazepine like mechanism of action.

Results. Based on a co-crystal structure with *Ls*-AChBP, homology modeling, quantum mechanical calculations and extensive mutational mapping the binding mode and the selectivity determinants of NS9283 was determined. In *Ls*-AChBP, NS9283 bridges the subunit interface and forms close contacts to residues known to govern agonist potency and efficacy. It even forms favorable, albeit non-classical contacts to Trp143 (*Ls*-AChBP numbering) which in the α4 subunit is involved in cation-π interactions and is key to agonist activity.

Discussion. We have previously shown that the α4α4 interface nests a third binding site for the endogenous transmitter acetylcholine. NS9283 competes for this site and has an agonist-like mechanism of action. To confirm the agonist-like mechanism we show that NS9283 on a receptor engineered to contain more than one NS9283 compatible binding site is capable of producing a partial agonist response. An interesting consequence of this study is that the term “allosteric modulator” does not apply to NS9283.

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CYP2C8 genotype significantly alters imatinib metabolism in Chronic Myeloid Leukaemia patients.

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Introduction: Between 25 and 49% of patients treated with imatinib for Chronic Myeloid Leukaemia (CML) withdraw from imatinib due to poor disease control or drug toxicity; large inter-individual variability in plasma concentration is a significant contributor (Gotta *et al.*, 2014). Imatinib is metabolised to N-desmethyl imatinib by CYPs 3A4 and 2C8, with evidence for CYP3A4 autoinhibition and increased CYP2C8 contribution at steady-state (Filppula *et al.*, 2013). We hypothesise that *CYP2C8* polymorphisms (*CYP2C8*3* and **4*) alter steady-state plasma imatinib concentrations and metabolic ratio (N-desmethyl imatinib:imatinib, MR) in CML patients.
Aim: Determine *CYP2C8* genotype, age-, sex-, time- and dose-effects on imatinib metabolism in CML patients.
Method: Retrospective analysis of 210 CML patients from the TIDEL II trial (imatinib 600 mg/day with dose changes based on response). Trough plasma imatinib and N-desmethyl imatinib concentrations, dose, age and sex data on days 8, 22 and 90 of treatment were available. Patients were genotyped for *CYP2C8*3* (rs11572080, rs10509681) and **4* (rs1058930). Imatinib concentration and MR were compared between genotypes for patients on 600 mg/day at day 90 only (Kruskal-Wallis, Dunn's post-hoc). Linear mixed effects (LME) modeling was used to investigate dose, time, age, sex and genotype effects on imatinib concentration and MR using day 8, 22 and 90 data.
Results: At 600 mg/day on day 90, genotype differences were significant (P=0.02) for MR [median±SD (n) **1/*1* =0.22±0.09 (111); **3* carrier=0.26±0.06 (23); **1/*4*=0.23±0.08 (15). Post-hoc P<0.05 **1/*1* vs **3* carrier], but not imatinib concentrations (P=0.9). LME showed only dose predicted imatinib concentration ($P=3x10^{-9}$), but dose, time and genotype predicted MR (nested ANOVA P=0.003, $2x10^{-5}$, $8x10^{-5}$ respectively). Controlling for dose and time, **3* carriers had 12% higher, and **1/*4* 14% lower, MR compared to **1/*1*.
Discussion: *CYP2C8* genotype alters imatinib metabolism, but not trough plasma imatinib concentrations, in CML patients. Clinical impact will thus depend on presently unclear contributions of active metabolite(s) to response.

Filppula A M et al (2013) *Drug Metab. Dispos.* 41, 50-59.
Gotta V et al (2014) *Leukemia Res* 38, 764-772.

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Impact of *CYP2C8*1/*3* polymorphism on *in vitro* metabolism of imatinib to its active metabolite N-desmethyl imatinib

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Introduction: Imatinib is metabolised to its active metabolite N-desmethyl imatinib by CYP3A4 and CYP2C8. *In vitro* using human liver microsomes (HLMs) imatinib was found to be an autoinhibitor of its own CYP3A4 metabolism suggesting a more significant role of CYP2C8, especially on chronic dosing (Filppula, 2013). Functional effects of the major polymorphism *CYP2C8*3* on N-desmethyl imatinib formation are unknown.
Aim: To investigate the impact of *CYP2C8*3* polymorphism on imatinib N-demethylation in HLMs.
Methods: Kinetics of imatinib metabolism were determined using *CYP2C8*1/*1* (n=5) and **1/*3* (n=4) HLMs and 20 imatinib concentrations (1-200 µM). Eadie-Hofstee plots directed the choice of kinetic model. Analysis of the data was compared between models for one enzyme Michaelis-Menten (MM), one enzyme substrate inhibition, two enzymes MM, and one enzyme MM with one enzyme substrate inhibition.
Results: One enzyme substrate inhibition was detected in all *CYP2C8*1/*1* HLMs, but not in three out of four *CYP2C8*1/*3* HLMs with K_m values significantly lower than the wild type (Table). Substrate inhibition was found in one *CYP2C8*1/*3* HLM with K_m, V_{max} and K_i values of 28 µM, 183 pmol/min/mg and 30 µM respectively.
Discussion: Our kinetic data could only find the involvement of one enzyme and that *CYP2C8*3* appears to lead to enhanced functional activity suggesting that autoinhibition might be mediated via CYP2C8 and not CYP3A4.

HLMs	V_{max} (pmol/min/mg)	K_m (µM)	K_i (µM)	Cl_{int} (µL/min/mg)
*CYP2C8*1/*1* (n=5)	130 ± 38	9.1 ± 2	157 ± 69	14±4
*CYP2C8*1/*3* (n=3)	107 ± 31	6.2 ± 0.75*		18±4

* Mann-Whitney U test P=0.03 versus *1/*1. Values are median ± SD.

Filppula, A M et al (2013) Drug Metabolism and Disposition, 41:50-59.

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Forensic Pharmacogenetics, Psychiatry and Public Health

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Introduction: we wished to assess the forensic utility of CYP450 testing in serious adverse drug reactions to mental health drugs and combinations.
Aims: To investigate drug-drug, drug-gene and drug-drug-gene interactions that caused over 100 people with no previous history of mental illness, suicidality or violence to become disabled with akathisia, aggression and suicidality from drugs metabolized by CYP450.
Setting: A community where prescribers are educated by clinical practice guidelines. Prescribing did not accord with advice in Product Information or with Public Health Advisories from the United States Food and Drug Administration.
Patients and methods: Over 130 subjects were referred author's medicolegal practice largely because of new and continuing disability. Adverse drug reactions, drug-drug and drug-gene interactions slowing metabolism and were documented in conjunction with their genotypes at CYP450 2D6, 2C9 and 2C19.
Results: Compared with primary care patients tested at the same facility, variant, diminishing and multiple polymorphisms at CYP450 were significantly higher in subjects with adverse drug reactions. Only four had wild type genes throughout and they were over medicated. Only nine started on a single drug at a standard dose. The rest were on multiple doses, interacting drugs, herbs, illicit substances and prescribing cascades followed. Some withdrew medication slowly and recovered but 50 percent were lost to follow up. Four developed delayed post-withdrawal akathisia which was intractable.

Conclusion: Much "psychiatric" disability is caused by toxicity from medications and incorrectly attributed to mental illness. Psychiatric drugs, then polypharmacy are being prescribed to a large, genetically diverse, population for stressful situations not accessible to pharmacological remedy. The principles of personalized medicine, applied forensically, elucidate this problem and can prevent these debilitating and dangerous conditions. Regulatory authorities and most doctors in Australia remain unresponsive. The problem is internal to psychiatric practice and can only be put right only by education.

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Introduction of a multi-gene pharmacogenomic test and an electronic support system into aged persons mental health inpatient care

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Introduction. Pharmacogenomic tests are not used often in public psychiatry inpatient care and this study explored the effect of introducing an 6 gene multi-gene pharmacogenomic test into inpatient care together with the introduction of an electronic support system to order tests and retrieve the results.
Aims. To demonstrate the effect of introduction of a multi-gene pharmacogenomic test and an electronic support system into aged persons mental health in 6 different hospitals.
Methods. A secure intranet- based ordering and results retrieval system was introduced to support a 6 gene pharmacogenomic test into two wards in acute hospitals and 4 residential units. 177 patients were compared with 86 patients in a third ward where the pharmacogenomics testing was not offered. Also a further 82 patients were studied in residential units.
Results. Some subsets of the tested patients were found to have significantly increased length of stay Patients found to be CYP2C19 ultrarapid and heterozygous ultrarapid metabolisers had a 12 day increase in median length of stay. For the intervention group the mean number of psychotropic medications fell by 31.4% however in the control group the number of psychotropic medications increased by 10%. The previous year's medication use was studied in all 3 hospitals and in these control groups the medications increased by 26%. The number of patients with at least one fall in the intervention group was reduced (18.6 per 100 versus 34,2 per 100 patients).
The decision support system was widely used and 90% of the clinicians surveyed acknowledged that having the electronic support system had increased their confidence in interpreting pharmacogenomic results.
Discussion. The pharmacogenomic decision support software was successfully used to introduce a multi-gene pharmacogenomic test into acute and residential aged persons mental health care. Some of the results have important economic implications such as increased stay in certain groups recognised by the test and reduced number and drugs and falls. This has the potential to save the health system millions of dollars.

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Characterisation of novel benzamide derivatives as P2X7 receptor antagonists.

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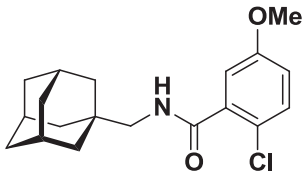
Introduction. The P2X7 receptor is an ATP ligand-gated ion channel found predominantly on immune cells. It has the rare ability to form a pore upon prolonged activation. Recent work has implicated the P2X7 in a number of maladies, such as depression, neuropathic pain and rheumatoid arthritis, making it an important therapeutic drug target (Skaper et al, 2010).



Compound 1

Aims. To improve the physicochemical properties of the lead P2X7 receptor antagonist, compound **1**, and to enhance its therapeutic efficacy. Derivatives varying in the number of fluorine atoms at the bridgehead positions of the adamantane were synthesised, resulting in mono-, di- and tri- substituted analogues. Evaluation of these derivatives for potency at the human and mouse P2X7 receptor will also provide further information on their biological profile.

Methods. HEK cells, transfected with the human P2X7 receptor, were used to examine the binding affinities, as assessed by radioligand binding, and calcium influx using Fluo-4AM was employed to assess potency of ion channel antagonism on both human and mouse HEK-transfected cells.

Results. For calcium influx, the fluorinated derivatives of compound **1** were equally potent, with pIC_{50}'s±SD ranging from 7.59±0.18 to 8.00±0.22. All compounds exhibited diminished potency at the mouse P2X7 receptor, with pIC_{50}'s ranging from 5.72±0.28 to 6.80±0.14.

Discussion. The compounds tested showed potent antagonism of P2X7 receptor-stimulated calcium influx, consistent with previous results for pore formation using dye uptake on THP-1 cells. By fluorinating the adamantane moiety, the newly synthesised compounds not only displayed a reduced lipophilicity enabling passage across the blood brain barrier, but retain their potency at the human P2X7 receptor, making them key candidates for therapeutic use in CNS disorders.

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Assessment of Janus protein tyrosine kinases as potential regulators of epithelial-mesenchymal transition using a model of epidermal growth factor induced breast cancer epithelial-mesenchymal transition

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Introduction. Epithelial-mesenchymal transition (EMT) is the process of epithelial cells acquiring a mesenchymal phenotype, with increased migratory and invasive properties, and is thought to be involved in metastasis. Regulators of EMT may represent novel therapeutic targets in invasive breast cancer subtypes. Previously we have implicated calcium signalling in the activation of signal transducer and activator of transcription 3 (STAT3) and the expression of specific EMT markers in a MDA-MB-468 breast cancer cell line model of epidermal growth factor (EGF) induced EMT. However, the exact mechanism of this regulation is unknown.

Aims. To investigate upstream regulators of STAT3 activation and their role in the expression of the EMT marker vimentin using a model of EMT in MDA-MB-468 breast cancer cells.

Methods. MDA-MB-468 cells were pre-treated for 1 h with the pan Janus tyrosine kinase (JAK) inhibitor, JAK inhibitor I (1, 10 μM) or the Src family tyrosine kinase inhibitor, PP2 (0.1, 1, 10 μM). Total cellular protein was isolated following stimulation with EGF (50 ng/mL) for 10-20 min and levels of the activated, phosphorylated STAT3 were analysed using immunoblotting. Vimentin protein and mRNA expression was assessed 24 h after EGF treatment (50 ng/mL) in the presence or absence of JAK inhibitor I or PP2. Vimentin protein was assessed using immunoblotting and vimentin RNA using real time RT-PCR.

Results. Both JAK inhibitor I and PP2 significantly decreased EGF-induced STAT3 phosphorylation. JAK inhibitor I also appeared to decrease vimentin protein and mRNA expression at 24 h.

Discussion. Using the MDA-MB-468 model of EGF-induced EMT we have identified a potential role for Janus protein tyrosine kinases in the upstream regulation of STAT3 phosphorylation and vimentin expression. Further studies will be undertaken to identify the specific JAK isoforms involved and the intricacies of the relationship with calcium transporters.

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Assessment of the consequences of silencing of the secretory pathway calcium ATPase 1 in MDA-MB-231 breast cancer cells.

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Introduction. Secretory pathway calcium ATPases actively transport Ca^{2+} into the Golgi. Secretory pathway calcium ATPase isoform 1 (SPCA1) is elevated in the poor prognosis, basal-like breast cancer molecular subtype. The silencing of SPCA1 reduces the proliferation of basal-like MDA-MB-231 breast cancer cells. However, the mechanism for this effect is not fully understood. We have recently used 2D-DIGE and MS/MS to identify proteins sensitive to SPCA1 silencing. These studies identified downregulation of heat shock protein 60 (HSP60) protein and mRNA with SPCA1 silencing in MDA-MB-231 cells.
Aims. To compare mRNA levels of proteins whose levels were identified using 2D-DIGE as potentially altered upon SPCA1 silencing in MDA-MB-231 cells.
Methods. MDA-MB-231 cells were treated with SPCA1 siRNA or non-targeting siRNA (control), 24 h after plating. RNA was isolated 48 h post siRNA treatment and levels of mRNA were assessed using real time RT-PCR. Thirteen targets were assessed (including HSP60).
Results. SPCA1 silencing significantly reduced the level of HSP60 mRNA (~60%, $P < 0.05$). Junction plakoglobin/gamma-catenin mRNA levels significantly increased (~80%, $P < 0.05$) with SPCA1 silencing, as did mRNA levels of tubulin beta-4B chain (22%, $P < 0.05$).
Discussion. These studies have identified that silencing of SPCA1 produces a significant increase in the mRNA levels of junction plakoglobin/gamma-catenin and tubulin beta-4B chain in MDA-MB-231 breast cancer cells. Further studies are required to define the mechanism and significance of these changes when SPCA1 is silenced in MDA-MB-231 cells.

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Expression of copper transporters in colorectal cancer cells and its relation to oxaliplatin

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Introduction. Copper transporters are important membrane-bound proteins to maintain mammalian copper homeostasis, including copper transporter 1 (CTR1), $copper^{++}$-transporting P-type ATPase 7A (ATP7A) and 7B (ATP7B). Recent preclinical studies suggest their roles in regulating the uptake and export of platinum-based anticancer drugs. Resistance in colorectal cancer cells is attributable to reduced cellular concentration of oxaliplatin. We hypothesize that copper transporters may play important roles in the transport and sensitivity of oxaliplatin in colorectal cancer cells.
Aims. To characterize the expression of copper transporters in colorectal cancer cells and determine the effect of Cu chelator, varying levels of Cu and oxaliplatin on their expression pattern and to measure the sensitivity of colorectal cancer cells to oxaliplatin.
Methods. Cytotoxicity was measured for oxaliplatin in human colorectal cancer cells (COLO205, SW620, HCT-15 and DLD-1) using a MTT assay. Single and double fluorescence immunocytochemistry were carried out using anti-CTR1, ATP7A, ATP7B and Na/K ATPase primary antibodies and Alexa Fluor 488 or 494-conjugated secondary antibodies on cells treated with different levels of $CuCl_2$ and oxaliplatin. Digital images were acquired and processed using confocal microscope and ImageJ software.
Results. The sensitivity to oxaliplatin differed by 15-fold among these cell lines, with IC_{50} values ranging from 0.82±0.22 to 15.4±2.2 μM. Cellular immunoreactivity to CTR1, ATP7A and ATP7B was observed in all cell lines. CTR1 displayed stronger membrane staining in SW620 cells than other cell lines. Compared to CTR1, ATP7A and ATP7B expression were more abundant across all cell lines with apparent plasma membrane staining. Treatment of cells with Cu, Cu chelator and oxaliplatin did not cause significant changes in the expression pattern of transporters.
Discussions. Our data have demonstrated that copper transporter CTR1, ATP7A and ATP7B are expressed by these human colorectal cancer cells with stronger membrane presence for efflux transporters under basal culture conditions. The effect of extracellular Cu conditions and oxaliplatin on their expression pattern is minimal, but it needs to be investigated further at mRNA and protein levels. This work is supported by Cancer Council Tasmania.

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Comparative anti-proliferative and pro-apoptotic actions of a novel ω-3 fatty acid analogue in human breast cancer cell lines.

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Introduction. The CYP-derived epoxide formed at the ω-3 olefinic bond in ω-3 PUFAs inhibits tumour cell viability. However, because epoxides are metabolically unstable, we have developed a series of urea isosteres suitable for *in vivo* evaluation of anticancer activity. One agent - termed CTU - rapidly and effectively kills MDA-MB-231 cells *in vitro* and *in vivo*. It is well documented that breast cancer heterogeneity is an important factor in the *in vivo* efficacy of anticancer drugs. Cancer cell profiling could help identify tumour characteristics that promote susceptibility to new agents such as CTU in order to direct their optimal clinical use.
Aims. This study evaluated the comparative cytotoxic properties of CTU in four representative breast cancer cell lines: MDA-MB 231 (231; ER-), MDA-MB 468 (468; ER-), MCF-7 (ER+) and T-47D (ER+).
Methods. Cell proliferation was estimated by mitochondrial reduction of MTT, viability by ATP production, cell cycle progression by flow cytometry, apoptosis by caspase-3/7 activity and mitochondrial targeting with JC-1.
Results. CTU decreased MTT reduction in 231 cells after 24 and 48 hr of treatment to 58±2% and 25±1% of control at 10 μM (mean±SEM). ATP production in 231 cells was decreased to 38±3% and 29±4% of control after 24 and 48 hr of treatment, respectively (10 μM CTU). 231 cells were found to accumulate in G_0/G_1 phase when treated with 5 μM CTU, and fewer cells completed mitosis. Caspase-3 activity was strongly activated in 231 cells to 4.3±0.4-fold of control after 24 hr treatment at 10 μM. Using fluorescence microscopy, JC-1 staining indicated that the mitochondrial membrane potential in 231 cells was disrupted after only 4 hr of CTU treatment. In comparison, 468 cells and, in particular, MCF-7 and T-47D cells were less responsive to CTU.
Discussion. CTU was effective against the aggressive 231 breast cancer cells, exhibited intermediate activity against 468 cells and had lower activity in the MCF-7 and T-47D cell lines. Mitochondrial targeting by CTU, as reflected by JC-1 staining, decreased ATP production and increased caspase-3 activity, was most pronounced in 231 cells.

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Genetically-encoded calcium indicators for the assessment of intracellular calcium signalling in MDA-MB-231 breast cancer cells.

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Introduction. Genetically-encoded calcium indicators offer potential advantages over small molecule calcium sensors such as Fura-2 and Fluo-4 and are not susceptible to elimination by multidrug resistance ATPase I. These sensors are amendable to the assessment of calcium signals over long periods of time and in *in vivo* assays. GCaMP6 are ultra-sensitive genetically targeted calcium sensors, which can detect Ca^{2+} changes due to single action potentials *in vivo*. Few studies have assessed the use of genetically-encoded calcium indicators in cancer cells.
Aims. To compare the properties of the genetically-encoded calcium indicators GCaMP6s, GCaMP6m and GCaMP6f for the assessment of Ca^{2+} signals in MDA-MB-231 breast cancer cells.
Methods. MDA-MB-231 cells were plated into 96 well microplates and transfected with GCaMP6 using lipofectamine 2000. Adenosine trisphosphate (ATP) (0.01 μM - 10 μM) was used to elevate cytosolic free Ca^{2+} levels ($[Ca^{2+}]_{CYT}$). GCaMP6 fluorescence was assessed using an ImageXpress Micro (Molecular Devices) automated epifluorescent microscope with 472/30 excitation and 520/35 emission filters with images acquired at approximately 1/s. Fluorescence was quantified in 12.9 μm squares in the cytosol of 10 cells in each field.
Results. Optimised transient transfection conditions produced a transfection efficiency of approximately 7.5%. Addition of ATP produced increases in $[Ca^{2+}]_{CYT}$, which were detected by GCaMP6s, GCaMP6m and GCaMP6f. GCaMP6 calcium sensors allowed visualization of the heterogeneity of Ca^{2+} responses between individual MDA-MB-231 cells with differences in the time to reach peak $[Ca^{2+}]_{CYT}$ and recovery rates.
Discussion. The development of breast cancer cell lines stably expressing GCaMP6s, GCaMP6m and GCaMP6f holds promise in the assessment of calcium signals in breast cancer cells *in vitro* and *in vivo* during cellular migration and invasion. Further studies are required to define if particular processes in breast cancer cell lines are best assessed by GCaMP6s, GCaMP6m or GCaMP6f.

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Tumour microenvironment factors and the promotion of epithelial to mesenchymal transition in MDA-MB-468 breast cancer cells.

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Introduction. Epithelial to mesenchymal transition (EMT) in breast cancer cells involves the acquisition of a more invasive phenotype and is believed to be important in the development of metastasis. EMT results in the upregulation of mesenchymal cell markers including vimentin, N-cadherin, Twist, and the ratio of the stem cell markers CD44/CD24. Inducers of EMT include growth factors and conditions present in the tumour microenvironment such as epidermal growth factor (EGF). A recent study using conditioned media from wounded MDA-MB-468 breast cancer cells suggests that some tumour microenvironment factors may augment EGF-mediated increases in vimentin protein expression in MDA-MB-468 cells. However, effects on other EMT markers are unknown.
Aims. To determine whether conditioned media from wounded MDA-MB-468 breast cancer cells increases the sensitivity of MDA-MB-468 breast cancer cells to EGF-induced changes in EMT markers.
Methods. MDA-MB-468 cells were plated in 96 well plates and allowed to attach overnight. Cells were serum starved (24 h) before treatment with EGF (0 - 50 ng/mL) in the presence of conditioned media (from a wounded monolayer of MDA-MB-468 cells) or control media (from an intact monolayer of MDA-MB-468 cells). RNA was isolated 24 h after cell treatments. Real-time RT-PCR was used to compare vimentin, N-cadherin, Twist and CD44/CD24 mRNA levels in all samples.
Results. EGF treatment of MDA-MB-468 cells produced increases in the mRNA levels of the EMT markers vimentin, N-cadherin and Twist, and resulted in an increase in the CD44/CD24 mRNA ratio. EGF-mediated increases in vimentin mRNA levels appeared to be promoted by wound-conditioned media at submaximal concentrations of EGF (3 ng/mL). Some markers of EMT appeared to be insensitive to this induction (e.g. N-cadherin).
Discussion. These studies suggest that some markers of EMT may be differentially sensitive to agents that promote EGF-induced EMT in MDA-MB-468 breast cancer cells.

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Cruciferous vegetables and cancer prevention - A critical review

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Introduction. Breast cancer is the second leading cause of cancer death in Australia. Nutrition, particularly intake of cruciferous vegetables has been shown to reduce the risk of developing different types of cancers, including breast cancer. The anticancer effect of cruciferous vegetables has been attributed to their rich source of isothiocyanates (ITCs) and research over the last three decades has highlighted extensive scientific evidence for the efficacy of various ITCs against breast cancer from animal and *in vitro* studies.
Aims. The aim of the current study was to systematically review and evaluate the evidence derived from all types of studies published over the last two decades that examined the protective effects of ITC constituents from cruciferous vegetables on breast cancer.
Methods. The Medline and PubMed databases were searched for studies published in English, from January 1994 to May 2014. These studies examined the effects of cruciferous vegetables, such as broccoli, watercress, and their ITC constituents on breast cancer. The search terms used were: cruciferous vegetables, *Cruciferae*, Brassica vegetables, isothiocyanate, allyl isothiocyanate, phenethyl isothiocyanate, benzyl isothiocyanate, sulforaphane, mammary, breast, cancer, cancers, prevention, chemoprevention, human, animal, randomised controlled trials, controlled clinical trial, random allocation, clinical trials, case control, cohort, in vivo, and in vitro studies.
Results. A total of 58 studies were reviewed, of which 8 animal and 50 *in vitro* studies were identified. Literature evidence demonstrates the anti-breast cancer effects of benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PITC), and sulforaphane (SFN). These are the most extensively investigated compounds. Allyl isothiocyanate, erucin, and iberin have been reported to inhibit breast cancer cell proliferation in one *in vitro* study. No symptoms of ITC toxicity have been reported in the literature. Of the 8 animal studies identified, 5 studies demonstrated a significant reduction in the incidence and/or multiplicity of mammary tumours by SFN, BITC and PITC. No published human clinical studies on cruciferous vegetables and breast cancers were identified in the last two decades. Description of the identified studies were summarised in Tables (1 and 2), which can be provided on request.
Discussion. There is consistent scientific evidence derived from animal and cell culture work demonstrating protective effects of SFN, BITC, PITC on breast cancer. The reported mammary cancer protective properties are dose related, and there is substantial evidence which highlighted the importance of ER in the development of breast cancer. In summary, the findings suggested that the different molecular targets modulated by SFN, BITC, PITC compounds are useful indicators of success in future human breast cancer prevention trials.

Aras U et al (2013) Biopharm Drug Dispos. 34(2):98-106.
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Comparative Investigation of Plasma Glucose Lowering Activity and Effect on Liver, Kidney and Antioxidant Enzymes Parameters of Palmatine and Palmatine –like alkaloid Isolated from Stem of Consinum Fenestratum Plant on STZ Induced Diabetic Rat Model.

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Introduction: *Coscinium fenestratum(CF)* is a medicinal plant in the family of Menispermaceae. It is found in Southeast Asia and has been widely used as a medicinal plant. Our earlier report on crude and partially purified fraction E from the DCM stem extract of *CF*, has been shown to lower plasma glucose level, normalized lipid profile and antioxidant property. The HPLC-MS-UV profile of fraction E revealed the presence of Palmatine- like alkaloid (PLA). Palmatine is a protoberberine alkaloid which is commonly found in plants. It has been studied for its potential use in the treatment of jaundice, dysentery, hypertension, inflammation, and liver-related diseases. So far there is no any report on plasma glucose lowering, liver, kidney parameters and antioxidant enzymes effect of palmatine. Aims: Our study was aimed at comparing the effect of palmatine and palmatine- like alkaloid isolated from CF, on plasma glucose, haematology, biochemistry, lipid, SOD, GSH, LPO and CAT parameters on stz-induced diabetic rat model**.** Methods: Sprague dawley rats (250g) purchased from the Institute for Medical Research (IMR) Kuala Lumpur Malaysia, were injected with 35mg/kg of streptozocin (stz) to induce diabetes. After 1 week of observation, with plasma glucose level of 11mmol/L or 200mg/dL were orally administered with palmatine (2mg/kg) and PLA (100mg/kg) for 60 days, with the plasma blood glucose and body weight measured every week. After treatment, the rats were sacrificed and the blood and liver were harvested for general haematology, lipid profile, biochemistry and antioxidant enzyme parameter test Results: There was a significant reduction in plasma blood glucose level and increased in the body weight of the rats in the palmatine and PLA treated group. It was also observed that palmatine and PLA treatment normalised the haematology, lipid, biochemistry and antioxidant enzymes profile of the rat model compared to negative control. Discussion: The effect of palmatine and PLA were similar and maybe because of their antioxidant property.

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The expression of plasma membrane Ca^{2+} ATPase isoform, PMCA2, in breast tissue with lactational change and in breast cancer cells and the consequences of its silencing in MDA-MB-231 breast cancer cells.

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Introduction. The plasma membrane Ca^{2+} ATPase isoform, PMCA2, extrudes Ca^{2+} from the cytosol to the extracellular space. The expression of PMCA2 is mostly limited to cerebellar Purkinje cells and cochlear hair cells. In mouse and rat mammary glands, PMCA2 is highly expressed during lactation and is involved in the transport of Ca^{2+} into milk; however PMCA2 expression has not been assessed in human mammary glands during lactational change. PMCA2 mRNA levels are elevated in some breast cancer cells.
Aims. To assess PMCA2 expression in human breast tissue with lactational change and in breast cancer tissue. To investigate PMCA2 as a potential therapeutic target in breast cancer.
Methods. PMCA2 expression was assessed in breast tissues by immunohistochemistry. PMCA2 was silenced in the MDA-MB-231 breast cancer cell line using siRNA. The percentage of S-phase positive cells and cell number were evaluated by staining the siRNA treated cells with EdU (Click-iT EdU kit) and DAPI and using high-content imaging (ImageXpress Micro). The proliferation of cells treated with siRNA and doxorubicin was assessed by measuring the cell area using a kinetic imaging system (IncuCyte).
Results. PMCA2 expression was observed in the luminal epithelium of breast tissue exhibiting lactational change. Approximately 10% of breast tumours (9/96) exhibited PMCA2 staining on the plasma membrane. No correlation was seen with PMCA2 expression and the expression of estrogen, progesterone or HER2 receptor. Silencing of PMCA2 in MDA-MB-231 cells reduced total cell number and the percentage of cells in S-phase. PMCA2 silencing also promoted the anti-proliferative effects of doxorubicin in MDA-MB-231 breast cancer cells.
Discussion. Our findings suggest that PMCA2 protein expression is increased in human mammary glands during lactation and is a feature of some breast cancers. Inhibitors of PMCA2 may increase the effectiveness of therapeutics currently used to treat basal breast cancers and represent a new therapeutic approach for some breast cancers.

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Exploring the structure activity relationships of a series of agonists targeting the α4β2 nicotinic acetylcholine receptor

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Introduction. The nicotinic acetylcholine receptor α4β2 is the most abundant receptor subtype of the excitatory Cys-loop family of receptors in the mammalian central nervous system. Among other things, it is implicated in attentive and learning processes, and there is ample evidence of pro-cognitive effects of drugs targeting α4β2 in preclinical as well as clinical studies. The α4β2 receptor is known to express in two different stoichiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$, which sjow distinct pharmacology. The underlying structural reason for the stoichiometry-specific pharmacology was recently resolved with the discovery that the $(\alpha4)_3(\beta2)_2$ stoichiometry holds a third orthosteric binding site for ACh in the α4-α4 subunit interface. Hence, full activation of a $(\alpha4)_3(\beta2)_2$ receptor is achieved by ACh binding in three interfaces (two at α-β and one at α-α interfaces) whereas the $(\alpha4)_2(\beta2)_3$ receptor is fully activated by binding to only two interfaces (the two α-β interfaces).
Aims. In the present study, a series of closely related agonists ranging from partial to full agonists are tested for their activities at both stoichiometries of α4β2 receptors. In combination with computational docking studies the overall aim was to broaden the structure-activity understanding of how agonists interact with the two types of interfaces.
Methods. Functional activities of the agonists were measured in electrophysiological measurements using two-electrode voltage-clamp electrophysiology in *Xenopus laevis* oocytes. Docking studies were used to suggest binding modes and interpret the biological results
Results. Concentration-response relationships for the agonists will be presented along with docking results.
Discussion. The results reveal that seemingly small chemical differences in a highly related series of compounds can significantly alter how compounds interact with α-β and *vs.* α-α interfaces. The implication of this is that the structure-activity understanding at α4β2 receptors seen as comprehensive just few years ago is in fact incomplete. This underscores the importance of determining all parameters of novel compounds in future drug-discovery programs.

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Allosteric interactions of indole and benzimidazole derivatives at the human translocator protein

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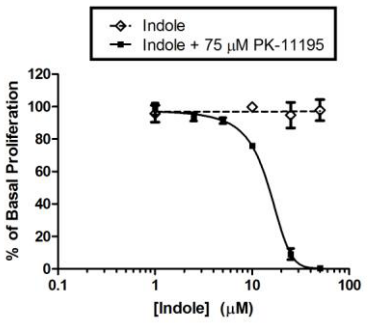
Introduction. The 18 kDa translocator protein (TSPO) has become a target for the development of drug treatments for anxiety and cancer. Limited knowledge of TSPO binding sites has hindered development of these drugs, however recent work suggests pyrazolo [1,5-*a*] pyrimidine acetamides may be a promising class of TSPO ligands (Scarf et al, 2009).
Aims. We aim to examine the effect of altering the number and position of nitrogen atoms around the pyrazolo [1,5-*a*] pyrimidine acetamide heterocyclic core on affinity and efficacy at the human TSPO.
Methods. Affinity was examined by competitive radioligand binding on human T98G glioblastoma cells using [^{3}H]-PK 11195, while a bromodeoxyuridine ELISA was used to examine anti-proliferative action.



Results. When nitrogen atoms were reduced to either 1 in the case of indoles or 2 in the case of benzimidazole, complex binding was displayed (n_H = -2.11±0.05 & -0.49±0.15 respectively, $p<0.05$ for $n_H \neq 1$). Compounds displaying complex binding also positively modulated the anti-proliferative ability of an IC_{10} concentration of PK 11195 without themselves affecting proliferation (Fig1). This behavior was absent in the pyrazolopyrimidine, imidazopyridine and purine heterocylic cores.
Discussion. We found evidence that changing the number and position of nitrogens in the heterocyclic core of pyrazolo [1,5-*a*] pyrimidine acetamide influences the ability of these derivative compounds to interact allosterically at the TSPO. This is the first evidence of allosteric behavior at the human TSPO and opens an avenue for the production of novel allosteric anxiolytics, anti-cancer and neuroprotective drugs.

Scarf A et al (2009) J Med Chem 52:581-592

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AMTB, a TRPM8 channel blocker also inhibits voltage gated sodium channels (Na_v) in breast cancer cells

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Introduction. Calcium permeable ion channels are aberrantly expressed in many cancers, including breast cancer. The calcium permeable ion channel, transient receptor potential cation channel subtype M, member 8 (TRPM8) has been identified as a potential therapeutic target in prostate cancer, however, few studies have looked at the effect of pharmacological inhibitors of TRPM8 ion channels in breast cancer cell lines.
Aims. To determine the effect of the TRPM8 inhibitor AMTB in MDA-MB-231 and SK-BR-3 breast cancer cells.
Methods. MDA-MB-231 and SK-BR-3 breast cancer cells were treated with 0.1 μM - 100 μM of the TRPM8 inhibitor, N-(3-aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)-benzamide hydrochloride hydrate (AMTB) for 72 hours. Viable cell number was approximated using an MTS assay. The effect of AMTB on voltage gated sodium channel (Nav) activity was assessed using a high-throughput FLIPR membrane potential assay in HEK-293 cells stably expressing hNav isoforms using the Na_v channel activator, veratridine. Real-time RT-PCR was used to determine mRNA levels of TRPM8 and Na_v subtypes 1.1-1.9 in breast cancer cell lines.
Results. AMTB (100 μM) decreased cell viability by greater than 70% in both MDA-MB-231 and SK-BR-3 cells. However, very low mRNA expression of TRPM8 transcripts was detected in these breast cancer cell lines. FLIPR assay analysis of AMTB, showed it inhibited voltage gated sodium channels. Real time RT-PCR assessment detected the presence of mRNA for Nav subtypes 1.3, 1.5, 1.7 in MDA-MB-231 and Nav 1.4, 1.6 in SK-BR-3 breast cancer cells.
Discussion. The TRPM8 inhibitor, AMTB inhibits the activity of voltage gated sodium channels expressed in breast cancer cells. The effects of AMTB in breast cancer cells is unlikely to be mediated by TRPM8. Further studies are required to assess the consequences of voltage gated sodium channel inhibition in MDA-MB-231 and SK-BR-3 breast cancer cells.

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High antioxidant Australian plant extracts: anti-proliferative activities against CaCo2 and HeLa cell lines

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Introduction. High levels of antioxidant phytochemicals have been linked to the treatment and prevention of several cancers. Recent reports have identified a number of native Australian fruits as having high antioxidant capacities. Despite this, several of these species are yet to be tested for anticancer activity.
Methods. Solvent extracts were prepared from high antioxidant native Australian plants and their antioxidant capacities were determined by the DPPH free radical scavenging assay. Anti-proliferative activities against CaCo2 and HeLa cancer cells were determined by an MTS based cell proliferation assay. Toxicity was determined using the *Artemia franciscana* nauplii bioassay.
Results. The methanolic and aqueous extracts of all plant species displayed high antioxidant contents (equivalent to 16-95 mg of vitamin C per gram of plant material extracted). In contrast, the ethyl acetate extracts for all species had relatively low antioxidant contents (generally below 5 mg of vitamin C equivalents per gram of plant material extracted). The antioxidant contents correlated with the ability of the extracts to inhibit proliferation of CaCo2 and HeLa cancer cell lines. The high antioxidant methanolic and aqueous extracts of all species were potent inhibitors of cell proliferation, with IC50 values generally below 1000 μg/mL. The aqueous *S. australe* fruit extracts were particularly effective, with IC50 values of 27 and 172 μg/mL against CaCo2 and HeLa cells respectively. In contrast, the lower antioxidant content ethyl acetate extracts generally did not inhibit cancer cell proliferation. Indeed, exposure of the cancer cells to most of the ethyl acetate extracts induced potent cell proliferation. The methanolic and aqueous Syzygium extracts displayed significant toxicity in the *Artemia franciscana* bioassay, with LC50 values below 1000 μg/mL. All other extracts were nontoxic.
Discussion. The antiproliferative activity of the high antioxidant plant extracts against HeLa and CaCo2 cancer cell lines indicates their potential in the treatment and prevention of some cancers.

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Macadamia nut as an anti-Giardial agent

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Introduction *Macadamia integriflora* (family Proteaceae) is an endemic Australian plant that has been used for thousands of years as a food. It is known to contain high levels of medium chain saturated fatty acids, raising the possibility that they may be useful in the treatment of Giardiasis

Methods. Macadamia nuts were extracted with various solvents and tested for inhibitory activity against the gastrointestinal protozoal parasite *Giardia duodenalis* using colorimetric cell proliferation assays. Toxicity was evaluated using an *Artemia franciscana* nauplii bioassay.

Results. Methanol, water and ethyl acetate extracts of macadamia nuts significantly inhibited *G. duodenalis* proliferation. The water extract was particularly potent, with an IC50 of 13.7 μg/ml. The methanol and ethyl acetate extracts, whilst less potent, also displayed good anti-Giardial activity (with IC50 values of approximately 125.2 and 143.5 μg/ml respectively). The chloroform and hexane extracts were ineffective as proliferation inhibitors, with no significant difference to the untreated control levels. With the exception of the water extract, all extracts were non-toxic or of low toxicity in the Artemia nauplii assay.

Discussion. The antiproliferative activity and low toxicity of these extracts indicate that macadamia nut may be useful in the treatment of Giardiasis.

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Biopharming *Carica papaya* compounds with anti-Proteus activity: The potential for the treatment and prevention of rheumatoid arthritis

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Introduction. Previous studies have reported field grown *Carica papaya* leaves to have antibacterial activity. However, *Carica papaya* has not been studied for the ability to block the bacterial triggers of autoimmune inflammatory diseases. Furthermore, studies into the bioactivity of the roots are lacking.

Aims. The current study was undertaken to test the ability of *Carica papaya* root extracts for the ability to block the microbial triggers of autoimmune inflammatory diseases and to use metabolomics fingerprint analysis to detect anti-inflammatory compounds.

Methods. *Carica papaya* roots were grown *in vitro* under controlled/standardised conditions. The roots were extracted with solvents of varying polarity and investigated for the ability to inhibit the growth of several bacterial triggers of autoimmune inflammatory disorders. The most promising extract was further analysed by RP-HPLC coupled to high accuracy TOF mass spectroscopy.

Results. The *Carica papaya* root extracts displayed potent inhibitory activity against the bacterial trigger of rheumatoid arthritis (*P. mirabilis*). However, no inhibition of the growth of the bacterial triggers of any other autoimmune disease was noted. The ethyl acetate, chloroform and hexane extracts were the most potent *P. mirabilis* inhibitors. Subsequent analysis of the *Carica papaya* root extracts by RP-HPLC coupled to high resolution TOF mass spectroscopy enabled the putative identification of a high proportion of the compounds present in the most potent ethyl acetate extract.

Discussion. The growth inhibitory bioactivity of *Carica papaya* root extracts against Proteus spp. indicates their potential in blocking the onset of rheumatoid arthritis.

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Tasmannia lanceolata extracts: anti-Proteus activity and potential for the treatment and prevention of rheumatoid arthritis

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Introduction. A wide variety of herbal medicines are used in indigenous Australian traditional medicinal systems to treat RA and inflammation. *Tasmannia lanceolata* (Tasmanian pepper) has received recent attention as a potential medicine due to its high antioxidant content (Cock 2013) and antibacterial activity (Winnet et al 2014).
Aims The current study was undertaken to test the ability of a panel of Tasmanian pepper extracts for the ability to block the microbial trigger of RA and to use metabolomics fingerprint analysis to detect anti-inflammatory compounds.
Methods. Tasmanian pepper berry and leaf were extracted with solvents of varying polarity and investigated for the ability to inhibit the growth of the bacterial trigger of RA (*P. mirabilis*). The extracts were tested for toxicity in the Artemia nauplii bioassay. The most potent inhibitor of *P. mirabilis* growth was further analysed by RP-HPLC coupled to high accuracy TOF mass spectroscopy.
Results. The Tasmanian pepper berry extracts were determined to be the most effective inhibitors of *P. mirabilis* growth, with MIC values as low as 11 and 126 μg/ml for the methanolic and aqueous extracts respectively. Subsequent analysis of the *T. lanceolata* fruit extracts by RP-HPLC coupled to high resolution TOF mass spectroscopy detected the resveratrol glycoside piceid and 2 combretastatin stilbenes in both *T. lanceolata* fruit extracts. All extracts were also shown to be non-toxic in the Artemia nauplii bioassay.
Discussion. The low toxicity of these extracts and their inhibitory bioactivity against Proteus spp. indicate their potential in blocking the onset of rheumatoid arthritis.

Cock IE (2013) Pharmacog Commn 3(4) pp 1-13.
Winnett et al (2014) Pharmacog Commn 4(1) pp. 42-52.

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Multi-dimensional IMAC: The capture of multiple bacterial metabolites from culture

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Introduction. Immobilised metal affinity chromatography (IMAC) is an aqueous-compatible separation technique. In previous studies (Braich et al, 2008; Gu et al, 2012), we demonstrated the utility of single-column IMAC for purifying metal-binding metabolites with important clinical applications, including the anticancer drug bleomycin and the siderophore desferrioxamine B (DFOB, used in iron chelation therapy).
Aims. The aim of this study was to improve upon IMAC for capturing metabolites so that multiple classes of metal-binding metabolites could be purified simultaneously. We have called this new approach 'multi-dimensional IMAC' (MD-IMAC). MD-IMAC involves joining in series two single IMAC columns, each containing resin charged with a different metal ion, with the goal of simultaneously separating and capturing two classes of bacterial metabolites. This has the potential to increase the efficiency of current methods of metabolite purification and discovery.
Methods. The first part of this study involved the development of the MD-IMAC setup to find the optimal resin conditions (chelator and metal ion) that would maximise the capture of each metabolite. The second part of this study involved culturing *Streptomyces verticillus* and using MD-IMAC to capture native bleomycin and DFOB.
Results. The optimised MD-IMAC setup consisted of a Yb(III)-charged upper resin and a Cu(II)-charged lower resin. Using this setup, two distinct metabolites (bleomycin and DFOB) were successfully captured from *S. verticillus* bacterial culture: DFOB on the Yb(III)-resin and bleomycin on the Cu(II) resin.
Discussion. Selectivity of the IMAC resin for a metal-binding metabolite is affected by the metal ion on the resin and the nature of the covalently-bound chelator attached to the resin beads. By varying these factors, we were able to tune each resin in the MD-IMAC setup to capture a different metabolite. We have been the first to develop MD-IMAC for the simultaneous capture of multiple metabolites. MD-IMAC could be used to purify useful metal-binding metabolites other than bleomycin and siderophores. This work has implications for green chemistry, streamlining pharmaceutics processing and metabolite discovery.

Braich N and Codd R (2008) Analyst 133: 877-880
Gu J and Codd R (2012) J Inorg Biochem 115: 198-203

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Isolating native doxorubicin from *Streptomyces peucetius* var. *caesius* culture using immobilised metal ion affinity chromatography

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Introduction. Doxorubicin is an important anticancer agent isolated from the culture of *Streptomyces peucetius* var. *caesius* (Arcamone et al, 1969). Current methods for the purification of this agent consists of a labour-intensive, multi-step process involving the use of large volumes of organic solvents (Arcamone et al, 1974). Due to the metal binding properties of doxorubicin (Beraldo et al, 1985, Guin et al, 2012), it was considered a potential candidate for capture using immobilised metal ion affinity chromatography (IMAC) – traditionally used in the isolation of recombinant proteins.
Aims. To capture doxorubicin from bacterial culture using IMAC.
Methods. The first part of this study involved developing optimum conditions on an IMAC column for capturing doxorubicin using a standard. The second part consisted of culturing *S. peucetius* on a solid agar plate, lysing the colonies and running the solution through an IMAC column. The presence of doxorubicin was determined using UV-Vis absorption spectroscopy (490 nm) and liquid chromatography/mass spectrometry.
Results. The optimised conditions for doxorubicin capture used a Ni(II)-charged resin with binding and elution buffers at pH values of 7.5 and 5.5, respectively. The binding capacity of doxorubicin was about 4.5 µmol/mL of resin. Doxorubicin was successfully isolated from bacterial culture using the IMAC methods in a yield of approximately 70%.
Discussion. This work was the first to use IMAC as a way of isolating doxorubicin, which is in restricted supply worldwide. This method has potential in industry, as it uses less steps and aqueous buffers, complying with the need for green chemistry.

Arcamone F et al (1969) Biotechnol Bioeng 11: 1101-1110
Arcamone F et al (1974) US-Pat. 3803124 A
Beraldo H et al (1985) Biochemistry 24:284-289
Guin PS et al (2012) J Coord Chem 65: 705-721

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Inhibitor activity of *Urena lobata* leaf extract on Dipeptidyl Peptidase IV (DPP-IV)

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Introduction. Recently, treatment for type 2 diabetes is focused to incretin hormone but this hormone is metabolized by Dipeptidyl Peptidase IV (DPP-IV) excessively into inactive form. The inhibition of DPP-IV can enhance incretin bioavaibility and regulate blood glucose level, therefore it would be beneficial in the treatment of type 2 diabetes. In the other hand, synthetic drugs of DPP-IV inhibitor for long term use has not been obtained complete data especially the safety. It induces the search of DPP-IV inhibitor compounds from herbs having less side effect. *Urena lobata* is plant growing in Indonesia and has been used to cure many diseases due to their biology activity.
Aims. To determine anti diabetic effect of *U. lobata* leaf extract on DPP-IV inhibitor activity.
Methods. In vitro study use *Gly-pro-p-nitroanilide* (GPPN) as substrate and Vildagliptin as a reference standard. Both ethanolic and water of *U. lobata* leaf extract were tested the activity of DPP-IV inhibitor. p-nitroanilida as product of reaction was observed with microplatereader wavelength 407 nm. All data are expressed as the mean ± SD and the IC-50 value was determined by linear regression curve.
Results. The ethanolic extract of *U. lobata* showed DPP-IV inhibitor activity stronger than water extract with an Inhibitory Concentration-50 (IC-50) value of 2355.43 and 7361.89 µg/ml respectively. Vildagliptin used as reference standart of DPP-IV inhibitor activity have IC-50 value 57.44 µg/ml.
Discussion. Water extract of *U. lobata* more applicable in use but the activity of DPP-IV inhibitor lower than ethanol extract. *U. lobata* leaf extract have inhibitory effect on DPP-IV related to active compound in this extract such as fitosterol and flavonoid groups. The inhibition of DPP-IV will increase bioavailability of incretin hormone that contribute to regulate blood glucose level through stimulation of insulin secretion, β cells proliferation and inhibition of glucagon. It might be potential to be an candidate of fitotherapy for the diabetes mellitus tipe 2.

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New histone deacetylase inhibitors from innovations in chemical biology

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Introduction. Inhibitors of the histone deacetylase enzyme family are an emerging class of anticancer drugs that act by increasing the transcription of genes associated with cell differentiation and apoptosis (Marks et al, 2009). This project aims to source new drug leads as inhibitors of Zn(II)-containing histone deacetylase from the parent compound desferrioxamine B (DFOB). Selected bacteria that produce DFOB for iron acquisition, including *Streptomyces pilosus*, co-exist with *Azospirillum irakense*, which is able to catabolise DFOB as a carbon source (Winkelmann et al, 1999). While DFOB itself does not display any inhibition of histone deacetylase, we have identified catabolic fragments including *N*-5-aminopentyl-*N*-(hydroxy)-succinamic acid (AHS) and *N*-5-aminopentyl-*N*-(hydroxy)-acetamide (AHA), as potential candidates for histone deacetylase inhibitors due to structural similarity to currently used inhibitors.
Aims. To biochemically source new histone deacetylase inhibitors by exploiting the catabolic potential of *A. irakense*.
Methods. Cultures of *A. irakense* were supplemented with 10 mg DFOB as the sole carbon source, with catabolism of DFOB confirmed by the diminution of absorbance values characteristic of Fe-DFOB and the metabolites of DFOB characterised by analytical HPLC-MS. Semi-preparative HPLC was used with a 6-20% ACN:H_2O gradient over 30 min and a 0.5 mL min^{-1} flow rate or purification.
Results. AHS and AHA were detected in the *A. irakense* culture supernatant after supplementation of 10 mg DFOB, with close-to-maximal concentrations of each at 42 hours. *A. irakense* cultures supplemented with 20 mg or 50 mg DFOB generated greater quantities of AHS and AHA, indicating potential to upscale this system for a greater yield. AHS and AHA were successfully purified from the *A. irakense* supernatant. These fragments were then assessed for the *in vitro* inhibition of HDAC group 1 enzymes.
Discussion. Overall, this study developed a biochemical method for the production of AHS and AHA that can be upscaled in future to generate greater yields of these potential histone deacetylase inhibitors.

Marks PA, Xu WS (2009) J Cell Biochem 107:600-608
Winkelmann G et al (1999) Biometals 12:255-264

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The potential role of nonulosonic acid analogues in drug discovery: The road to new antibacterial agents

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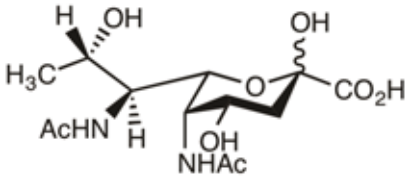
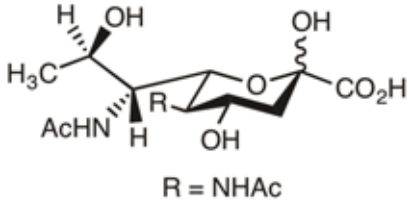
Bacterial infections by multi-drug resistant pathogenic bacteria are a global issue that not only affect people of all socio-economic backgrounds and age, but also place a major burden on the health systems of all countries[1]. Pseudaminic acid and legionaminic acid are 5,7-diamino-3,5,7,9-tetradeoxy-nonulosonic acids that play an essential role in the pathogenic capabilities of numerous clinically relevant drug resistant Gram-negative bacteria. The enzymes that process these important carbohydrates are attractive potential drug targets as they are found only within micro-organisms.
Currently, research findings within the literature clearly show that interfering with the biosynthesis of these carbohydrates is a potential opportunity for developing new anti-bacterials[2,3].
Therefore our research group has been actively investigating novel pathways for the synthesis of such compounds, which includes the synthesis of selectively functionalised analogues of pseudaminic acid and legionaminic acid which are intended for use are small molecule biological probes to gain a more thorough understanding of how Gram-negative pathogenic bacterium utilise these important endogenous compounds in order to become pathogenic.



Pseudaminic acid



R = NHAc

Legionaminic acid

1. Charles, P.G.P. & Grayson, M.L. (2004) *Med. J. Aust.* 181: 549-553.
2. McNally, D.J., *et al* (2008) *Chem. Med. Chem.* 3: 55–59.
3. Zunk, M. & Kiefel, M.J. (2014) *RSC Adv.* 4: 3413-3421.

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Differential regulation of the μ-opioid receptor by distinct agonists

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Introduction. The μ-opioid receptor (MOPr) has been extensively studied due to its importance as the main target of opiate analgesics. Recent data from our group shows differential regulation of the MOPr in a ligand-dependent manner and suggests that opioid agonists have distinct effects on the dynamics and signalling of this receptor. The spatiotemporal distribution of both receptor and signalling components is key for the generation of distinct and highly specialized GPCR-mediated responses.
Aims. It has recently been reported that morphine-activated MOPr is retained within a defined membrane microdomain resulting in a sustained increase in cytosolic extracellular signal-regulated kinase (ERK). In contrast, activation of MOPr by a prototypical high-efficacy agonist DAMGO ([D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin) causes receptor redistribution within the plasma membrane, transient increases in cytosolic and nuclear ERK, and receptor internalization. However, it remains unknown whether these effects are also observed when stimulating MOPr with endogenous opioids such as endomorphin or other opiate analgesics such as oxymorphone.
Methods. Biophysical techniques (Förster Resonance Energy Transfer (FRET) and Bioluminescence Resonance Energy Transfer (BRET)) and imaging approaches were used to investigate the trafficking and spatiotemporal signalling of MOPr upon activation by different agonists.
Results. Endomorphin-1 displays signalling and regulation profiles similar to DAMGO responses, promoting receptor internalization. In contrast, oxymorphone follows a pattern similar to morphine, restraining the receptor within the plasma membrane and inducing a similar ERK activation profile.
Discussion. Our results suggest that the ability of MOPr to translocate across the plasma membrane is ligand-dependent and linked to distinct signalling outcomes. Further studies to determine the protein complexes controlling differential MOPr signalling and regulation are required.

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Comparative pharmacology of allosteric modulators at the M_5 muscarinic acetylcholine receptor

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Introduction. Studies analysing M_5 muscarinic acetylcholine receptor (M_5R) knockout mice have indicated that this protein can regulate brain reward circuitry. As such, the M_5R may be an attractive target for drug dependence. However, the discovery of suitably selective orthosteric ligands has been limited to date by the high homology between muscarinic receptor subtypes.
Aims. The aim of the study was to characterise the pharmacology of recently identified positive (ML129 and ML380; Bridges *et al*., 2009; Gentry *et al*., 2014) and negative (ML375; Gentry *et al*., 2013) allosteric modulators (PAM and NAM) of the M_5R using radioligand binding and cell based functional assays.
Methods. ML129, ML380 and ML375 were assessed alone and in combination with orthosteric agonists in assays of inositol phosphate (IP) accumulation and [^{3}H]-*N*-methyl scopolamine ([^{3}H]-NMS) binding in whole FlpIn-CHO cells stably expressing the human M_5R (hM_5R). Data were analysed according to an allosteric ternary complex model (binding) and operational model of allosterism (functional assays; Leach *et al*., 2007).
Results. M_5R PAMs caused a concentration-dependent, saturable increase in the affinity of the orthosteric agonists ACh, carbachol or oxotremorine-M for the hM_5R (Logα values range 0.4 ± 0.2-1.3 ± 0.2). Such effects accounted for their ability to potentiate functional IP responses to the same agonists. Additionally, ML129 and ML380 displayed partial or full allosteric agonism, respectively, in their own right. Conversely, both compounds displayed neutral cooperativity with respect to pilocarpine affinity, but caused a 20-fold increase in pilocarpine signalling efficacy. ML375 acted as a NAM with respect to orthosteric agonist affinities and functional potencies in the IP assay. Despite increasing agonist affinity, allosteric modulators inhibited binding of the inverse agonist, [^{3}H]-NMS.
Discussion. ML129 and ML380 exert probe dependent potentiation of agonist-mediated hM_5R function, consistent with a two-state model. However, ML375 inhibits the binding of both agonists and inverse agonists suggesting a more complex mode of interaction.

Bridges *et al*. (2009) *Bioorg Med Chem Lett* **23**: 2996-3000
Gentry *et al*. (2013) *J Med Chem* **56**: 9351-5
Gentry *et al*. (2014) *J Med Chem* (in press)
Leach *et al*. (2007) *TiPS*. **28**: 382-89

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Identifying novel small molecules that enhance the bronchodilation and anti-inflammatory effect of Vasoactive Intestinal Peptide

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Introduction. Chronic Obstructive Pulmonary Disease (COPD) is a lung disease characterised by chronically poor airflow and is caused by persistent inflammatory responses to inhaled irritants. It is forecast to be the third leading cause of death by 2030 and current treatments are ineffective. Vasoactive intestinal peptide (VIP) is a widely distributed neuropeptide that has potent bronchodilation and anti-inflammatory effects. It is hypothesised that an agonist or positive allosteric modulator (PAM) of the VIP receptor (VPAC1-R) could lead to an increase in bronchodilation and reduction of inflammation in COPD patients.
Aims. To screen a focused set of 2,000 proprietary compounds supplied by Alchemia that were selected based on prior knowledge from previous exploratory research, to identify positive allosteric modulators of the VPAC1-R that will be triaged through a screening cascade, to test the efficacy of lead compounds in an *ex vivo* guinea pig trachea model and an *in vivo* cell-based inflammation model.
Methods. Cell-based high throughput cAMP accumulation assays (PerkinElmer Ultra LANCE, LANCE and AlphaScreen kits) were developed to screen compounds in VPAC-1R expressing CHO FlpIn cell lines. A kinetic CAMYEL biosensor assay was used as an orthologous screen to confirm hits, before the lead compounds were tested in an *ex vivo* guinea pig trachea relaxation model and an *in vivo* macrophage inflammation model measuring TNFα release.
Results. We identified a number of compounds that positively modulate the VPAC1-R using cAMP accumulation assays that were further ranked using a more sensitive kinetic assay. Positive modulation of the VIP response was confirmed using an *ex vivo* guinea pig trachea relaxation model measuring bronchodilation, and an anti-inflammatory model measuring inhibition of TNFα release.
Discussion. We have identified the first known positive allosteric modulators of VPAC-1R. The compounds positively modulate VIP relaxation in an *ex vivo* guinea pig bronchodilation model and enhance the inhibitory effect of VIP on TNFα release in an *in vitro* model, indicating anti-inflammatory effects. Further medicinal chemistry SAR studies are underway to identify more potent analogues.

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Phase I metabolism study of bacterial NorA efflux pump inhibitors

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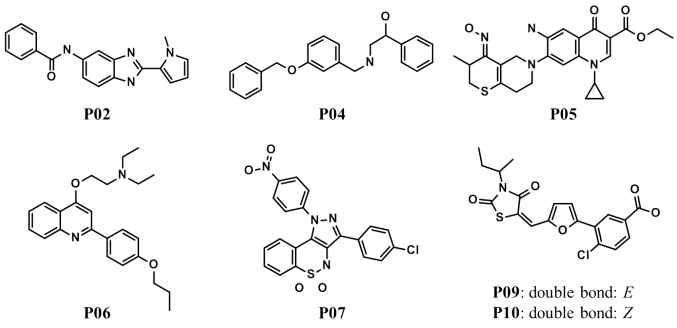
Introduction. Bacterial multidrug resistance (MDR) can be achieved by reduction of intracellular drug concentration through overexpression of efflux pumps such as *S. aureus* NorA, a key protein that reduces the concentration of fluoroquinolone drugs inside pathogens (Poole K et al, 2006). Molecules able to inhibit the activity of NorA have been recently discovered (Pieroni M et al, 2010; Brincat JP et al, 2011; Sabatini S et al, 2012).



Aims. Seven promising EPIs have been further developed, by studying in vitro their phase I metabolism.
Methods. Metabolic stability on Human liver microsomes was assessed through in vitro experiments. For those compounds with low half-life we also identified the major metabolites.
Results. The seven compounds showed different metabolic profile, from stable (P07 and P09) to intermediate (P02, P06, P10) and even largely unstable (P04, P05). Various metabolites have been identified for those that are unstable.
Discussion. Metabolite identification (MetID) was carried out both manually and with the use of new recent software, that is supposed to speed up the MetID process on pharmaceutical industry. The comparison between MassMetasite (Molecular Discovery Ltd) and UNIFI (Waters) is provided.

Poole K et al. (2006) Drug Discov Today: Ther Strategies, 3: 145-152.
Pieroni M et al.(2010) J Med Chem, 53: 4466-4480.
Brincat JP et al (2011) J Med Chem, 54: 354-365.
Sabatini S et al (2012) J Med Chem, 55: 3568-3572.

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Rosiglitazone induction of mouse brite adipocytes derived from subcutaneous white adipose tissue

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Introduction. Whereas classical white adipose tissue (WAT) stores chemical energy, brown adipose tissue (BAT) releases energy as heat. BAT thermogenesis thus represents a target for therapies aimed at reducing weight gain. Numerous studies have revealed functional BAT in adult humans, including inducible "brite" (brown in white) adipocytes that express uncoupling protein UCP1 and the BAT transcriptional regulator Prdm16, but also the WAT marker Hoxc9. Brite differentiation can be induced by the PPARγ activator rosiglitazone (Petrovic et al, 2010).
Aims. We determined the effect of rosiglitazone (Rosi) on gene expression profiles and UCP1 protein distribution in primary mouse adipocytes derived from the stromal vascular fraction of interscapular BAT, subcutaneous inguinal WAT (iWAT) and epididymal WAT (eWAT).
Methods. Adipocytes were cultured for 7 days in the presence of 2.4 nM insulin, plus or minus 1 μM Rosi. Gene expression was measured using a custom qPCR array and UCP1 protein was detected by immunocytochemistry.
Results. UCP1 mRNA was 375-fold higher in Rosi versus control BAT cultures (98 vs 0.3% relative to Actb), and 1200-fold higher in Rosi-treated iWAT cultures (4.6 vs 0.04% relative to Actb). Immunocytochemistry indicated that Rosi-treated iWAT cultures consist of a mixed cell population with varying levels of UCP1 protein. eWAT cultures displayed much lower induction of UCP1 mRNA or protein. Despite the heterogeneity of Rosi-treated iWAT cultures with respect to UCP1, both iWAT and BAT cultures displayed similar expression of key adipocyte and thermogenic markers, including Fabp3, Fabp4, Cpt1b, Pgc-1α, PPARα, Acadl, Sirt3 and Trib3. Overall, Rosi-induced changes in iWAT gene expression were biased toward genes encoding mitochondrial proteins and transcription factors critical to thermogenesis, fatty acid uptake and metabolism, and energy production.
Discussion. Our data indicate that cells from subcutaneous iWAT undergo Rosi-induced brite differentiation, but that high-level, homogeneous UCP1 expression requires further activating stimuli.

Petrovic N et al (2010) J Biol Chem 285: 7153-7164

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Discovery and optimization of novel, highly selective M_5 mAChR allosteric modulators

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Introduction. The muscarinic acetylcholine receptors (mAChR) are a family of class A G protein-coupled receptors comprised of five subtypes (M_{1-5}) expressed throughout the CNS. These receptors play a vital role in regulating a wide range of physiological functions. The design of M_5 mAChR selective ligands is challenging due to the conserved nature of the orthosteric pocket across mAChR subtypes. Activation of this receptor has been particularly implicated as a potential target in the treatment of chronic cerebrovascular diseases, whereas inhibition of the M_5 mAChR may provide novel therapies for the treatment of addictive behavior. Aims. The objective of this project was to obtain potency optimized, highly selective allosteric modulators for the M_5 mAChR in order to provide novel chemical probes for the elucidation of this receptor's role in the CNS. Methods. A high throughput screen for M_5 mAChR-specific ligands was performed on the Molecular Libraries Probe Center Network screening deck. This effort revealed a weakly active M_5 mAChR positive allosteric modulator (PAM) scaffold along with a scaffold identified as an M_5 mAChR inhibitor. The structure-activity relationships (SAR) of the lead chemical scaffolds were subsequently optimized using iterative parallel synthesis techniques. The activity of the synthesized compounds was tested via Ca^{2+} mobilization assays. Results. SAR optimization of the PAM scaffold, VU0472882 (hM_5 EC_{50} > 10 μM), yielded an M_5 mAChR-preferring PAM with a >50-fold improvement in potency, ML380 (hM_5 EC_{50} = 190 nM, hM_3 EC_{50} = 2.1 μM, hM_1 EC_{50} = 5.4 μM, $hM_{2,4}$ EC_{50} > 30 μM). The inhibitor scaffold, VU0352221 (hM_5 IC_{50} = 3.5 μM) was similarly optimized, resulting in ML375 (hM_5 IC_{50} = 300 nM, hM_{1-4} IC_{50} > 30 μM). Furthermore, radioligand binding experiments have revealed ML375 to possess an allosteric mode of pharmacology; thus, ML375 represents the first M_5 mAChR-selective negative allosteric modulator (NAM). Discussion. The M_5 mAChR PAM, ML380, and NAM, ML375, represent novel, highly potent, and selective probes for the study of this receptor. The selectivity and unique modes of pharmacology of these probes make them invaluable for a variety of in vitro studies into M_5 mAChR structure and function. At present, further chemical optimization efforts are underway for both scaffolds with the goal of producing a second generation of M_5 mAChR-selective allosteric modulators with optimized PK properties in order to furnish tool compounds for in vivo studies.

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Probing the structure and dynamics of a class A G protein-coupled receptor using small-angle X-ray scattering.

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Introduction. A novel GPCR drug screening approach using a nanofluidic device is currently being developed. This approach involves the use of a nanofluidic device to measure translocation of target molecules (GPCRs) through a nanopore approximately 10-50 nm diameter. The translocation time of the molecule through the nanopore is determined by its size, charge and shape, thus it is imperative that the size and shape of the target molecule is known. The neurotensin 1 receptor (NTS_1) has been engineered to be stable in detergent, making it ideal for structural studies. X-ray crystallography and NMR spectroscopy yield high-resolution information for the receptor, but structural information of the receptor/detergent complex as a whole is lost. For use in nanofluidic devices as proposed here, this information is crucial. Small-angle X-ray scattering (SAXS) is an ideal technique to investigate the size and shape of the NTS_1 solubilised in detergent as the whole receptor/detergent complex can be observed.
Aims. To characterize the protein-detergent complex (PDC) formed when GPCRs are extracted from the cell membrane and solubilized in detergent. Different detergents produce varying PDC shapes and sizes, and understanding the size and shape of PDC is crucial when introducing GPCRs into the nanopore.
Methods. NTS_1 solubilised in *n*-decyl-β-D-maltopyranoside has been purified using affinity chromatography and size-exclusion chromatography. Affinity chromatography coupled to SAXS at the Australian Synchrotron was then used to investigate the size and shape of the PDC. Coupling affinity chromatography directly to a SAXS beam line is completely new and is the perfect technique for the NTS_1 system.
Results. The scattering profile of the PDC showed a large micellar species with a characteristic peak at 0.18 $Å^{-1}$, indicative of a DM micelle. Additionally, SAXS has shown that micelles are stable at acidic and basic pH, but unstable in high salt.
Discussion. Further studies in this area will extend to the use of other detergents such as n-octyl-β-D-glucoside, which will be important for crystallisation experiments concurrently underway in the lab.

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Identifying novel small molecules that enhance the insulinotrophic effect of glucagon-like peptide-1 (GLP-1)

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Introduction. The actions of GLP-1 on the GLP-1 receptor (GLP-1R) play a major role in regulating insulin secretion and thus maintaining glucose homeostasis within the body. The physiological effects of GLP-1R activation have made the receptor a major therapeutic target for treatment of type 2 diabetes mellitus (DM) and associated obesity. There are currently 3 FDA-approved peptides that act via the GLP-1R available for the treatment of DM however their use has been limited to injection and the appearance of adverse side effects in some patients. An orally active small molecule that acts as an allosteric modulator of the GLP-1R, thereby enhancing insulin secretion only in the presence of the endogenous GLP-1 peptide, would be a major advance for the treatment of DM.
Aims. Screen a 20,000 compound diversity scanning library supplied by Alchemia to identify positive allosteric modulators of the cAMP signaling pathway in GLP-1R expressing cells. Confirm hit compound activity against insulin release from a pancreatic β cell line (INS). Assess top hits in isolated mouse islet cells.
Methods. cAMP accumulation assays were carried out in a GLP-1R expressing CHO Flp-In cell line and INS cells using the PerkinElmer LANCE kit. Insulin release assays were carried out in INS cells and isolated mouse islet cells using the CisBio HTRF insulin detection kit.
Results. Hit compounds identified in the screen showed only modest effects on cAMP release in GLP-1R expressing cell lines and no effect on cAMP release in INS cells. Top hits were tested for their effects on glucose-dependent insulin release in INS cells. Several agonists of insulin release were identified and two compounds showed positive allosteric modulation of the GLP-1(7-36)-induced insulin release from INS cells. Compounds that increased cAMP release but inhibited insulin release were also identified.
Discussion. We have identified a suite of compounds with differing effects on glucose-dependent insulin release. They will provide a valuable tool for the investigation of the complex signaling pathways underlying the therapeutically beneficial effects of GLP-1 and contribute towards the development of an orally available treatment for type 2 diabetes.

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A novel role for mammalian target of rapamycin complex 2 (mTORC2) in brown adipose tissue (BAT) glucose uptake mediated by β-adrenoceptors (AR).

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Introduction: BAT is the primary site for thermogenesis and can consume, in addition to free fatty acids, a high amount of glucose from the blood that can both acutely and chronically affect glucose homeostasis. Therefore BAT may be a potential therapeutic target for a number of metabolic disorders dependent on glucose homeostasis, such as type 2 diabetes. Recent data indicates a role of mTORC2 in glucose homeostasis, with adipose-specific ablation of rictor, a mTORC2component, depressing insulin-stimulated glucose uptake *in vitro* and impairing glucose tolerance *in vivo* (1). Mice with adipose specific deletion of raptor, a mTORC1component, are resistant to diet-induced obesity and are insulin sensitive (2), indicating vastly different roles for mTORC1 and mTORC2 in adipose tissues.
Aims: To investigate the role of mTOR on sympathetic stimulation of glucose uptake in BAT.
Methods: Mouse brown adipocytes or human multipotent adipose-derived stem cells were used. The role of mTOR in β-AR mediated increases in glucose uptake, GLUT1 transcription and translocation was assessed by ablation of rictor or raptor using siRNA or pharmacological inhibition of mTOR by specific mTOR inhibitors.
Results: β-ARs increase glucose uptake in BAT via two different signaling pathways: one part is dependent upon cAMP mediated increases in GLUT1 transcription and translation, and another part is dependent on mTORC2 stimulated translocation of GLUT1 to the plasma membrane, leading to increased glucose uptake. Both parts are essential for β-AR-stimulated glucose uptake. Importantly, β-AR effects via mTORC2 are separate from the classical insulin-phosphoinositide 3-kinase-Akt pathway, highlighting a novel mechanism of mTORC2 activation.

(1) Kumar et al Diabetes 59:1397-1406 (2010)
(2) Polak et al Cell Metab 8:399-410 (2008)

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Monitoring angiotensin II receptor complexes using bioluminescence resonance energy transfer

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Introduction. The renin-angiotensin system is involved in the regulation of blood pressure and fluid balance. Its major effector, the hormone angiotensin II, acts upon the angiotensin II type 1 receptor (AT_1 receptor) and the angiotensin II type 2 receptor (AT_2 receptor). The AT_1 receptor is a well-characterised G protein-coupled receptor that mediates most of the well-known biological effects of angiotensin II such as hypertension and antinatriuresis. In contrast, the molecular and physiological functions of the AT_2 receptor remain poorly understood, though it is often believed to counteract many AT_1 receptor-mediated effects. In order to understand the molecular and physiological properties of these receptors, it is necessary to understand how they interact, and the functioning of the resultant complex, within the cellular environment.
Aims. The aim of this study was to investigate the interactions of the AT_1 and AT_2 receptors, and elucidate the properties of the resultant multi-component complex.
Methods. Bioluminescence resonance energy transfer (BRET) was used to investigate interactions. BRET is a widely used technique for monitoring very close proximity in live cells in real time. Receptors and their interactors were tagged with either BRET donor luciferase enzymes or BRET acceptor fluorophores and transiently transfected in HEK293FT cells.
Results & Discussion. This study has enabled elucidation of the AT_1-AT_2 receptor complex and its potential role in the context of broader functional interactions with various partners.

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Rapid Selection and Development of GPCR expressing Mammalian cell lines using novel ClonePix Technology

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The G-protein-coupled receptor (GPCR) family represents the largest and most versatile group of cell surface receptors. GPCRs play a pivotal role in cell signaling and are targets of approximately 50% of all pharmaceutical drugs in the marketplace. Identifying key modulators of GPCRs and developing cell based screening technologies aimed to identify novel drugs against both known and orphan GPCRs are of utmost significance in drug discovery. The endogenous expression of GPCRs in mammalian cells is typically very low with generally no more than 3,000 copies per cell and thereby presents a challenge with respect to current GPCR screening efforts. Most screening assays require a much higher concentration of functional GPCRs presented on the cell surface. Attempts to create expression systems in “lower” organisms have been met with limited success due to inefficient folding (bacteria), low yield (yeast) or incorrect post-translation modification (baculovirus). These challenges incite the market need to utilize mammalian expression systems capable of providing requisite GPCR protein expression levels. However, developing mammalian cell lines to express target proteins using traditional methods such as limiting dilution is a laborious process. Further functional validation of the cell lines poses additional time and resource challenges. We present here a one-step solution and automated high-throughput platform technology that would aid in the identification and isolation of clones from large heterogeneous pools of transfected cells. Utilizing white light and fluorescent based imaging in situ, the clone selection system has both the sensitivity and specificity to quantitatively detect the endogenous cell surface protein expression levels of respective GPCRs. The fluorescent based automated system can be efficiently used to detect and pick respective GPCR expressing clones, and thereby provide a unique source of GPCR proteins for variety of applications, including antibody generation using antigens with natural epitopes at high expression level and cell-based functional assays for hard-to express GPCRs. Moreover, establishing reliable cell lines expressing GPCRs of interest would provide great advantage in characterization studies of receptor mediated signaling and screening campaigns of novel therapeutic drug candidates.

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Expression and Purification of Human Calcitonin Receptor

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Introduction. G protein-coupled receptors (GPCRs) play vital roles in a range of biological processes and are attractive targets for therapeutic drugs. In recent years, there have been significant advances in both structural and biophysical techniques that have lead to high-resolution structures of an increasing number of GPCRs. Despite these recent advances, structural studies of GPCRs remain a significant challenge due to difficulties in producing high levels of stable receptor. To date, there are no full length Family B GPCR structures and only two family B GPCR transmembrane domain structures have been reported. The human calcitonin receptor (hCTR) is a family B GPCR that mediates responses to the peptide hormone calcitonin. To advance our understanding of the mechanism of family B GPCRs, we have optimised conditions to overexpress and purify hCTR for structural studies.
Aims. To overexpress and purify hCTR for structural studies
Methods. A hCTR construct bearing an N-terminal FLAG-tag and a C-terminal His-tag was cloned into a baculovirus vector for insect cell expression. Receptor expression was determined by radioligand binding. The receptor was purified by immobolised metal affinity chromatography followed by FLAG antibody affinity chromatography in the presence of dodecyl-d-maltopyranoside and cholesterol. Receptor purity was analysed by SDS-PAGE and receptor monodispersity was assayed by size exclusion chromatography
Results. hCTR expression in insect cell was optimised with yields of approximately 10 nmol/L. hCTR can be purified to near homogeneity using affinity chromatography followed by size exclusion chromatography. Purity was found to be >95 % assessed by SDS-PAGE. Preliminary results revealed purified hCTR was able to couple to Gαs heterotrimer the presence of peptide agonist.
Conclusion. The ability to produce high amount of receptor and G protein complex for structural studies will aid in understanding family B GPCR function.

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Pharmacological characterization of a muscarinic acetylcholine M_1 receptor positive allosteric modulator MIPS1463

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Introduction. Activation of the muscarinic acetylcholine M_1 receptor (M_1R) is a potential approach for the treatment of cognitive impairment, such as in Alzheimer's disease. Due to the high homology in the orthosteric binding site of muscarinic receptor subtypes, selective M_1R activation can be obtained more easily by targeting less conserved allosteric binding sites.
Aims. This study aimed to characterize the *in vitro* pharmacology of a novel M_1R positive allosteric modulator, MIPS1463.
Methods. [^{3}H]N-methylscopolamine binding and inositol phosphate accumulation assays were utilized to study the pharmacology of MIPS1463 at M_1R expressed heterologously in CHO cells, or endogenously in murine primary neuronal cells.
Results. MIPS1463 displayed binding affinity with a pK_b value of 5.56±0.10 and selectively potentiated the binding affinity of acetylcholine at M_1R (binding cooperativity with a log α value of 2.84±0.13) as well the potency of acetylcholine in inducing the accumulation of inositol phosphate. Furthermore, it displayed direct allosteric agonism in both recombinant and neuronal cells, in addition to modulating the cognate agonist.
Discussion. MIPS1463 is a new selective M_1R allosteric positive modulator (PAM) that is more potent than the current exemplar M_1R PAM, benzyl quinolone carboxylic acid (BQCA). Having shown activity in murine neuronal cells, MIPS1463 represents a potentially useful pharmacological tool for further M_1R *in vivo* studies.

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Investigating the molecular determinants of allosteric modulation at the adenosine A_1 receptor.

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Introduction. The adenosine A_1 receptor (A_1AR) is an important therapeutic target for a number of cardiovascular and neuronal conditions (Jacobson *et al*, 2006). However, effective targeting has remained elusive due to its widespread distribution and high conservation of the orthosteric adenosine-binding pocket. A_1AR allosteric modulators interact with a topographically distinct binding site from that of adenosine and offer a number of theoretical advantages, including increased subtype selectivity and preservation of the spatial and temporal pattern of endogenous agonist signalling. To facilitate the rational design of more selective and efficacious A_1AR allosteric ligands, greater structural knowledge of the allosteric binding site is required.
Aims. To probe the key residues involved in conferring A_1AR allosteric ligand affinity and cooperativity.
Methods. Mutant A_1ARs containing single alanine substitutions were stably expressed in FlpINCHO cells. Radioligand binding interaction studies between the orthosteric agonist, NECA, and the allosteric ligand, PD81723, quantified the influence of mutations on allosteric ligand affinity and cooperativity. Molecular dynamic simulations were performed using a 3D model of the human A_1AR based on an active $A_{2A}AR$ structure (PDB ID: 3QAK).
Results. Substitution of $F171^{ECL2}$, $E172^{ECL2}$ for alanine resulted in a significant reduction in PD81723 affinity. The mutations $V87^{3.32}A$, $Q92^{3.37}A$, $N184^{5.42}A$, $K168^{ECL2}A$, $E170^{ECL2}A$, $K173^{ECL2}A$, and $H251^{6.52}A$ significantly reduced the allosteric cooperativity between NECA and PD81723. The mutations, $V87^{3.32}A$ and $Q92^{3.37}A$ also significantly decreased NECA affinity.
Discussion. A_1AR mutagenesis and molecular modelling suggest that $V87^{3.32}$, $Q92^{3.37}$, $N184^{5.42}$ and $H251^{6.52}$ have an important role in conferring conformational rearrangements upon activation through the formation of hydrophobic and hydrogen bonds with NECA, while $E172^{ECL2}$ forms a hydrogen bond and $E171^{ECL2}$ forms an aromatic stacking interaction with PD81723. The extracellular residues, $K168^{ECL2}$, $E170^{ECL2}$, $E172^{ECL2}$, $K173^{ECL2}$, form hydrogen interactions with one other, which may explain their role on the transmission of allosteric cooperativity.

Jacobson K et al (2006) Nat Rev Drug Discov 5:247-264

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Potent non-peptide agonists for human C3a receptors

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Introduction. C3a is a product of complement activation and has been implicated in the pathogenesis and progression of numerous inflammatory conditions such as asthma, allergies, arthritis and others[1]. To date, there is no potent and selective non-peptide agonist or antagonist of C3a receptor (C3aR), which can be used as a pharmacological probe for interrogating the roles of human C3a in physiology and disease.
Aims. To explore new compounds containing an oxazole ring that can potentially impart and stabilise a turn structural motif by replacing an amino acid and the N-terminus of this scaffold.
Methods. Human monocytes were isolated using Ficoll-paque density centrifugation from buffy coat of human blood. $CD14^+$ monocytes were positively selected using $CD14^+$ and differentiated to macrophages (HMDMs) using M-CSF. Compound activity was assayed by radioligand binding for affinity and selectivity and by intracellular calcium release for function.
Results. A study of N-acyl amino acid-oxazole-arginine compounds showed that the optimal amino acid side chain for attachment to the oxazole scaffold was leucine (56) or isoleucine with only slightly different binding affinity (pIC_{50}=7.71±0.08 versus 7.53±0.10) and potency (pEC_{50}=7.72±0.27 versus 7.57±0.11). Further modification of the leucine terminus (56) with an indole ring gave the greatest improvement in affinity and potency (pIC_{50}=8.03±0.05; pEC_{50} =8.15±0.10) in calcium mobilisation assay. In addition, one of the most potent C3a agonists had an isoleucine side chain and a bromine substituent on the pyridine ring. This significantly improved binding affinity and was one of the most potent agonists in this series with pIC_{50} = 8.30±0.10 and pEC_{50} = 7.76±0.36.
Discussion. Introduction of a rigid turn-like conformation enforced by an oxazole heterocycle has produced multiple potent and selective C3aR non-peptide agonists (EC_{50}< 20 nM, HMDMs). These compounds could be useful for probing the physiological roles of C3a, which is highly unstable *in vivo* losing its C-terminal arginine and no longer binding to C3aR. By contrast the small molecule agonists reported here are extremely stable.

[1]Haas PJ and Van Strijp J (2007) Immunol Res 37(3):161- 175

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Fluorescently labelled ligands targeting the dopamine D_2 receptor

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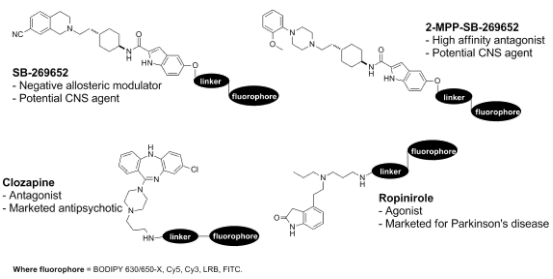

Introduction. Fluorescently labelled ligands (FLLs) are useful tools for exploring G Protein-Coupled Receptor (GPCR) function such as receptor- ligand interactions, receptor internalization, compartmentalization and trafficking (Vernal et al, 2014). FLLs can be adapted towards competition-based binding assays that may be used for screening for new compounds and scaffolds (Stoddart et al, 2012). The dopamine D_2 receptor (D_2R) is implicated in multiple CNS disorders. However, there is a lack of fluorescent ligand tools with which to investigate the function of this important therapeutic target.
Aims. To functionalize a range of ligands of differing pharmacology with organic fluorophores and pharmacologically evaluate them at the D_2R.
Methods. Chemical synthesis of FLLs. Pharmacological evaluation in radioligand binding assays and confocal microscopy using FlpIn CHO cells stably expressing dopamine D_{2L} receptors.
Results. The 2-MPP-SB269252 FFLs maintained high binding affinity for the D_2R (best analogue pK_i = 7.88 ± 0.20 (13 nM)). Three FFLs showed selective binding to D_{2L} receptors using confocal microscopy.
Discussion. We identified three novel FFLs with high binding affinities that could be utilised in applications such as competition based binding assays as an alternative to radioligand binding assays for the D_2R.

Vernal A. J. et al (2014) Brit. J. Pharmacol. 171:1073-1084.
Stoddart L.A. et al (2012) Chem. Biol. 19:1105-1115.

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Designed multiple ligands targeting the dopamine D_2 and muscarinic M_1 receptors.

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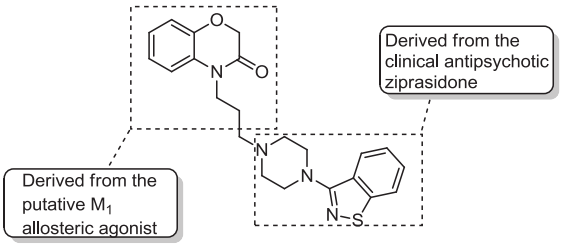

Introduction. Atypical antipsychotics display marked polypharmacology. Although this is thought to confer favourable efficacy, it was achieved through serendipitous discovery rather than by a rational drug design process. Activity at dopamine D_2 and serotonin 5-HT_{2A} receptors has been implicated as useful for antipsychotic efficacy. Additionally, the M_1 muscarinic acetylcholine receptor (mAChR) is highlighted as important for the cognitive deficits in key CNS disorders. The concept of designed multiple ligands (DMLs) takes two pharmacophores and integrates the two into a single molecule (Morphy et al, 2009). Therefore this approach can be used as a rational approach to achieving desirable polypharmacology.
Aims. To use D_2 privileged structures derived from putative and clinically relevant antipsychotics and hybridize with a M_1 scaffold to develop a series of DMLs.
Methods. Chemical synthesis and pharmacological evaluation of ligands in both functional (D_2 and M_1) and radioligand binding assays (D_2, M_1 and 5-HT_{2A}).
Results. The most promising DML displayed an activity profile at all three receptors; M_1 (pEC_{50} = 5.98 ± 0.25 (1 µM), E_{max} = 64 ± 10), D_2 (pK_i = 7.75 ± 0.10 (18 nM)) and 5-HT_{2A} (pK_i = 8.24 ± 0.37 (5.8 nM)).
Discussion. We designed and characterized a novel DML using a privileged structure derived from the antipsychotic ziprasidone, that retained strong binding and functional activity at the D_2R, weak partial agonism at the M_1 mAChR and a high affinity for the 5-HT_{2A}R. This approach highlights the utility of privileged structures in drug discovery.

Morphy R et al (2009) Curr. Pharm. Design 15:587-60

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Non-selective opioid receptor modulation of Beta-endorphin1-11 in cAMP accumulation

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Introduction: Opioid receptors are G protein-coupled receptors that modulate cAMP through action on adenylyl cyclase. Beta-endorphin (BE1-31) is a major endogenous opioid peptide with affinity for both mu and delta opioid receptors (MOP and DOP). This peptide is biotransformed rapidly *in-situ* producing an array of bioactive fragments. Beta-endorphin 1-11 (BE1-11) is one of the major peptide fragments produced from this biotransformation and may itself be involved in an array of biological actions. Opioid peptide fragments of BE1-31 have been shown to provide less selectivity for MOPs, potentially indicative of different pharmacological effects dependent upon the nature of the specific receptor interactions. This study examined the effect of BE1-11 on the inhibition of cAMP production in Human Embryonic Kidney 293 (HEK) cells over expressing MOP and DOP, (HEK-MOP,HEK-DOP) and a native cell line (retinoic acid differentiated SHSY5Y) with phenotypically neuronal properties.
Aim: To investigate the inhibitory action of BE1-11 through MOP and DOP action in HEK-MOP, HEK-DOP and differentiated SHSY5Y cells on the forskolin induced cAMP accumulation assay.
Method: Changes in cAMP accumulation were assessed at a range of concentrations of BE1-11 (0.1nmol/L – 1µmol/L). Forskolin was used to stimulate cAMP accumulation, assessed using the Alpha-screen cAMP assay (Perkin Elmer™). The antagonism effects of the opioid antagonists, CTAP (MOP antagonist) and naltrindole (DOP antagonist) were examined for confirmation of cAMP modulatory effect of BE1-11 on MOP and DOP.
Results: A concentration-dependent decrease in cAMP accumulation was observed with increasing BE 1-11 concentrations in HEK-MOP, HEK-DOP and differentiated SHSY5Y cells. The EC50 values of BE1-11 in HEK-MOP and HEK-DOP cells were determined to be 18 and 21 nmol/L respectively. In addition, the EC80 for BE1-11 in differentiated SHSY5Y cells was 1µmol/L; cAMP inhibition action of BE 1-11 was antagonised by CTAP and naltrindole independently.
Discussion: In this study we have shown that BE1-11 not only has equal efficacy in modulating cAMP in HEK-MOP and HEK-DOP cells, but also reduces cAMP accumulation in a native cell line, differentiated SHSY5Y cells, through both receptors.

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Modulation of the M4 Muscarinic Acetylcholine Receptor Regulation by Allosteric Ligands

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Introduction. The M4 muscarinic acetylcholine receptor (mAChR) is predominantly expressed within the CNS. Importantly, M4 mAChRs represents a potential pharmacological target for drug therapies in schizophrenia and Alzheimer's disease (Langmead, 2008). More recently, M4 mAChR drug discovery has focused on the development of allosteric modulators of this receptor as means to achieve more selective drugs.
Aims. To investigate the effects of typical orthosteric ligands and novel allosteric ligands on M4 mAChR regulatory processes.
Methods. β-Arrestin-YFP recruitment upon incubation with different orthorsteric and allosteric ligands to a c-terminus tagged Renilla Luciferase (RLuc) M4 mAChR will be measured by BRET in transfected CHO and NG-108 cells treated with and without pertussis toxin (PTx).
Results. Preliminary results show that while canonical M4 mAChR signalling pathways are dependent on Gαi proteins, β-Arrestin-1 and -2 recruitment, is independent of Gαi protein in both CHO and NG108-15. This finding indicates that β-Arrestins recruitment does not require activation of Gαi proteins. Furthermore, the allosteric M4 mAChR ligand (LY2033298) has the capacity to potentiate the potency of ACh for these assays in a dose-dependent manner.
Discussion. Given the therapeutic potential of M4 mAChR in locomotion, cognition and psychosis-associated disorders, understanding the mechanisms behind the regulation of this receptor upon orthosteric and allosteric ligands is of key importance. Our results demonstrate that β-Arrestin recruitment to M4 mAChR, which traditionally precedes receptor internalization, is independent of Gαi protein activation. Future studies will focus on a detailed characterisation of the role of β-Arrestin in M4 mAChR signalling.

Langmead C J et al (2008) Pharmacology & Therapeutics 117:232–243

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Mechanistic Insights into Allosteric Structure-Function Relationships at the M_1 Muscarinic Acetylcholine Receptor

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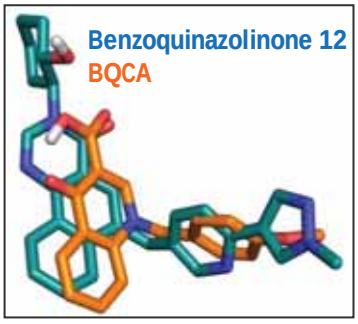
Introduction. The M_1 muscarinic acetylcholine receptor (mAChR) is an attractive therapeutic target for CNS disorders such as Alzheimer's disease and schizophrenia. Benzylquinolone carboxylic acid (BQCA) is the first highly selective positive allosteric modulator (PAM) for the M_1 mAChR, but possesses low affinity for the allosteric site of the receptor. Recent drug discovery efforts identified benzoquinazolinone 12 as a more potent M_1 mAChR PAM that is structurally related to BQCA.



Aims. To characterise the pharmacology of benzoquinazolinone 12 and define the molecular basis for its improved affinity at the M_1 mAChR.
Methods. Radioligand binding and Inositol-1-phosphate accumulation assays on cells expressing either WT or mutant M_1 mAChRs in addition to molecular modelling studies.
Results. The improved potency of benzoquinazolinone 12 is derived from a 50-fold increase in affinity for the allosteric site, as compared with BQCA, while it retains a similar level of positive cooperativity with acetylcholine (ACh). Our results validate the allosteric binding pocket previously described for BQCA as a shared site for benzoquinazolinone 12, and provide a molecular basis for its improved activity at the M_1 mAChR. This includes a key role for hydrophobic and polar interactions with residues Tyr179, in the second extracellular loop (ECL2), and $Trp400^{7.35}$ in transmembrane (TM) domain 7, and additional interactions of benzoquinazolinone 12 with TM2 residues $Tyr82^{2.61}$ and $Tyr85^{2.64}$.
Discussion. This study highlights how the properties of affinity and cooperativity can be differentially modified on a common structural scaffold, and identifies molecular features that can be exploited to tailor the development of M_1 mAChR-targeting PAMs.

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Signal Transduction Pathways Activated by Insulin-like peptide 5 at Relaxin Family Peptide Receptor 4

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Introduction. Insulin-like peptide 5 (INSL5) is a two-chain, three-disulphide bonded peptide belonging to the insulin/relaxin superfamily that is uniquely expressed in the enteroendocrine L-cells of human and mouse colon (Grosse et al, 2014). It is the cognate ligand for the G protein-coupled relaxin family peptide receptor 4 (RXFP4) that is mainly expressed in the colorectum and the enteric nervous system. Wild-type mice injected with INSL5 show an increase in food intake, an effect that was abolished in RXFP4 knockout mice, demonstrating that INSL5-RXFP4 forms a novel gut-hormone axis that regulates appetite (Grosse et al, 2014). However, currently there is little known regarding the signal transduction pathways activated by RXFP4.
Aims. This study examined intracellular signalling pathways activated by human INSL5 (hINSL5) and mouse INSL5 (mINSL5) acting at the human RXFP4 stably expressed in CHO cells.
Methods. The effect of hINSL5 and mINSL5 on cyclic AMP (cAMP), mitogen-activated protein kinase (MAPK) and protein kinase B (PKB/Akt) pathways was investigated using AlphaScreen® proximity assays. Intracellular Ca^{2+} flux was monitored in a Flexstation® using X-rhod-1 AM.
Results. mINSL5 inhibited forskolin-stimulated cAMP accumulation and activated ERK1/2, p38MAPK, Akt-Ser473 and S6 ribosomal protein (S6RP) more potently than hINSL5; however both peptides were equipotent for Akt-Thr308. No JNK1/3 activity or intracellular Ca^{2+} mobilisation was observed.
Discussion. INSL5 negatively regulates cAMP production and activates multiple signalling pathways important for diverse cellular functions including growth, differentiation and proliferation (ERK1/2, p38MAPK, Akt) and protein synthesis (S6RP). Information on signalling pathways activated by INSL5 at RXFP4 is essential for understanding the biological roles of this novel gut hormone.

Grosse et al (2014) PNAS. 111:33-38

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Investigating the signalling bias of GLP-1(9-36)amide at the GLP-1 receptor

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Introduction. Glucagon-like peptide 1 (GLP-1), an incretin synthesised in the intestine, is released in response to food ingestion to stimulate insulin secretion via binding to the GLP-1 receptor (GLP-1R). Secreted GLP-1(7-36)amide, is rapidly cleaved by the serine protease, dipeptidyl peptidase-4 (DPP-4), to produce GLP-1(9-36)amide, the predominant form of GLP-1 in postprandial plasma. Despite GLP-1(9-36)amide being at a concentration 5- to 10-fold greater than GLP-1(7-36)amide, no consensus on its biological role has been identified in humans.
Aims. The GLP-1R has been reported to signal via several, stimulatory and inhibitory G proteins and we have previously shown that different ligands influence this coupling. Here, we aim to address the effect of GLP-1(9-36)amide on GLP-1R signalling through the stimulatory G protein, and the inhibitory Gαi and Gαz subunits.
Methods. To compare the effect of GLP-1(9-36)amide on Gαs, Gαi and Gαz signalling, we expressed the GLP-1R in a yeast reporter assay, allowing the study of individual G protein subunit activation. We then validated our results through the use of pertussis toxin (PTX) on GLP-1R transfected cells lines.
Results. Consistent with previous findings reported in the yeast system [1] results indicated that GLP-1(9-36)amide is a partial agonist of the GLP-1R-Gαs response (pEC_{50}= 7.4±0.2 and EMax =48±1.4), and a full agonist of Gαi (pEC_{50} =7.7±0.3). Further, we demonstrated that GLP-1(9-36)amide is a full agonist of GLP-1R coupled Gαz, but with a much lower potency (pEC_{50} =6.5±0.2), when compared to Gαi. Using GLP-1R expressing HEK 293T cells treated with PTX (200ng/ml for 16 h), we confirmed that the GLP-1R is coupled to inhibitory G proteins in mammalian cells. GLP-1R stimulation with GLP-1(9-36)amide showed a more pronounced effect when compared to GLP-1(7-36)amide.
Discussion. Our data provide further evidence that GLP-1(9-36)amide is an active metabolite and we suggest that it may act to modulate the GLP-1(7-36)amide activated signalling pathway, through inhibitory G proteins. We are currently investigating the Gαi signalling bias in mammalian cells to clinically prescribed GLP-1 mimetics.

[1] Weston *et al.*, (2013) *Proc Br. Pharmaco Soc.*

Inhibition of human neuronal $Ca_v2.3$ channels via μ-, δ- and κ-opioid receptor activation

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Introduction. G protein-coupled (GPC) opioid receptors mediate the pain relief offered by opioid analgesics such as morphine. Presynaptic voltage-gated $Ca_v2.1$ and $Ca_v2.2$ calcium channels are efficiently inhibited by GPC opioid receptor activation in mammalian neurons and heterologous expression systems (Altier and Zamponi, 2008). This mechanism is at least partly responsible for the analgesic effects of opioids. Neuronal voltage-gated $Ca_v2.3$ channels are widely expressed in the central and peripheral nervous system where they contribute to neurotransmission and pain sensation. However, modulation of the $Ca_v2.3$ channel through μ- and δ-opioid receptors is poorly defined and has not previously been reported for κ-opioid receptors.

Aims. We hypothesised that activation of human μ-, δ- or κ-opioid receptors modulate human $Ca_v2.3$ channels via G protein signalling.

Methods. Whole-cell Ba^{2+} currents were recorded in human embryonic kidney (HEK293T) cells co-expressing human $Ca_v2.2$ or $Ca_v2.3$ channels and μ-, δ- or κ-opioid receptors. The voltage dependence of $Ca_v2.2$ or $Ca_v2.3$ channel inhibition was investigated with depolarizing paired-pulse protocols, whereby depolarizing pre-pulses to +80 mV or +120 mV were used to test relief from inhibition. Selective opioid receptor agonists and antagonists were used to study receptor modulation. The involvement of intracellular signalling pathways was investigated using specific inhibitors of GPC receptor-G protein coupling.

Results. Activation of μ-, δ- or κ-opioid receptors inhibited $Ca_v2.3$ or $Ca_v2.2$ channel current amplitude by ~45% and ~60%, respectively. Inhibition of the $Ca_v2.3$ channel was not dependent on the type of calcium channel β subunit co-expressed. Inhibition of the $Ca_v2.3$ channel was primarily voltage independent, as depolarizing pre-pulses could not relieve the inhibited current. This was in marked contrast to the primarily voltage dependent modulation of $Ca_v2.2$ channels that showed classical characteristics of G protein βγ subunit interaction with the channel and nearly complete recovery of the current by depolarizing pre-pulses. For all three types of opioid receptors, the pathway leading to $Ca_v2.3$ channel inhibition was sensitive to pertussis toxin and to intracellular application of non-hydrolysable GDP analogue GDP-β-S. Similarly, the overexpression of a G protein βγ subunit scavenger, myristoylated-phosducin, significantly reduced the magnitude of $Ca_v2.3$ channel inhibition.

Discussion. It is generally believed that $Ca_v2.3$ channels are relatively insensitive to modulation by GPC receptors. We demonstrate that $Ca_v2.3$ channels are efficiently inhibited by activation of μ-, δ- or κ-opioid receptors. Inhibition occurs via voltage-independent G protein signalling mechanisms. These results suggest opioid receptors control specific members of the Ca_v2 channel family via differential signalling pathways. Neuronal $Ca_v2.3$ channels are therefore potential targets for opioid analgesics.

Altier C and Zamponi GW (2008). J Recept Signal Transduct Res. 28:71-81

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Functional analysis of the N-terminal residues of human amylin

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Introduction. Amylin is a 37-amino acid peptide hormone involved in metabolism, glucoregulation and satiety. Accordingly, amylin has been investigated as a therapy for diabetes and obesity. Amylin is a Family B G protein-coupled receptor (GPCR) peptide ligand, proposed to activate its receptors via a two-domain model of binding whereby the peptide N-terminus is important for receptor activation. The calcitonin family, of which amylin is a member, all possess a highly conserved N-terminal disulphide loop which is critically important for receptor activation. Nevertheless, very little is currently known about the significance of the individual N-terminal residues of amylin in receptor activation.
Aims. To investigate the role of the N-terminal loop residues of human amylin in receptor activation by completing an alanine scan of amino acids in the loop.
Methods. Fmoc solid-phase peptide synthesis was used to make peptide analogues, followed by cleavage, precipitation, oxidation and purification of the peptides. All were amidated with the disulphide bond. Biological functional assays were carried out in COS7 cells transiently transfected with the human calcitonin receptor $hCT_{(a)}$ or the amylin 1 receptor $hAMY_{(1a)}$ consisting of $hCT_{(a)}$ with human RAMP1. Alpha Screen detection of cyclic AMP formation upon peptide stimulation was measured as an indicator of receptor activation.
Results. Alanine substitution of lysine at position 1 or asparagine at position 3 did not significantly alter human amylin activity at either receptor $hCT_{(a)}$ or $hAMY_{(1a)}$ suggesting that these residues are not critical for receptor activation. The substitution of threonine to alanine at position 4 significantly decreased pEC_{50} at $hCT_{(a)}$ but not $hAMY_{(1a)}$ with no changes in E_{max}. Threonine substitution with alanine at position 6 caused a significant reduction in E_{max} at both receptors with no change in pEC_{50}.
Discussion. It appears that the N-terminal residues which encompass the peptides “activation loop” have disproportionate and differential impacts on receptor activation. The strongly conserved threonine at position 6 is clearly a key residue in the N-terminal loop with threonine 4 showing slightly more importance in activation of $hCT_{(a)}$ over $hAMY_{(1a)}$.

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The third intracellular loop of the human L-amino acid receptor GPRC6A controls cell surface expression and receptor function.

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Introduction. Human G protein-coupled receptor, family C, group 6, subtype A (GPRC6A) was cloned by us 10 years ago (Wellendorph et al. Gene. 2004) and great effort has since been dedicated to characterize the receptor. One challenge has been to understand why the two close orthologs (human vs. mouse, 80% seq. identity) are differently expressed on the cell surface when expressed recombinantly (Wellendorph et al. Mol. Pharmacol. 2005). Previous work with chimera constructs between human and goldfish GPRC6A has shown that the transmembrane and/or C-terminal part of human GPRC6A prevent surface expression (Wellendorph et al 2005).
Aims. The aim of the study was to identify the amino acid motif responsible for the intracellular retention and lack of functional response of the human GPRC6A receptor.
Methods. Chimeric receptors where parts of the 7-transmembrane domain in human GPRC6A had been replaced by the corresponding mouse sequence were generated by PCR. The cDNA encoding the bonobo chimpanzee GPRC6A sequence was synthesized by Genscript. Mutations were generated using the QuikChange protocol. Cell surface expression was determined by ELISA of a N-terminal c-myc epitope and functional responses were determined by measurement of inositol monophosphate using the IP-ONE time-resolved FRET assay (Cisbio).
Results & Discussion. The chimeric human/mouse receptors demonstrated that the third intracellular loop (ICL3) controls cell surface expression and function given that the human receptor containing the mouse ICL3 was trafficked to the surface and could be activated by the GPRC6A receptor agonists L-arginine and L-ornithine. Next we showed that the GPRC6A receptor from bonobo, which is 99% identical to the human receptor, trafficks to the cell surface. This allowed us to narrow down the motif controlling surface trafficking to a "RKLP" motif in the bonobo ICL3, which is shortened and changed to "K--Y" in the human sequence. Subsequent introduction of the RKLP motif in the human GPRC6A receptor generated a fully functional receptor trafficking to the cell surface.
Conclusion. We have identified the motif in the human GPRC6A receptor causing intracellular retention and lack of function. Our current efforts focus on delineating the impact of the ICL3 variation on human physiology.

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Allosteric coupling in the calcium-sensing receptor

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Introduction. The calcium-sensing receptor (CaSR) is a GPCR that supports whole body calcium homeostasis. Ligand binding in its extracellular Venus Flytrap Domain (VFTD) induces intracellular signalling via a Cysteine-Rich domain (CRD) coupled to a heptahelical transmembrane domain (HHD). However, the mechanism that supports this interdomain coupling has not been identified.
Aims. To identify the role of the CRD in receptor activation and establish whether residues Cys236 in the VFTD and Cys561 in the CRD participate in a functionally critical interdomain disulfide.
Methods. CaSR constructs with C236S and C561S mutations were evaluated for receptor expression by an ELISA-based method. The impact of the mutations on signalling was assessed via assays measuring Ca^{2+}_i mobilisation or IP_1 accumulation. Furthermore, to test the hypothesis that a disulfide bond provides a second physical connection between the VFTD and the CRD, a thrombin cleavage site was inserted into Wt CaSR and C236S/C561S CaSR. Following purification and thrombin digestion, the resulting fragments were analysed by western blotting. Additionally, following molecular modeling, cysteine residues were introduced in the proximal region of the CRD and the constructs were assessed by IP_1 accumulation to establish the role of the CRD across the dimer interface.
Results. C236S/C561S receptors failed to respond to Ca^{2+}_o in the presence of the VFTD modulator L-Phe, whereas receptor activation was retained in response to cinacalcet, which binds in the HHD, bypassing the loss of disulfide-dependent receptor activation. Fragmentation analyses, following thrombin digestion, showed $CaSR_{THR}$ constructs were held as intact proteins in non-reducing conditions but DM_{THR} constructs, with the potential disulfide linkage removed, separated into smaller fragments. Preliminary IP_1 accumulation studies of the proximal mutants suggest introduced cysteine residues may form disulfide links across the dimer interface and enhance receptor activation.
Discussion. Cys236 and Cys561 are critical for coupling between the calcium-binding VFTD and the signalling HHD. Western blot analyses are consistent with the hypothesis that a disulfide linkage supports ligand-dependent coupling between the VFTD and the HHD. Mutational work at the proximal region of the receptor shows the crucial role this region plays in receptor activation.

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Deciphering the complex mode of relaxin receptor (RXFP1) activation

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Introduction. The receptor for the peptide hormone relaxin, RXFP1, and its related receptor RXFP2 are unique among GPCRs in that they contain a large ectodomain consisting of 10 leucine-rich repeats (LRRs) and an N-terminal low-density lipoprotein type A (LDLa) module. Activation of RXFP1 by relaxin requires coordination of ligand binding contacts with multiple receptor domains with the LDLa module driving the final receptor active conformation(s) via interactions with other domains by an unknown mechanism.
Aims. In this study, we explore the role of the linker between the LDLa and LRR domain in binding and activation utilizing functional analysis of receptor mutants and nuclear magnetic resonance (NMR) experiments on recombinant proteins of the LDLa module with or without linker.
Methods. Protein constructs were expressed using fermentation and purified on an IgG sepharose column. ^{15}N-labeled LDLa proteins were analysed by HSQC NMR with varying concentrations of H2 relaxin as well as a synthetic H2 relaxin analog with a paramagnetic label on the N-terminus of the A-chain. Site-directed mutagenesis was performed on RXFP1 and once verified, mutants were transfected transiently into HEK cells and submitted to relaxin binding, cAMP signalling and cell-surface expression assays.
Results. Mutations of residues in the linker region showed a marked loss of function as well as reduced binding, despite cell surface expression being equivalent to wild-type levels. Chemical shifts in residues seen in NMR data suggested that relaxin binds to the linker with μM affinity, and experiments with paramagnetically labelled relaxin implicate the A-chain of relaxin in this interaction. Furthermore, the broadening of resonances in the NMR spectrum is non-sequential, indicating a specific, binding-induced conformational change is occurring.
Discussion. Taken together, these experiments implicate the linker region between the LDLa module and LRRs in the activation process of RXFP1. The linker is hypothesized to be a secondary binding site, and by undergoing a binding-related structural change, the LDLa and linker together interact with the TM domain to induce activation. The understanding of the mechanics of this activation will help in the discovery of alternative means to control RXFP1 activity as well as enhancing the knowledge of this interesting and unusual GPCR.

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Characterising the Conformational Diversity of the Neurotensin Receptor 1

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Introduction. The neurotensin receptor 1 (NTS_1) is a GPCR for the tridecapeptide neurotensin (NT). Activation of NTS_1 by NT has been implicated in schizophrenia, Parkinson's disease and cancer cell growth. Crystal structures of NTS_1 bound to fragments of NT, as well as mutagenesis data, have defined intermediate states of NTS_1 along with the high affinity binding site for NT. It is desirable to complement these achievements by probing conformational dynamics and identifying conformations mediating particular signaling responses. Furthermore, little is known about transient interactions that may underlie NT binding. Cellular high-throughput encapsulation, solubilisation and screening (CHESS)-based directed evolution, yielded stable mutants of NTS_1 suitable for solution NMR studies.
Aims. We aim to assess the dynamic landscape of site-specific $^{13}CH_3$-methionine labeled NTS_1 and to characterise low affinity binding sites for NT using solution NMR techniques.
Methods. NTS_1 mutants are expressed to the *E. coli* inner membrane and reconstituted in detergent micelles. Isotopic labeling using $^{13}CH_3$-methionine facilitates NMR based dynamics studies of NTS_1 mutants that may reveal motions at the ns scale. Saturation transfer difference NMR (STD NMR), a technique commonly used for fragment screening against soluble proteins is applied to detect low affinity peptide interactions to samples of NTS_1 mutants.
Results. The high stability of these receptors in detergent allowed us to repeatedly measure the binding of NT fragments over 24 h periods at 25°C without significant loss of the STD signal. Regions of NT involved in receptor interactions could be mapped, exhibiting agreement with the crystal structures. We could further show that signals originating from low affinity ligands are abolished in the presence of a higher affinity competitor. Using ^{1}H-^{13}C-HMQC NMR we identified the 9 $^{13}CH_3$-methionine resonances that are well dispersed across the whole sequence of one of our labeled NTS_1 mutants. These $^{13}CH_3$ groups will act as local probes in NMR based dynamics studies.
Discussion. This work will help to understand the dynamics of NTS_1 upon ligand interaction and to uncover binding pathways of NT fragments to high affinity binding sites in NTS_1. This is the first time, to our knowledge, that STD NMR was successfully applied to show interaction of a GPCR with its ligand.

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Identifying novel sites of ligand interaction on the α_1 adrenoceptors.

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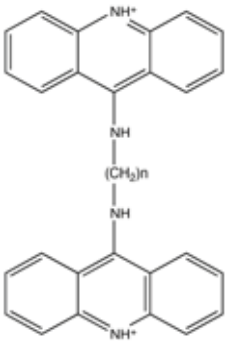

Introduction. The homobivalent bisacridines (see figure, "Cn" bisacridine) are ligands for the α_1 adrenoceptors (ARs) with steep binding curves and the potential to interact bitopically with the α_1 ARs. C4 is >16X selective for the α_{1A} AR and C2 is >8X selective for the α_{1B} AR over the other α_1 ARs
Aims. To identify novel binding sites on the α_1 ARs.
Methods. The bisacridines were characterised using competition and dissociation radioligand binding assays. Homology models and in silico docking were used to identify potentially novel interactions between the bisacridines and the α_1 ARs.
Results. The bisacridines increase the dissociation rate of [^{3}H]prazosin from the α_{1A} and α_{1B} ARs showing increasing effect with increasing linker length(α_{1A}; r^2: 0.27, $p<0.001$, α_{1B}; r^2:0.22, $p<0.001$). 100 µM C9 can increase [^{3}H]prazosin dissociation 17.6 ± 2.5X from the α_{1A} AR ($p<0.001$) and 62.3 ± 19.3X from the α_{1B} AR ($p<0.001$). Docking revealed interactions with the second extracellular loop (ECL2) and a small cluster of residues at the extracellular end of transmembrane helix II (TMII). Mutation of ECL2 residues in the α_{1A} AR (Q177G.I178V.N179T) reverses the selectivity of C4, but does not alter affinity of C2. Functional effects of bisacridines and kinetic effects of mutants are being characterised.
Discussion. The α_{1A} AR is of particular clinical interest as it is considered an efficacious target in the treatment of benign prostatic hyperplasia (Lepor, 2007), a common disease in older men, as well as a potentially useful target in the use of seizure disorders (Hillman et al. 2009). Current, clinically used antagonists do not possess high selectivity over both the α_{1B} and α_{1D} ARs. Identification of novel sites of interaction of the receptor may yield alternative, selective sites of interaction, or non-orthosteric modes of antagonism allowing for the design of drugs with higher selectivity or improved side effect profiles.

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Signalling of indole-2-carboxamides as allosteric modulators of the cannabinoid receptors.

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Introduction. The GPCR cannabinoid receptor type 1 (CB_1) has an allosteric binding site. One of the most studied of the CB_1 allosteric modulators is ORG27569 which we have recently shown to produce a complex, concentration and time-dependent modulation of agonist-mediated regulation of cAMP levels, as well as an increased rate of desensitisation of CB_1-mediated cellular hyperpolarisation and a decrease in agonist-induced receptor internalisation (Cawston et al, 2012).
Aims. To characterise the cAMP signalling response of indole-2-carboxamides structurally correlated to ORG27569 and previously characterised for ability to modulate binding of [3H]CP55,940 to hCB_1 (Piscitelli et al., 2012).
Methods. Using the real-time kinetic BRET CAMYEL assay we assessed the forskolin stimulated cAMP response of 10 µM indole-2-carboximide compounds in both the presence and absence of EC90 CP55,940 in HEK 3HA-hCB_1 cells and Flp-In 293 HA-3TCS-hCB_2 cells. Compounds that displayed altered cAMP responses were then characterised further with regards to potency and cAMP signalling characteristics.
Results and Discussion. The initial screening of forskolin stimulated cAMP responses of 14 indole-2-carboximide compounds identified three compounds that antagonized the CP55,940 mediated inhibition of cAMP more rapidly than ORG27569, suggestive of higher potency in HEK 3HA-hCB_1 cells. More detailed analysis confirmed this enhanced potency, and demonstrated that these compounds all displayed equivalent inverse agonism to ORG27569. Three further compounds displayed allosteric antagonism of the CP55,940 mediated effect with no inverse agonism. Four compounds displayed agonist activity at the hCB_2 receptor. As these compounds were previously described to decrease [3H]CP55,940 binding, further studies are required to determine if this effect is allosteric or orthosteric. Data from this study will be used to further define the appropriate chemical structures for targeting therapeutically useful pathways in relation to CB_1 and CB_2.

Cawston EE et al, 2013 Br J Pharmacol 170(4):893-907;
Piscitelli F et al 2012. J Med Chem 55**:** 5627-31.

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GPCR MAS modulates purinergic P2Y2 and GLUT1 activities via interacting with MBP7-like motif

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Introduction. Using phage-displayed peptide library, our lab identified a surrogate ligand MBP7 (MAS Binding Peptide 7) for GPCR MAS. Intriguingly, a MBP7-like motif was found in hamster purinergic P2Y2 receptor and glucose transporter 1 and 7 (GLUT1 and 7).

Aims. To examine the possibility that GPCR MAS modulates the activities of purinergic P2Y2 receptor and glucose transporter via interacting with the MBP7-like motif.

Methods. P2Y2-mediated calcium mobilization and Glut1-mediated glucose uptake were measured in stable *Mas*-overexpressing CHO cell clones. Direct physical interaction of MAS and GLUT1 were examined by co-immunoprecipitation and immunohistochemistry.

Results. Several stable CHO cell lines expressing *Mas* at different levels were used. Of interest, the higher the levels of *Mas* expression, the larger the right shift of the dose-response curve of ATP-stimulated calcium mobilization. GLUT1-mediated glucose uptake in *Mas*-overexpressing cells was lower than that of cells stably transfected with empty vector. However, GLUT1-mediated glucose uptake was elevated in *Mas*-overexpressing cells in the presence of MBP7 peptide. Furthermore, MAS and GLUT1 were found to be co-localized and co-immunoprecipitated.

Discussion. These results suggest GPCR MAS suppresses P2Y2-mediated calcium mobilization and GLUT1-mediated glucose uptake via interacting physically with the MBP7-like motif.

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Architecture and function of signalling complexes assembled by β_2-adrenergic and M_3 muscarinic receptors

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Introduction. Protein complexes have diverse structure and function within cells. Recently, a newly discovered signalling complex (signalosome) built around RXFP1 conferred some unique properties to this GPCR. Assembly of the RXFP1 signalosome, comprising several scaffolding and signalling proteins, enabled ultrasensitive responses to sub-picomolar relaxin (Halls et al, 2010). Although the closely related receptor RXFP2 does not form a signalosome, we have confirmed this phenomenon for other GPCRs, namely the β_2-adrenoceptor (β_2AR) and M_3 muscarinic receptor (M_3R).

Aims. To investigate the architecture of β_2AR and M_3R signalosomes, to identify domains that are important for protein-protein interactions, and to investigate the cellular function of these novel complexes.

Methods. FRET biosensors transiently expressed in HEK293 cells were used to measure responses to sub-picomolar concentrations of isoprenaline/carbachol, mediated by endogenous β_2AR or M_3R respectively. GST immunoprecipitation (IP) and co-IP were used to determine the architecture and interacting domains of these complexes. Quantitative proteomics was employed to decipher the function of the signalosomes.

Results. We show that β_2AR signalosome activation involves $G_s/G_{\beta\gamma}$ stimulation of adenylyl cyclase (AC). Scaffolding is provided by Gravin and β–arrestins and negative regulation of the cAMP signal is mediated by $G_{i/o}$, PKA and PDE4D5. M_3R signalosome activation involves $G_q/G_{\beta\gamma}$ stimulation of PKC that leads to increased AC activity. The complex is scaffolded by AKAP79 and β-arrestins. Negative regulation of cAMP signalling occurs by PKA and PDE4D3. The C-terminal fragment (Helix 8) of β_2AR is important for these interactions, whilst M_3R binds its interacting protein partners via the intracellular loop 3. Activation of either the β_2AR or M_3R signalosome results in compartmentalised downstream signalling (nuclear ERK or cytosolic PKC, respectively), and quantitative proteomics revealed both up- and down-regulation of sets of proteins unique for each receptor signalosome.

Discussion. We show that β_2AR and M_3R can form signalosomes, sense sub-picomolar concentrations of ligand and activate nuclear ERK or cytosolic PKC, respectively. This leads to up/down-regulation of distinct subsets of the proteome. Future experiments will aim to elucidate a physiological role for these unique GPCR complexes.

Halls ML & Cooper DM (2010) EMBO J 29:2772-87.

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Multi-pathway analysis of allosteric modulation of the calcium-sensing receptor by calcilytics

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Introduction. Negative allosteric modulators (NAMs) of the calcium-sensing receptor (CaSR) offer potential for the treatment of autosomal dominant hypocalcaemia, Bartter's syndrome type V and osteoporosis. However, to date CaSR NAMs have shown limited clinical efficacy, which may reflect the recruitment of undesirable as well as beneficial cellular pathways. Indeed, both orthosteric agonists and allosteric modulators of the CaSR can engender biased signalling (Thomsen et al., 2012; Davey et al., 2012; Cook et al., 2014); this represents a potential mechanism for tissue selectivity. However, further understanding of the desired bias profile of calcilytics is required for selective CaSR targeting.

Aims. To investigate the pharmacology of ATF936, the most recently described calcilytic to enter clinical trials (John et al., 2013), in both heterologous and endogenous CaSR expression systems using both endogenous (Ca^{2+}_o) and exogenous (Sr^{2+}_o) orthosteric agonists.

Methods. The ability of ATF936 and NPS-2143 to modulate Ca^{2+}_o and Sr^{2+}_o-induced Ca^{2+}_i mobilisation, ERK1/2 phosphorylation and IP_1 accumulation was investigated in HEK293-CaSR cells and human thyroid carcinoma (TT) cells. CaSR expression was confirmed with western blotting and RT-qPCR.

Results. ATF936 acts as a NAM of Ca^{2+}_o and Sr^{2+}_o-induced signalling, with similar estimated affinity and cooperativity values for each endpoint evaluated. These data suggest that, unlike NPS-2143, ATF-936 is not biased in its modulation of Ca^{2+}_i versus ERK1/2 phosphorylation.

Discussion. Understanding the bias profile of calcilytics and the pathways utilised by endogenously expressed CaSR may enable better development of parathyroid-specific NAMs for osteoporosis and CaSR disorders.

Thomsen ARB et al. (2012) JPET 343:638–649
Davey AE et al. (2012) Endocrinology 153:1232-41
Cook AE et al. (2014) Br J Pharmacol In Press
John MR et al. (2013) Bone 49:233-241

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Improving G protein-coupled receptor virtual screening outcomes: method for binding pocket refinement

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Introduction. G Protein-Coupled Receptors (GPCRs) are a superfamily of transmembrane proteins that mediate cellular responses to their environment upon binding of an effector to their extracellular-facing binding pocket. With over 800 human GPCRs playing key roles in modulating tissue/cell physiology and homoeostasis, they represent a major target for pharmaceutical intervention. Recently, an increasing number of GPCR X-ray crystal structures were solved; Computer-Aided Drug Discovery (CADD) methods such as Virtual Screening (VS) leverage that growing structural information. VS by docking attempts to identify new drug leads, ranking libraries of small molecules based on the predicted complementarity between small molecules and the target GPCR binding pocket. The success of VS is highly dependent on the conformation of the target binding pocket, therefore, a key step of CADD is to refine the binding pocket within a protein structure.
Methods. Here we present a new computationally efficient Ligand Directed Modelling (LDM) method for GPCR binding pocket refinement. This method aims to establish the global energy minimum of a GPCR binding pocket in complex with a single known active ligand for that GPCR. Our LDM method uses protein sampling, ligand docking and scoring of ligand/binding pocket complexes in recursive steps.
Results. We benchmarked the method in a range of different scenarios for which there is an available GPCR X-ray crystal structure bound to the ligand that was used for LDM refinement. The LDM refined structures were compared to both the starting and final X-ray crystal structures for their capacity to distinguish known ligands from decoys in a small scale VS.
Discussion. This benchmark provides a guideline for the application of this LDM method in future CADD projects.

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Discovery of novel selective C5a2 ligands that modulate IL-6 release from macrophages

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Introduction. The complement cascade is a highly sophisticated network of proteins that are well regulated and directed in response to invading pathogens or tissue injury and forms a major component of our innate immune response (Monk *et al.*, 2007). Complement C5a binds to two G-protein coupled receptors (GPCR); namely the C5a receptor (C5a1) and C5a receptor like-2 receptor (C5a2). C5a2 is a non-signalling GPCR and its role has been difficult to validate due to the unavailability of selective ligands (Li *et al.*, 2013).
Aims. Discover novel C5a2 ligands that allow us to probe and explore the functional role of C5a2.
Methods. Radioligand binding assays were used to screen a small peptide based library of 61 ligands designed to act at C5a1 for the ability to displace [125I]-C5a from CHO-K1 cell membranes expressing C5a2. A novel β-arrestin 2 recruitment via C5a2 BRET assay was developed to test for ligand activity at C5a2 and ligands were counter screened for β-arrestin 2 recruitment via C5a1. ELISA was used to measure cytokine release from human monocyte derived macrophages (HMDM).
Results. Radioligand binding testing of the 61 ligands revealed two ligands (P32 & P59) which showed ≥ 40% inhibition of ^{125}I-C5a binding to C5a2 membranes at 100 µM. P32 and P59 were able to dose dependently recruit β-arrestin 2 via C5a2 but not C5a1 each with an EC_{50} of 7.6 µM. Neither P32 nor P59 was able to recruit β-arrestin 2 to a similar level as C5a, with P32 able to recruit to ~55% and P59 ~30% of the level of C5a. Both P32 and P59 dose dependently inhibited the release of IL-6 from HMDM when co-stimulated with LPS but had no effect on TNFα or IL-10 release.
Discussion. P32 and P59 are the first reported selective ligands for C5a2 and show novel pharmacology with the ability to modulate IL-6 release from HMDM. The discovery of these selective ligands will allow us to better investigate the role of C5a2 signalling in many inflammatory and immune disorders.

Li R et al (2013). FASEB J 27**:** 855-864.
Monk PN el al (2007). Br J Pharmacol 152**:** 429-448.

701

Functional expression of modified calcitonin receptor in nanodics for biophysical measurements of family B GPCR conformational changes.

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Introduction. The complex mechanism of family B GPCR activation that allows ligand binding to the extracellular N terminal domain to activate signalling effectors within the cell is currently not understood at a structural level. Although this mechanism is not completely understood for family A GPCRs, there is much more information available for this class due to recent methodological advances. These include the crystallisation of receptors in inactive, partially and fully active conformations, the incorporation of GPCRs into nanodiscs, that allow biophysical studies in a near native environment and biophysical measurements using environmentally sensitive probes introduced at various positions in the GPCR to measure conformational changes.
Aim. This project aims to identify a model system to investigate conformational changes that occur in family B GPCRs upon activation by ligands.
Methods. Cysteine reactive probes will be used to measure conformational changes in the calcitonin receptor (CTR) expressed in nanodiscs. We will use (i) mass spectrometry: to identify reactive cysteine residues in the native CTR. (ii) Molecular modelling: to identify potential residues in the CTR that may undergo conformational changes. (iii) Mutagenesis: to mutate reactive cysteines to serines, and to introduce cysteine residues in positions that may undergo conformational changes, that can then be coupled with cysteine reactive probes. (iv) cAMP AlphaScreen and whole cell binding assays: to assess the pharmacology and cell surface receptor's expression of native and mutant CTRs in COS7 cells. (v) CTR purification and nanodisc production: to assess CTR function in nanodiscs.
Results. Mutation to serine of solvent accessible cysteine residues and introduction of cysteines in the positions predicted by the molecular model did not alter the CTR pharmacology. Conditions for assembling nanodiscs containing functional CTR were identified.
Conclusions. We have successfully inserted CTR into nanodiscs to create a system to perform biophysical measurements that may provide insights into the activation mechanism of family B GPCRs.

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Comparative pharmacology of structurally distinct calcimimetics acting at the calcium-sensing receptor

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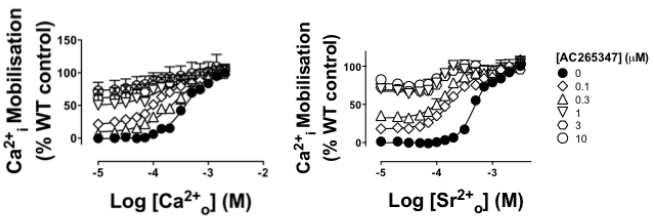
Introduction: The calcium-sensing receptor (CaSR) is activated by many divalent cations including the cognate ligand, Ca^{2+}_o and Sr^{2+}_o, which is used in the treatment of osteoporosis. Calcimimetics, including cinacalcet, BTU compound-13 (Deprez *et al*., 2013) and AC-265347 (Ma *et al*., 2011), are all allosteric modulators of the CaSR. As such, they exert allosteric effects to positively modulate activation the actions of Ca^{2+}_o. However, characterisation of their effects on Sr^{2+}_o-mediated CaSR activation is yet to be investigated.
Aim: This study compares the pharmacology of the structurally distinct calcimimetics BTU compound-13, AC-265347 and cinacalcet to modulate Ca^{2+}_o and Sr^{2+}_o-dependent CaSR activation.
Methods: Compounds were characterised for their modulation of Ca^{2+}_o- and Sr^{2+}_o-mediated activation of well-characterised CaSR signalling pathways including phosphorylation of ERK1/2, intracellular Ca^{2+} mobilisation and IP_1 accumulation in HEK293 cells stably transfected and induced to express the human CaSR.



	log αβ	pK_B
Ca^{2+}_o	0.63 ± 0.08 (n=5)	6.42 ± 0.22 (n=5)
Sr^{2+}_o	0.62 ± 0.06 (n=3)	6.84 ± 0.16 (n=3)

Results: Comparative analysis of the effect of each of the calcimimetics on Ca^{2+}_o and Sr^{2+}_o signalling revealed no probe dependent modulation within the intracellular Ca^{2+} mobilisation assay, with similar affinity and cooperativity estimates in combination with both Ca^{2+}_o and Sr^{2+}_o (see Figure).
Discussion: Initial data suggests that calcimimetics exert similar effects on both Ca^{2+}_o and Sr^{2+}_o induced CaSR signalling.

Deprez P, *et al*. (2013). *Bioorg. Med. Chem. Lett.* **23**:2455-9
Ma J-N, *et al*. (2011). *JPET* **337**:275-84

704

Roles of the Calcium-Sensing Receptor intraloops -2 and -3 and the carboxy-terminus in signalling pathway selection

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Introduction. The calcium-sensing receptor (CaSR) is a Class C G-protein coupled receptor that is widely expressed. It contributes to the control of calcium metabolism and bone homeostasis via expression in the parathyroid, renal cortical thick ascending limb, calcitonin-secreting thyroid C cells, and in bone cells, including cells of the osteoblast lineage. The CaSR mediates diverse effects by selecting for signalling pathways in a ligand- and cell-type-specific manner. However, the mechanisms that underlie the selection of signalling pathways are not well understood.

Aim. To determine the roles of the intraloops and carboxyl-terminus of the CaSR in signalling pathway selection.

Methods. In the present study, alanine scanning site-directed mutagenesis has been used to identify subdomains and residues in intraloops -2 and -3 that are critical for the coupling of the extracellular Ca^{2+} (Ca^{2+}_o) -activated receptor to distinct pathways including downstream of PI-PLC (IP_1 accumulation), phosphorylated $ERK_{1/2}$ (pERK), intracellular Ca^{2+} (Ca^{2+}_i) mobilization, and suppression of forskolin-stimulated adenylyl cyclase (intracellular cAMP levels).

Results. The results demonstrate that distinct residues mediate coupling to distinct signalling pathways downstream of the receptor. Most strikingly, four mutants F706A (iL-2), L797A and E803A (iL-3), and a C-terminal truncation mutant R866X, which removes all but the proximal three residues of the C-terminus (863-865), none of which impaired cell surface expression, all markedly attenuated PI-PLC and pERK but had differential effects on Ca^{2+}_i mobilization and suppression of adenylyl cyclase. In particular, R866X exhibited complete loss of Ca^{2+}_i mobilization but retained intact suppression of adenylyl cyclase. In addition, E803A exhibited only partial impairment of Ca^{2+}_i mobilization but retained intact suppression of adenylyl cyclase.

Conclusion. The results demonstrate that pathway selection arises from distinct domains and sub-domains of the receptor's intraloops and C-terminus.

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***In silico* analysis and cardiac gene expression data provide novel insights into human and mouse taste receptor gene regulation**

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Introduction. G protein-coupled receptors (GPCRs) are important mediators of sweet, umami, bitter and fat taste qualities that serve as important discriminators of food intake and rejection. Taste receptors are also expressed outside of the oral cavity, including in the gut, airways, brain and heart, where they have additional functions and contribute to disease. However, there is little known about the mechanisms governing the transcriptional regulation of taste receptor genes.
Aims. Following our recent delineation of taste receptors in the heart, we sought to investigate the regulatory mechanisms that drive their cardiac expression.
Methods. Gene expression analyses of healthy and diseased human and mouse hearts were performed, in conjunction with *in silico* analyses of taste receptor *cis*-regulatory regions.
Results. We observed coordinated expression for a subset of chromosomally clustered taste receptors in heart tissues, consistent with a common gene regulatory control of the taste receptor locus. We also identified unique regulatory domains with strong evidence for regulatory potential in the vicinity of taste receptor genes. In addition, we identified several novel over-represented DNA-motifs in cardiac taste receptor gene promoters corresponding to ubiquitous and cardiac-specific transcription factor binding sites.
Discussion. This study represents the first investigation of the *cis*-regulatory landscape for taste GPCRs and opens a new area of research in this field. Given that GPCRs represent such tractable therapeutic targets, unravelling the upstream mechanism of taste receptor regulation, specifically in the heart, might reveal other means of targeting and controlling the expression of these receptors.

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Understanding the nature of efficacy at a model family B GPCR

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Introduction. Efficacy is a well-understood pharmacological term, however there is little data that addresses the biochemical mechanisms by which differential efficacy can occur. In the case of canonical G protein-coupled receptor (GPCR) signalling via heterotrimeric G proteins, the role of the GPCR may be considered as a guanine nucleotide exchange factor (GEF). The extension of this model is that the role of ligands is to allosterically modulate this (GEF) activity. β_2-adrenoceptors, bound with ligands of different efficacy show differential sampling of conformational space (Nygaard et al., 2013). Others have proposed that efficacy might be defined by relative contributions of entropy and enthalpy to binding energy, however this is not supported by data for the β_2-adrenoceptor and histamine H1 receptors (e.g. Strasser & Wittmann, 2012). Ultimately, efficacy differences must translate into differential GEF activity.
Aims. Our aim was to define how 2 ligands, possessing differential efficacy, achieve differential GEF activity using a model family B GPCR, the calcitonin receptor.
Methods. We used cAMP accumulation and fluorescent ligand binding to pharmacologically determine efficacy. GEF activity was defined using native PAGE, fluorescence anisotropy, BRET, TIRF and GSD super-resolution microscopy.
Results. Differential GEF activity was defined in terms of GTP affinity, GTP on rate and G protein residency.
Discussion. Defining the biophysical nature of efficacy is an important step forward in understanding the way in which GPCRs function.

Nygaard, R., Zou, Y., Dror, R. O., Mildorf, T. J., Arlow, D. H., Manglik, A., et al. (2013). The Dynamic Process of β_2-Adrenergic Receptor Activation. *Cell*, *152*, 532–542.
Strasser, A., & Wittmann, H.-J. (2012). Binding of Ligands to GPCRs–How Valid is a Thermodynamic Discrimination of Antagonists and Agonists? *Journal of Physical Chemistry & Biophysics*, *S:1*.

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Hypotonic stress indirectly activates TRPV4 via intracellular signalling

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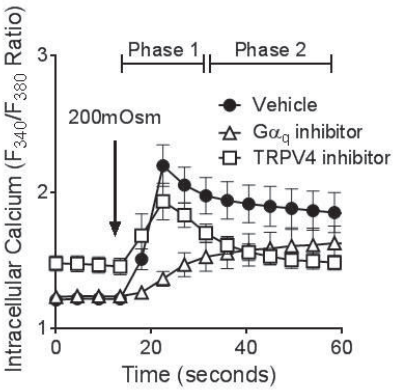
Introduction. The ability of cells to sense changes in osmolarity is fundamental to the body's ability to respond to its internal environment. The Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel has been identified as an important mammalian osmosensor; however, the mechanism(s) of TRPV4 activation are not well understood.
Aims. To investigate the mechanisms of activation of TRPV4 by hypotonic stress (HTS).
Methods. The response of primary human and bovine cells, and hTRPV4-transfected HEK293 cells to HTS (200 mOsm) were pharmacologically investigated using the calcium fluorescent dye Fura2.
Results. All cells responded to a TRPV4 agonist, but not all cells responded to HTS. hTRPV4-HEK cells showed a two-phase calcium response to HTS: phase one involving $G\alpha_q$-dependent intracellular calcium release; and phase two extracellular calcium influx through TRPV4 (Figure). PLA_2 signalling and Y110 phosphorylation were found to modulate both phases of the HTS response. The two phases of the response were independent, but both required the presence of the TRPV4 protein, as non-transfected HEK cells did not respond to a TRPV4 agonist or HTS.



Discussion. Not all TRPV4-responsive cells also responded to HTS, indicating that TRPV4 responsiveness to cell stretch is not an intrinsic property of the channel. Part of the hTRPV4-HEK cell response is mediated by a $G\alpha_q$ coupled GPCR which requires the presence of, but is not dependent on the opening of TRPV4. Further research is required to elucidate the relative importance of the biphasic TRPV4 response to hypotonic stress in generating cell depolarisation, or initiating further intra- or inter-cellular signalling cascades that allow the cells to respond appropriately to their local environment.

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Clickable photoaffinity labels for metabotropic glutamate receptor 5 based on allosteric modulator scaffolds

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Introduction. An array of pharmacologically, and chemically, diverse allosteric modulators have been identified for the metabotropic glutamate receptor (mGlu) family and the existence of multiple allosteric sites has been proposed. Despite the wealth of structure-activity relationships and mutagenesis studies on mGlu receptors, much remains to be understood regarding the dynamic ligand-receptor interactions that govern allosteric modulation.
Aims. We aimed to generate clickable photoreactive allosteric modulators for $mGlu_5$.
Results. Based on well-characterised allosteric modulator scaffolds, compounds were modified to contain a photoreactive moiety, to covalently modify the receptor, and a click chemistry handle to allow attachment of a fluorescent moiety for visualisation. We report on three probes that retain sub-micromolar affinity for $mGlu_5$ and retain negative cooperativity with glutamate in an intracellular Ca^{2+} mobilization assay. RVDU-3-130 contains a photoreactive benzophenone and has 4-fold lower affinity than the parent compound (MTEP). RVDU-3-154, also based on the MTEP scaffold, contains a photoreactive azide and has ~10-fold lower affinity than parent. RVDU-3-185 is based on the MPEP scaffold, contains a photoreactive azide and has 10-fold higher affinity than MPEP. RVDU-3-130 and RVDU-3-185 exhibit slow binding kinetics resulting in low affinity estimates derived from Ca^{2+} mobilization assays due to non-equilibrium conditions. All three $mGlu_5$ negative allosteric modulator probes irreversibly label the receptor following UV exposure. Furthermore, we demonstrate successful conjugation of fluorescent dyes using click chemistry onto the irreversibly bound photoprobes with in-gel fluorescence.
Discussion. Clickable photoprobes represent an innovative strategy to identify unknown binding sites and the work here is the first application of this approach to a G protein-coupled receptor. The common allosteric site photoprobes disclosed here represent novel tool compounds to study the structural basis of allosteric modulation of $mGlu_5$.

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Species-dependence of Cannabinoid Receptor 2 functional selectivity

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Introduction. Cannabinoid Receptor 2 (CB2) is a promising therapeutic target in immune-related diseases. Recently Atwood et al. (2012) compared the propensity of cannabinoid ligands to stimulate CB2 internalisation and reported functional selectivity for two potent agonists: CP55,940 (CP) internalised CB2, whereas WIN55,212-2 (WIN) did not.
Aims. We have observed a novel phenomenon whereby at the same time as inducing receptor internalisation, CB2 agonists also stimulate increased receptor delivery to the cell surface (likely via mobilisation of the CB2 intracellular pool). In internalisation assays designed to sample only the resulting surface expression level following drug stimulation (as utilised by Atwood et al.), the net combination of these pathways can result in the impression that no trafficking has taken place. We investigated whether this mobilisation could explain the previously reported difference in trafficking between CP and WIN.
Methods. HA-tagged human and rat CB2 stably expressed in HEK-293 cells were detected by fluorescent immunocytochemistry and intensity per cell quantified by automated imaging and analysis with an ImageXpress Micro XL automated microscope. Surface receptors were labelled with primary antibody either prior to drug stimulation (true internalisation) or following drug stimulation (net surface expression) and fluorescent secondary antibody applied under non-permeabilising conditions.
Results. We replicated the findings of Atwood et al. in terms of net receptor expression following drug stimulation. When internalisation was measured directly we observed that while WIN did not internalise rat CB2, WIN *does* internalise human CB2 with similar potency to CP, though slightly lesser efficacy. By comparison of internalisation with net surface expression we find that WIN is highly efficacious at stimulating delivery of hCB2 to the cell surface, producing the net result of apparently unchanged surface expression despite considerable receptor turnover.
Discussion. These results add to the evidence that there are substantial differences in the function of rat versus human CB2. Extreme care must be taken in interpreting and translating results from rodent model studies.

Atwood et al (2012) Molecular Pharmacology 81(2):250-63

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Do relaxin receptors function as obligatory dimers?

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Introduction. Relaxin is a peptide hormone that has recently passed a Phase III clinical trial for treatment of acute heart failure. RXFP1 is the cognate receptor of relaxin, and is a G protein coupled receptor (GPCR) with a complex mechanism of activation. RXFP1 forms constitutive dimers at the cell surface; however it is not known whether dimerisation is necessary for activation or if monomeric RXFP1 could be activated. A better understanding of the mechanism of RXFP1 activation will help drug design efforts to target this receptor.
Aims. To elucidate the functional importance of RXFP1 dimerization by disrupting the constitutive dimerisation of RXFP1 at the cell surface.
Methods. A BRET (Bioluminescence resonance energy transfer) assay was established as a measure of RXFP1 dimerisation. Receptor function was assessed using a cAMP reporter gene assay. DNA sequences representing single RXFP1 transmembrane (TM) domains and relevant control TM proteins were cloned into the pcDNA3.1 expression vector for co-expression assays. Synthetic RXFP1 TM peptides were also used to disrupt dimerization.
Results. Synthetic TM peptides were designed to block RXFP1 TM interactions, however they demonstrated no effect when tested in functional assays. In a parallel approach, single RXFP1 TM domain sequences have been cloned into pcDNA3.1 and will be tested for effects on dimerization and RXFP1 activation. A BRET assay was successfully established to show RXFP1 homodimerisation, using RXFP1-Venus and –Rluc8 pairing. Functional assays demonstrated the constructs retained activity and BRET saturation assays showed a $BRET_{MAX}$ of ~170 mBU for RXFP1 homodimers, in contrast with the TRHR1-Rluc8 control which demonstrated non-saturable BRET.
Discussion. Initial attempts to block RXFP1 dimerization with TM peptides did not affect RXFP1 function, probably due to poor solubility of the peptides. TM peptides have since been redesigned, containing poly-lysine tails to improve solubility. The established BRET assay will be used to assess disruption of RXFP1 homodimers by these peptides or by co-transfection of single RXFP1 TM domains in parallel to functional assays. Other means of disrupting RXFP1 dimerisation are also being investigated, and it is hoped that this work will lead to a greater understanding of RXFP1 activation and hence better drug targeting of this receptor.

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Biphasic Ca^{2+}-dependent control of 25-hydroxyvitamin D-1α-hydroxylase expression mediated by the calcium-sensing receptor

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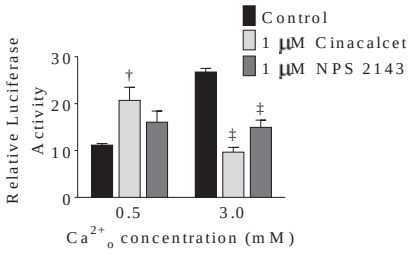
Introduction. Elevated extracellular Ca^{2+} concentration (Ca^{2+}_o) suppresses expression of 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the renal proximal tubule but promotes it in the parathyroid, thus exerting opposing effects on 1,25-dihydroxyvitamin D synthesis in these tissues. However, the underlying mechanisms are unknown.
Aims. In the present study, we investigated the mechanisms of Ca^{2+}_o-dependent modulation of CYP27B1 expression.
Methods. CYP27B1-luciferase constructs were transfected into HEK-293 cells stably expressing the CaSR (HEK-CaSR cells), as well as control HEK-293 cells. The cells were then exposed to different Ca^{2+}_o concentrations in the presence or absence of allosteric modulators for 24 h.
Results. At lower Ca^{2+}_o (0.5-3.0 mM), there was a Ca^{2+}_o-dependent increase in CYP27B1-luciferase expression in HEK-CaSR cells, which was absent in control HEK-293 cells. Interestingly, as Ca^{2+}_o concentrations increased further (5.0-6.5 mM), suppression was observed, to yield an overall biphasic Ca^{2+}_o-dependent response. The positive modulator cinacalcet (1.0 μM) that binds in the heptahelical domain of the CaSR enhanced both these responses, shifting the peak response from 3.0 mM to 0.5 mM, whereas the negative modulator NPS 2143 (1.0 μM) abrogated the Ca^{2+}_o-dependent response, indicating that both stimulatory and inhibitory effects are CaSR-mediated. However, the γ-glutamyl peptide S-methylglutathione (30 μM) that binds in the receptor's Venus flytrap domain had no effect.



Discussion. The results demonstrate the existence of ligand-biased, CaSR-mediated signalling control of gene expression. Experiments that identify the promoter response elements and upstream signalling pathways that support the stimulatory and inhibitory responses may provide a mechanistic basis for the activation of 1,25-dihydroxyvitamin D synthesis in the parathyroid, and inhibition of 1,25-dihydroxyvitamin D synthesis in renal proximal tubule cells.

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Identification of cAMP signalling phenotypes in subpopulations of cells using a flow cytometer

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Introduction. Mixed cell populations, containing a range of transgene expression levels, may be a more realistic model of GPCR function in neuronal cells. Traditional cAMP assays rely on averaging the cAMP in populations of cells (often >30,000 cells per sample), which can obscure interesting phenotypes within a mixed population. Fluorescence-based biosensors allow imaging of cAMP changes in single cells; however microscopy remains a labour-intensive way of collecting data when the population of interest occurs at a low frequency.
Aims. In order to further investigate a cell signalling phenomenon observed in a mixed population of cells, we aimed to develop a high through-put method of measuring cAMP which was suitable for looking at small subpopulations within mixed cultures.
Methods. The "cAMP sensor using YFP-Epac-Rluc" (CAMYEL) biosensor (Jiang et al., 2007) was modified to increase the biosensor signal by substituting the bioluminescence resonance energy transfer (BRET) pair to Venus and Rluc8; this construct was named "V8 CAMYEL". V8 CAMYEL was stably transfected into HEK293 cells, followed by transient transfection of the human cannabinoid CB1 receptor. Cells were stimulated using forskolin and CB1 agonist, then analysed using a Becton Dickinson FACSVantage cell sorter and a VictorXLight platereader.
Results. V8 CAMYEL provided a significantly improved bioluminescent output, allowing BRET detection from small samples of cells. Under different transfection conditions, CB1 signalling was seen as either "Gi-like", "Gs-like" or as giving no apparent cAMP signalling response, as measured by population average. The flow cytometry results further characterise this phenomenon in terms of surface CB1 expression.
Discussion. Mixed cell populations are a valuable source of physiologically-relevant data. This approach allows multiple cell phenotypes to be identified and characterised in the same assay. In the example of CB1 transient transfection, we have shown that a reading from a mixed population may not best reflect the phenotypes of the constituent subpopulations.

Jiang LI, Collins J, Davis R, et al. (2007). JBC 282(14): 10576-10584.

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Signalling pathway-selective consequences of the common μ-opioid receptor variants A6V and N40D

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Introduction. SNPs of GPCRs can affect receptor signalling. μ-Opioid receptor (MOPr) SNPs A6V and N40D are the most common variants, with allelic frequencies of up to 20% and 50% respectively within some populations. Considering their high prevalence, changes in MOPr signalling arising from these SNPs may explain some of the variability in clinical response to opioids.
Aims. We investigated agonist activation of signalling pathways by A6V and N40D MOPr variants compared to the wild-type (WT) MOPr. We used the endogenous opioid analogue DAMGO and the clinically important opioids morphine and buprenorphine (bup).
Methods. CHO and AtT20 cells were stably transfected with human WT, A6V or N40D MOP receptors. MOPr mediated adenylyl ciclase (AC) inhibition and ERK1/2 phosphorylation was assayed in CHO cells (Knapman et al, 2014). MOPr-induced K channel activation and desensitisation was assayed in AtT20 cells using membrane potential-sensitive dye in a Flexstation 3 and compared with agonist-induced MOPr phosphorylation at Ser377 determined by Western blot.
Results. At N40D-MOPr, bup efficacy was reduced by over 50% for AC inhibition (Emax (%) WT 35±6; N40D 16±4 (n =5, $P<0.05$)) and ERK1/2 phosphorylation (Emax (%) WT 35±7; N40D 14±6 (n =5, $P<0.05$)) with no effect on potency; while bup potency was reduced threefold for K channel activation (pEC50 WT 7.0±0.1; N40D 6.7±0.1 (n=6, $P<0.05$)) but efficacy was unchanged. At A6V-MOPr, bup inhibition of AC and stimulation of ERK1/2 phosphorylation was lost and DAMGO and morphine inhibition of AC and ERK 1/2 phosphorylation was significantly reduced; however activation of K channels was preserved. DAMGO induced phosphorylation of Ser377 at 30 min was significantly reduced at N40D (one-sample t-test 80±4% (n=5, P=0.008)), with morphine-induced phosphorylation unaffected while the modest bup-induced phosphorylation was increased at A6V.
Discussion. DAMGO and Morphine signalling through N40D MOP receptors was not changed, but buprenorphine signalled less effectively. At A6V, signalling to AC and ERK by all agonists was compromised but K channel activation was preserved. Agonist-induced MOPr phosphorylation at S377 was only modestly affected by the SNPs.
Knapman et al. (2014) Br J Pharmacol 171:4273-4288

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Signalling profile of CRIP1a: G-protein activation and signal transduction

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Introduction. Cannabinoid receptors are a family of GPCRs that are involved in a wide range of physiological functions and diseases. Key regulators unique to cannabinoid receptors are cannabinoid receptor interacting proteins (CRIPs). Among them, CRIP1a was found to decrease the constitutive activity of the cannabinoid type-1 receptor.
Aims. To gain an understanding of how CRIP1a modulates agonist-induced CB1 receptor function.
Methods. CB1R agonists, WIN55,212-2 (WIN), CP55,940 (CP), anandamide (AEA) and 2-arachydonylglycerol (2-AG) were used to investigate changes in signalling in the presence or absence of CRIP1a. Changes in K+ channel signalling were determined using a Membrane Potential Assay in AtT20-hCB1 cells inherently expressing CRIP1a. Changes in ERK1/2 phosphorylation and cAMP accumulation were determined using AlphaScreen assays in HEK293-hCB1±CRIP1a cells. These cells were also used to determine changes in G-protein activation using BRET technology following transient transfections with Rluc8- and Venus-tagged constructs.
Results. K+ channel activation: CRIP1a protein knockdown was observed in cells treated with CRIP1a-siRNA (20nM), 48 to 72 hours post-transfection ($p<0.001$). This siRNA-induced CRIP1a knockdown significantly increased both Anandamide and 2-AG-induced K+ channel activation ($p < 0.05$) whilst no change was observed in response to WIN and CP. ERK1/2 phosphorylation and cAMP accumulation: Downstream signalling studies found no significant difference in adenylyl cyclase or MAP kinase activity in response to WIN, CP, AEA and 2-AG in CRIP1a expressing HEK293-hCB1 cells compared with HEK293-hCB1 cells not expressing CRIP1a. G-protein activation: ERK1/2 phosphorylation assays, demonstrated that addition of Rluc8 to CB1 did not alter the potency or efficacy of G protein-coupling following activation by WIN and CP. BRET kinetic studies were used to show ability of the CB1R to signal via specific Gai proteins, including Gai1, 2 and 3, in response to WIN and CP. Ongoing BRET studies are looking at changes in agonist mediated Gi/o protein activation when CRIP1a is overexpressed.
Discussion. Overall these results suggest that CRIP1a modulates CB1 receptor signalling in the ligand- and pathway-specific manner.

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Novel agonist engagement and signalling bias by bitopic ligands at the M_1 muscarinic ACh receptor

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Introduction. Selective activation of the M_1 muscarinic ACh receptor (mAChR) may provide a therapeutic treatment of cognitive dysfunction (Langmead et al., 2008). The M_1 mAChR selective agonist TBPB [1-(1'-2-methylbenzyl)-1,4'-bipiperidin-4-yl)-1H benzo[d]imidazol-2(3H)-one] has recently been described to utilise a bitopic mechanism of action, engaging both the ACh binding (orthosteric) site and a topographically distinct allosteric site (Keov et al., 2013). We adopted a mutagenesis approach to identify key interactions that TBPB makes with the receptor.
Aims. To characterise structural determinants underlying the mode of engagement of TBPB with the M_1 mAChR.
Methods. TBPB and other bitopic ligands were pharmacologically characterised via radioligand binding, intracellular calcium mobilisation and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation assays in cell lines expressing various M_1 mAChR mutants. The binding and functional profiles were compared with those of orthosteric ligands at the same mutants. Computational modelling of TBPB binding to the receptor was also performed to complement these pharmacological data.
Results. Compared to tested orthosteric ligands and consistent with a bitopic mode of binding, the binding affinities and signalling efficacies of bitopic ligands were similarly impaired at orthosteric site mutants. However, mutations outside of the orthosteric site discriminated between the two ligand classes. Strikingly, mutations in the second extracellular loop altered bitopic agonist bias profiles between the examined signalling assays. Computational modelling predicted a bitopic binding pose of TBPB that supports engagement with these identified regions.
Discussion. These findings and the proposed mode of binding further support TBPB's bitopic interaction with the orthosteric site and extracellular vestibule residues. We also identify a novel role for the second extracellular loop in dictating receptor activation states. This information can aid the development of bitopic and/or allosteric ligands selective for the M_1 mAChR.

Langmead et al (2008) Pharmacol Ther 117:232-243 Keov et al 2013 Mol Pharmacol 84:425-437

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The modulatory effect of morphine on matrix metalloproteinase-9 production in alternatively polarized macrophages

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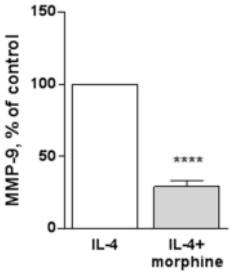
Introduction. Macrophages perform prominent immune activities such as antigen presentation and phagocytosis in the body. They can adopt a variety of phenotypes between two poles: the classically activated macrophage (M1) and the alternatively activated macrophage (M2). In many cancers, tumour-associated macrophages (TAMs) represent more than 50% of the tumour mass. During tumour progression, TAMs display a M2-like phenotype and promote tumour aggressiveness. TAMs secrete matrix proteases that break down the basement membrane and aid tumour cell movement and invasion. Opioids such as morphine, a potent analgesic for management of severe pain (including in cancer patients) have been shown to modulate tumour growth and metastasis.
Aim. To examine the effect of morphine on matrix metalloproteinase production by (i) RAW264.7 macrophages polarized towards M1 or M2 (using IFN-γ and LPS or IL-4, respectively) or (ii) RAW264.7 macrophages co-cultured with 4T1 breast cancer cells to induce a TAM phenotype *in vitro*.
Methods. The level of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) was measured using zymography of cell conditioned media. Moreover, the expression of markers of M1 (iNOS) and M2 (Arginase-1 and MRC-1) phenotypes were measured using immunoblot analysis or real time reverse transcriptase polymerase chain reaction (RT-qPCR). MMP-9 mRNA was also measured using RT-qPCR.



Results. MMP-9 production was increased significantly and dose-dependently in the conditioned medium of IL-4 treated macrophages but not in IFN-γ- and LPS-treated macrophages. MMP-2 production remained unchanged by all treatments. IL-4 further increased mRNA levels of the Arginase-1 and MRC-1 markers as well as MMP-9. Morphine reduced MMP-9 production in IL-4-treated macrophages in a naloxone- and methylnaltrexone-reversible manner. Morphine had no effect on MMP-9 production by M1 macrophages. Moreover, the co-culture of macrophages with breast cancer cells resulted in a significant increase in expression of arginase-1 and MMP-9 and this effect was reduced significantly by morphine.
Discussion. Our results indicate that morphine can modulate tumour aggressiveness and invasiveness by decreasing macrophage MMP-9 production and M2 polarization within the tumour microenvironment.

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Ligand-biased signalling and ligand-biased allosteric modulation at CB1 cannabinoid receptors

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Introduction. CB1 cannabinoid receptors (CB1Rs) are the most abundant G protein-coupled receptors in the brain and are potential targets for the treatment of numerous central nervous system disorders. Although a large number of CB1R ligands exist, their therapeutic applications are limited due to adverse on-target effects. Thus, selective activation of only receptor signalling events that mediate desired therapeutic effects would greatly improve CB1R-targeted therapies and may be achieved via a phenomenon referred to as ligand biased signalling (Kenakin and Christopoulos, 2013). There is growing evidence that CB1R agonists, and more strikingly, the CB1R allosteric modulator Org27569 (Price et al, 2005) may display pathway selective effects at CB1Rs.
Aims. The present study aimed to detect and quantify ligand-biased signalling and ligand-biased allosterism at CB1Rs using sophisticated analytical methods that may aid the development of selective CB1R-targeted therapies.
Methods. Flp-In CHO cells stably expressing hCB1Rs were used to determine effects of various cannabinoid ligands in the absence and presence of Org27569 on CB1R-mediated ERK1/2 phosphorylation and inhibition of cAMP signalling and on displacement of the CB1 inverse agonist [^{3}H]SR141716A.
Results. Cannabinoid agonists CP55940, THC, anandamide and in particular HU-210 and methanandamide were more biased towards inhibition of cAMP than activation of pERK1/2 whereas WIN55212-2 and 2-AG displayed little bias. Org27569 displaced [^{3}H]SR141716A, however it had minimal effects on binding of cannabinoid agonists. In functional assays, Org27569 completely abolished cannabinoid-mediated inhibition of cAMP, however, in pERK1/2 assays, while Org27569 abolished the response to HU-210 and CP55940, it had little or no significant effect on pERK1/2 activation by 2-AG, WIN55,212-2, anandamide, methanandamide or Δ9-THC.
Discussion. Our study quantifies, for the first time, ligand-biased signalling from the CB1R and provides striking evidence for probe-dependence and biased allosterism by Org27569.
Kenakin T and Christopoulos A (2013) Nat Rev Drug Discov 12(3):205-216
Price MR et al (2005) Mol Pharmacol 68(5):1484-1495

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Genetic Encoding of Unnatural Amino Acids in the Glucagon-like Peptide-1 Receptor (GLP-1R)

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Introduction. The glucagon-like peptide-1 receptor (GLP-1R) has a critical role in metabolic homeostasis, principally through increasing insulin biosynthesis and secretion, and is a key therapeutic target for type II diabetes mellitus. The GLP-1R can be activated by both endogenous (GLP-1(1-36)NH_2, GLP-1(1-37), GLP-1(7-36)NH_2, GLP-1(7-37), oxyntomodulin) and exogenous (exendin-4) peptide agonists, each of which display a unique functional profile.
Aims. The aim of this study is to elucidate direct interaction points between each peptide ligand and the GLP-1R to identify structural elements in the receptor core responsible for determining ligand-specific receptor activity.
Methods. We used amber codon suppression technology to site-specifically introduce unnatural amino acid residues (*p*-benzoyl-Phe, BzF) into select positions of the GLP-1R expressed in mammalian cells in culture. Functional BzF-labeled GLP-1R can subsequently be used in targeted UV photocrosslinking reactions to map sites of interaction between ligands and receptor. Sites chosen for introduction of BzF residues were determined based on existing functional data and molecular models of receptor-ligand complexes. The goal is to introduce BzF residues in such a way that ligand-dependent signaling is maintained in the BzF-receptor mutants, while placing the photoactivatable group close enough to the ligand to create a crosslink upon photolysis.
Results. A panel of site-specifically-labeled BzF GLP-1R mutants has been created and characterized pharmacologically. Functional BzF-GLP-1R mutants are being tested in crosslinking reactions with a series of fluorescently labeled agonist analogues.
Discussion. The differences in interactions between each peptide and the receptor that are established from application of this technology is beneficial in understanding the activation mechanisms involved in GLP-1R function, and will also provide valuable knowledge that will be advantageous to exploit in the design and application of superior therapeutics targeted to this receptor system.

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Disruption of GLP-1 receptor dimersation results in liraglutide sensitivity

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Introduction. Glucagon-like-peptide 1 (GLP-1) acts via a 7 transmembrane receptor that couples, predominantly, to the Gαs subunit stimulating insulin. Recently, treatment of obese or diabetic patients with GLP-1, or one of its longer lasting analogues has been of significant pharmaceutical interest although these drugs are not without servere adverse side effects.
Aims. Abolishing homodimerisation of the GLP-1 receptor has been demonstrated to significantly alter agonist dependent signalling [1]. Here, we determine the effects brought about by abolishing GLP-1 receptor dimerization on G protein selectivity, following stimulation with a range of GLP-1 mimetics (liraglutide and exenatide),.
Methods. The GLP-1 receptor couples to multiple G protein subunits including the stimulatory Gαs, the inhibitory Gαi families as well as Gαq. Previously we have described the use of a yeast system developed by GSK expressing individual Gα chimeras to investigate the G protein signalling bias of the human GLP-1 receptor [2]. Here we use the same yeast platform to determine the effects, on individual G protein couplings when GLP-1 receptor dimerization is abolished.
Results. We report that the dimer disrupting mutant, upon stimulation with GLP-1 (7-36) amide, displays a 100-fold reduction in potency (compared to wild type) when coupled to either the GPA1/Gαs (pEC50 =6.4±0.3) or GPA1/Gαq (5.2±0.9) subunits. Interestingly, no change in potency between the dimer mutant and wild type receptor was observed when coupled to Gαi. In addition, abolishing GLP-1 receptor dimerization appears to significantly reduce the ability of liraglutide to activate Gαs while potentiating the effects of exenatide.
Discussion. This study highlights the need to understand the molecular mechanisms modulating the GLP-1 receptor We have demonstrated the importance of GLP-1 receptor dimerization in modulating signalling by the clinically used GLP-1 mimetics, liraglutide and exenatide. We are currently validating these results in mammalian cell lines.

[1] Harikumar *et al.*, (2012) *Proc. Natl. Acad. Sci. (USA).* 109: 18607. [2] Weston *et al.*, (2014) *Br. J. Pharmacol.* 171: 3651.

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Determining the role of RAMP2 in modulating glucagon receptor G protein coupling

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Introduction. The glucagon receptor (GCGR) is a family B G protein-coupled receptor (GPCR) that predominantly couples to the Gαs subunit. Glucagon is its native ligand and is released from pancreatic α cells and serves to oppose the action of insulin thereby maintaining glucose homeostasis. GCGR like many other family B GPCRs has been suggested to couple through multiple Gαq subunits although the physiological consequences remain to be determined. Recently, building upon previous data [1] we have demonstrated that the GCGR interacts with receptor activity-modifying protein 2 (RAMP2) leading to significant alterations in G protein coupling.
Aims. To determine the effects on G protein coupling when GCGR interacts with RAMP2.
Methods. The human GCGR was expressed, in the presence or absence of RAMP2, in yeast strains specifically engineered to facilitate coupling to a growth reporter through the use of chimeric GPA1/Gα subunits [2]. This system facilitates the characterization of ligand-GPCR-G protein pharmacology in isolation. Stimulation of strains with glucagon enables pharmacological effects to be determined.
Results. We report that, analogous to Gαs, GCGR association with RAMP2 increases the potency of glucagon-stimulated signaling in the chimeric Gαq yeast strains without increasing the maximal response. Further we report that, unlike other family B GPCRs, GCGR does not appear to couple to Gαz, irrespective of the presence of RAMP2.

RAMP	GPA1/Gαs		GPA1/Gαq	
	pEC_{50}	Emax	pEC_{50}	Emax
Vector alone	7.9±0.1	98.0±3.4	7.7±1.5	69.2
RAMP2	8.4±0.2	189.7±8.7	8.3±0.6	67.0

Table 1. pEC_{50} and Emax of GCGR associated with vector alone and RAMP2 in response to stimulation with glucagon.

Discussion. Co-expression of GCGR with RAMP2, analogous to Gαs, increases the potency of glucagon-induced Gαq signalling. This data further demonstrates the modulating effects of RAMP2 on the GCGR-G proteins. We are currently confirming these observations in mammalian cells lines expressing GCGR and RAMP2.

[1] Christopoulos *et al.*, (2003) *J Biol Chem* 248: 3293 [2] Brown *et al.*, (2000) *Yeast* 16: 11.

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Gαq-mediated signalling bias by adrenomedullin2 at the calcitonin gene-related peptide receptor

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Introduction. The calcitonin receptor-like receptor (CLR) is a member of the secretin-like family of G protein coupled receptors. It associates with each of the three receptor activity-modifying proteins (RAMPs) to define 3 receptors with distinct pharmacology to the three native ligands; calcitonin gene-related peptide (CGRP), adrenomedullin (ADM1) and adrenomedullin2 (ADM2) [1]. Signaling of the CLR through Gαs and Gαi subunits has been well characterized, however RAMP-dependent signaling through the Gαq subunit has not been defined.
Aims. To investigate the Gαq-dependent signaling bias of the CGRP, AM1 and AM2 receptors.
Methods. The CLR was co-expressed with each RAMP in *Saccharomyces cerevisiae* yeast strains that facilitate coupling to the HIS3 growth reporter through the use of chimeric GPA1/Gα subunits [2]. The yeast strains were then stimulated with the three native peptides to determine their varying pharmacology.
Results. Expression of the CGRP receptor in the chimeric Gαq yeast strain, generated potencies of the three ligands CGRP>ADM=ADM2 and is in broad agreement with that for Gαs subunit. In contrast to that observed with Gαs, where the maximal response of ADM2 was observed to be 27.81±7.04 (relative to CGRP), ADM2 displayed an elevated maximal response (Emax =137±14.11) in the Gαq expressing strain. For the AM1 receptor, the rank potencies of the ligands were ADM=ADM2>CGRP in the Gαq strain (Gαs: ADM>CGRP=AM2). For the AM2 receptor there was no difference between the relative potencies ADM>ADM2>CGRP for the two Gα subunits.
Discussion. This study demonstrates the RAMP-dependent G protein signaling of the three receptors to the three ligands. Specifically we have demonstrated the ability of ADM2 to generate a high maximal response when used as the stimulating ligand for the CGRP receptor. This data may help to define the as yet undetermined biological role for ADM2. These data highlight the predictive nature of the yeast system and we are currently validating these experiments in mammalian cells.

[1] Poyner *et al.*, (2002) *Pharmacol Rev* 54: 233 [2] Brown *et al.*, (2000) *Yeast* 16: 11.

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Calcilytics and calcimimetics modulate calcium sensing receptor (CaSR) activity via overlapping but distinct allosteric binding sites

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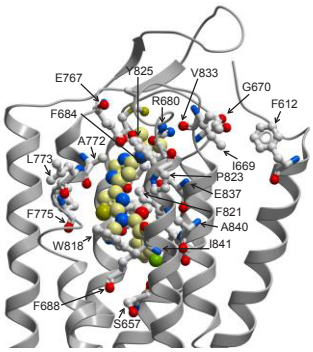

Introduction. The CaSR is a class C G protein-coupled receptor (GPCR) whose primary role is to maintain Ca^{2+}_o homeostasis throughout the body. Calcilytics and calcimimetics are allosteric modulators of CaSR function (Davey et al., 2012; Leach et al., 2013). They have been trialled, or are currently in clinical use, for the treatment of osteoporosis, hyperparathyroidism or disorders of calcium homeostasis. These drugs show diverse pharmacologies (Cook et al., Br. J. Pharmacol., In Press) but how they bind to the CaSR and exert their unique actions is unknown.
Aims. To probe residues in the transmembrane (TM) domains of the human CaSR important for calcilytic and calcimimetic binding and function.
Methods. The effect of up to 40 amino acid substitutions on the functional affinity of cinacalcet, NPSR568, AC265347 and NPS2143 was determined using calcium mobilisation assays. To map the location of amino acid residues identified to be important for modulator activity, a homology model of the CaSR based on the recent crystal structure of the family C metabotropic glutamate receptor 1 (mGluR1) was generated.
Results. Amino acid residues that altered the functional affinity or allosteric modulation (cooperativity) of calcilytics or calcimimetics were generally homologous to residues that line the allosteric binding sites in mGluR1 or mGluR5. However, whereas the binding of the calcilytic, NPS2143, was predicted to extend from the TM domains up towards the extracellular loops, the small calcimimetic, AC265347, was predicted to bind deeper in the TM domain. The larger calcimimetics, cinacalcet and NPSR568, likely bind in an intermediary location.
Discussion. Family C GPCRs share a common allosteric binding site that extends from the top to the middle of the TM domains. Calcilytics and calcimimetics can occupy different portions of this large binding cavity, which may partly explain their diverse pharmacology.

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Structurally distinct calcimimetics engender biased signalling from the human calcium sensing receptor (CaSR)

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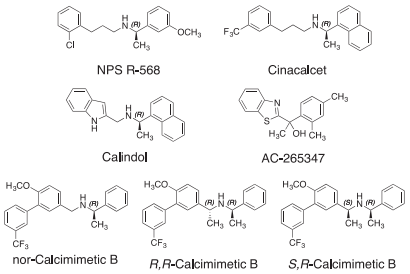

Figure 1 Structure of calcimimetics used in this study

Introduction. The clinical use of cinacalcet in hyperparathyroidism is complicated by hypocalcaemia, arising, at least in part, via activation of CaSRs in the thyroid and kidney and stimulation of calcitonin release and ion transport, respectively. Alternative calcimimetics that selectively bias signalling of the receptor towards pathways that mediate desired effects (e.g. PTH suppression) at the exclusion of those that mediate undesirable effects (e.g. hypocalcaemia), may offer superior therapies.
Aims. To determine the potential for structurally distinct calcimimetics to engender ligand-biased signalling from the CaSR.
Methods. The ligand-biased profile of calcimimetics (Figure 1) was characterised in HEK293 cells stably expressing the human CaSR and monitoring effects on Ca^{2+}_i mobilisation, IP_1 accumulation, pERK1/2 and receptor expression.
Results. Phenylalkylamine calcimimetics (cinacalcet, calindol, NPS R-568) were biased towards allosteric modulation of Ca^{2+}_i mobilisation and IP_1 accumulation. *S,R*-calcimimetic B was biased only towards IP_1 accumulation. *R,R*-calcimimetic B and AC-265347 were biased towards IP_1 accumulation and pERK1/2. Nor-calcimimetic B was unbiased. In contrast to phenylalkylamines and calcimimetic B analogues, AC-265347 did not promote trafficking of a loss-of-expression naturally occurring CaSR mutation ($G^{670}E$).
Discussion. The identification of allosteric modulators that bias CaSR signalling towards distinct intracellular pathways provides an opportunity to develop desirable biased signalling profiles *in vivo* for mediating selective physiological responses.

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Desensitization of α_{1A}-adrenoceptors by oxymetazoline

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Introduction and Aims. The α_{1A}-adrenoceptor has an important role in the regulation of smooth muscle contraction in the prostate and vasoconstrictor effects in resistance blood vessels. This receptor is also known to be less phosphorylated, desensitized and internalized upon exposure to endogenous catecholamines, including noradrenaline (NA) and adrenaline, than other adrenoceptor subtypes. Oxymetazoline (OXY) is a partial agonist at the α_{1A}-adrenoceptor that was recently found to induce phosphorylation, followed by rapid desensitization and internalization (Akinaga et al., 2013). The aim of this study is to investigate whether OXY-promoted desensitization has differential effects on a range of signalling pathways activated by the α_{1A}-AR.
Methods. Chinese Hamster Ovary (CHO) cells stably expressing the human α_{1A}-adrenoceptor were pre-incubated for 5 min with OXY (10 μM) or NA (10 μM), followed by rapid washing. Cells were then assayed for NA-stimulated intracellular calcium release, ERK phosphorylation and extracellular acidification rate (ECAR).
Results and Discussion. A decrease in the E_{max} of cells pre-treated with 10μM OXY compared to the control concentration-response curve was used as an indicator of receptor desensitization. A statistically significant decrease in the E_{max} for NA was observed following pre-exposure to OXY (10 μM) in assays for intracellular calcium release, ECAR and ERK phosphorylation with reductions of 17%, 37% and 100% respectively. No desensitisation was seen following exposure to NA (10 μM). These results indicate that desensitisation is closely linked with α_{1A}-adrenoceptor stimulation by OXY, although each pathway shows differential sensitivity, perhaps associated with coupling efficiency. This suggests that OXY is a biased agonist selective for the desensitization pathway of the α_{1A}-adrenoceptor, which may provide an alternative to the pharmacotherapeutic use of α_1-adrenoceptor antagonists.

Akinaga (2013) Mol Pharm 83: 870 – 881

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Lipid Conjugation for Targeting Endosomal Signalling

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Introduction. G protein-coupled receptors (GPCRs) can be regulated through internalization to the endosomal recycling pathway or lysosomal degradative pathway. Mounting evidence suggests that GPCRs can stimulate specific cell signalling events upon entering the endosomal network and that these are distinct from plasma membrane GPCR signalling events. The neurokinin 1 receptor (NK_1R) is a mediator of pain and inflammation. Upon stimulation by the neuropeptide Substance P, NK_1R undergoes arrestin-mediated internalization to endosomes and promotes sustained signalling as a part of a signalling complex. These internalized signalling complexes are a potential therapeutic target and hence, the rational targeting of drugs to the endosomal network offers the potential to selectively manipulate NK_1R signalling.
Aims. To evaluate drug lipidation as a method for targeting soluble drugs to intracellular compartments.
Methods. Constructs comprised of cholestanol (sterol group) for membrane anchoring, a polyethylene glycol (PEG) chain and either an NK_1R antagonist (spantide), agonist (GR73632) or cyanine5 (cy5) fluorophore reporter. As a control, the same constructs were synthesized without the cholestanol anchor. Cholestanol dependent trafficking of the Cy5 conjugate was investigated using high-resolution confocal microscopy. Forster resonance energy transfer biosensors were used to investigate spatially and temporally distinct signalling pathways initiated from cell surface and internalized NK_1R.
Results. Unlike the ethyl ester control, Cholestanol-cy5 rapidly associated with the plasma membrane of cells and internalized to discrete compartments. Cholestanol-cy5 trafficked to Rab5-positive early endosomes within 2 hours and to Rab7-positive late endosomes after 4 hours. When comparing soluble or membrane-anchored antagonists and agonists, changes in the magnitude and duration of endosomal signalling were observed.
Discussion. Lipid conjugation offers the potential for targeting GPCRs in specific intracellular locations and over extended periods, as a novel approach for selectively regulating spatially and temporally distinct signalling events.

Jensen *et al*. (2014) J Biol Chem 289:20283-94; Rajendran *et al*. (2008) Science 320:520–3.

729

Enhanced β_3-adrenoceptor expression and function in rosiglitazone-treated inguinal white adipocytes.

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Introduction: The high prevalence of obesity has provoked substantial interest in adipocyte thermogenesis as a means of expending excess energy. A potential therapeutic target is the β_3-adrenoceptor (AR), as sympathetic stimulation of brown adipose tissue (BAT) activates the β_3-AR to uncouple mitochondria via increased UCP1 expression and function. Recently functional BAT activity has been demonstrated in adult humans [1], but these cells may more closely resemble the brite (brown in white) adipocyte rather than classical BAT [2]. Brite adipocytes are derived from the conversion of a fat-storing white adipocyte into a brown-like genetic phenotype by multiple stimuli including the PPARγ activator rosiglitazone (ROSI) [3], but little is known of their thermogenic capacity.
Aims: To determine the effect of rosiglitazone on β_3-AR expression and function in mouse brite adipocytes.
Methods: Primary inguinal white preadipocytes from FVB mice were differentiated in vitro, and brite adipocyte expression induced by 1μM ROSI treatment. β_3-AR expression (RT-PCR, confocal microscopy) and function (cAMP production, [^{3}H]-2-deoxyglucose uptake, mitochondrial oxygen consumption) was assessed in response to norepinephrine (NE) in the absence/presence of ROSI. *p* values calculated using 2-way ANOVA or Student's t-tests.
Results: ROSI treatment of white adipocytes increased β_3-AR expression and NE-stimulated cAMP production (control: max 5.7±0.6% forskolin (100μM), pEC_{50} 7.2±0.4; +ROSI: max 12.9±1.3%, pEC50 7.0±.3, n=5, $p<0.05$). This coincided with an increase in UCP1 mRNA expression (1000-fold) and NE-stimulated (1μM) oxygen consumption (control: 127.7±12.5% basal; +ROSI 234.8±27.6% basal, n=12, $p<0.01$) and [^{3}H]-2-deoxyglucose uptake responses (control: 124.3±11.6% basal; +ROSI: 175.9±17.5% basal, n=7, $p<0.05$).
Discussion: ROSI-induced brite adipocytes show an increased capacity for glucose uptake and mitochondrial uncoupling compared to classical white adipocytes, taking on a brown adipocyte metabolic phenotype.

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[2] Wu, J et al (2012) Cell *150*: 366–76
[3] Petrovic, N et al (2010) JBC *285*: 7153-64

730

Binding pocket residue analysis of orphan G protein-coupled receptor, GPR37L1, a potential mediator of cardiovascular homeostasis: insights into ligand structural determinants

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Introduction. Over 100 orphan Class A G protein-coupled receptors (GPCR) remain an unlocked source of novel therapeutic targets. No endogenous ligand has been identified for GPR37L1, classified in the peptide β-subgroup of Class A GPCRs, although neuropeptides prosaptide and prosaposin have been proposed (Meyer et al, 2013).
Aims. To gain insights into interaction pharmacophores of GPR37L1 ligands based on pocket residue analysis.
Methods. For each unique Pocketome GPCR entry (www.pocketome.org), binding pocket residues were propagated through an alignment from the entry sequence to GPR37L1 using ICM v3.8-1 (Molsoft LLC, San Diego). A GPR37L1 homology model was built based on the neurotensin 1 receptor (PDB: 4GRV; 20% sequence identity). Prosaptide was tested for GPR37L1 activation using various assays *in vitro*.
Results. In at least 70% of GPCR Pocketome entries, conserved ligand-receptor interactions were seen with residues at positions 3.32, 5.43, 6.51, 6.52 and 7.39 (Ballesteros and Weinstein numbering). This indicates their importance for ligand binding and activation. For GPR37L1, these residues are E3.32, Y5.43, E6.51, N6.52 and Q7.39, which create an overall negative charge in the pocket. From our homology model, the E3.32 represents an uncompensated charge, indicating preference for interaction with positively charged or amine-containing ligands.
Discussion. An acidic residue in position 3.32 is observed in only a few receptor subfamilies in the entire GPCR family. Biogenic amine receptors have an aspartic acid at 3.32 that enables recognition of N+ of the endogenous amine. For GPR37L1, the glutamic acid at 3.32 makes it suitable to interact with basic residues or the N-terminus of a peptide. Furthermore, E6.51, located directly opposite E3.32, may be compensated by K7.45 but 'switch' interacting partners upon ligand binding. Supporting our 'pocket' analysis, no activation of GPR37L1 by prosaptide was observed *in vitro* likely because the prosaptide peptide sequence does not contain basic residues. We are currently looking for ligands that can complement the unique binding pocket features of GPR37L1.
Meyer RC et al (2013) PNAS 110: 9529-9534

731

Structural Elucidation of The Venus Fly Trap Module In The Calcium-Sensing Receptor

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1. School of Molecular Bioscience, University of Sydney, NSW 2006.

Introduction. The Calcium-Sensing Receptor (CaSR) is a Class C G-protein coupled receptor (GPCR), structurally characterised by the presence of a large nutrient sensing N-terminal extracellular region consisting of a bilobed Venus Fly Trap (VFT) module and a cysteine-rich (CR) domain. Up to five primary binding sites for the endogenous ligand Ca^{2+} and a binding pocket for positive allosteric modulator L-phenylalanine have been identified in the VFT. In addition, the VFT module is known to be involved in receptor dimerisation and activation. However, there are currently no available structures for CaR's major domains.
Aims. The aim of the current project is to determine the structure of the VFT module in the N-terminal extracellular region of the Calcium Sensing Receptor. The structural elucidation of the extracellular domain of the Calcium sensing receptor is anticipated to provide critical insights into mechanisms underlying ligand binding and promiscuity as well as receptor dimersation and cooperativity.
Methods. Based on secondary structure predictions, disorder analysis and sequence alignments of the CaSR across species and Class C GPCRs, six variants of CaSR extracellular domain (ECD) inserts were prepared and ligated into bacterial protein expression vectors pRSET, pGEX, pMal c2x and pMal p5x for expression in Rosetta and BL21 *E.coli* cells. These vectors were His, GST and MBP tagged respectively.
Results. Preliminary results from protein expression trials showed overexpression of the His-tagged CaSR ECD constructs confirmed by a western blot using anti-His antibody. However, interestingly most of the His-tagged protein appeared to be cleaved into two fragments although full-length protein and oligomers of the protein were visible in non-reducing conditions. Overexpression of GST tagged CaSR ECD constructs were also observed in Coomassie blue R250-stained SDS-PAGE and confirmed by western blotting using a CaSR ADD polyclonal antibody. However, the protein does not seem to be soluble in any of the conditions tested.
Discussion. Successful overexpression of His- and GST-tagged constructs have been observed. However, the low yield and solubility of the expressed proteins are limiting factors in further structural studies. Therefore, other expression systems including mammalian and insect cell systems are currently being investigated.

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Characterizing Novel Aspects of Signaling Bias from GLP-1 Receptor using RNA-Seq.

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Introduction The glucagon-like peptide 1 receptor (GLP-1R) is a key physiological regulator of β-cell function and survival and currently targeted for treatment of type II diabetes mellitus (TIIDM). TIIDM is characterized by hyperglycemia, insulin resistance and β-cell dysfunction. Long acting GLP-1R agonists, exendin-4 and liraglutide are approved therapeutics for this disease. The molecular mechanisms underlying GLP-1R physiology are highly complex and poorly understood, but $G_{\alpha s}$-mediated cAMP production and signaling via β-arrestins is implicated in augmentation of glucose sensitive insulin secretion, enhanced β-cell proliferation and anti-apoptotic effects. Limitations of GLP-1 peptidomimetics have led to a continued interest in the search for small molecule GLP-1R agonists as therapeutics.
Aims This study evaluates biased signalling at the GLP-1R mediated by peptide ligands with/without allosteric modulators in an endogenously expressing cell system. Subsequently, global cellular changes are assessed to identify various signaling proteins accountable for the positive profile of GLP-1R ligands in INS-1 832/3 cells.
Methods Biased signalling by different ligands was assessed by cAMP accumulation, insulin secretion, ERK1/2 phosphorylation, iCa^{2+} mobilization, proliferation and apoptosis in both high and low glucose. In- depth RNA-Seq was also performed on RNA extracted from cells treated in presence of different orthosteric ligands at different time.
Results We show glucose-dependent effects of GLP-1R ligands on insulin secretion, ERK1/2 phosphorylation, iCa^{2+} mobilization, proliferation and survival that in some cases were modulated in presence of small compounds. RNA-Seq identified differentially expressed genes in response to GLP-1R peptides that were associated with insulin secretion and cell survival mechanisms, some of which were subsequently validated using an RT-qPCR array.
Conclusion These results reveal biased-signalling profiles of multiple ligands in an endogenously expressing GLP1R system. They also elucidate modulation of GLP-1R signaling in presence of small molecule allosteric modulators. This may help in designing small molecule agonists with potential to either augument/mimic the positive actions of GLP-1, while reducing side-effect profiles thus identifying better therapeutics for the treatment of TIIDM.

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Determination of signaling signature of 40 SNPs found in melatonin type 2 receptor: from ligand-biased signaling to mutation-bias signaling.

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Introduction. Melatonin binds two high affinity G protein-coupled receptors (GPCRs): MT1R and MT2R. Genome-wide association studies revealed 40 single nucleotide polymorphisms (SNPs) of MT2R. Some SNPs are found exclusively in patients suffering from type 2 diabetes (T2D) or having preclinical signs of T2D while others are found in both T2D and normal subjects or in normal subjects only. All mutant MT2R exhibit normal receptor expression and only 4 of them show loss of melatonin-binding.
Aims. Given that GPCRs can engage multiple signaling pathways, the objective of this study is to assess the effects of each SNP on the activation of different pathways to generate a «signaling signature».
Methods. Using bioluminescence resonance energy transfer (BRET)-based and protein-complementation assays, we monitored the basal and melatonin-mediated Gαi1 and β-arrestin activation for all SNPs. The relative melatonin-induced responses were determined using the operational model and expressed as Δlog(τ/Ka), while the constitutive receptor activation was expressed as normalized difference between wild-type and each SNP. By plotting these data on radial graphs, we obtained each mutant receptor-signaling signature.
Results. Our results indicate that 14 mutants display drastic overall signaling impairment. Half of these impaired mutations are only found in the T2D patients or individuals with preclinical signs of T2D. Most importantly, their radial graphs are asymmetric.
Discussion. This asymmetry underscores the mutation-induced bias signaling. Indeed, some mutations preferentially reduce Gαi1 activation over β-arrestin responsiveness while other mutations have the opposite effect. Asymmetry also indicates that constitutive and agonist-mediated activities are not always similarly affected by SNPs. Together, our signaling signatures reported for MT2R SNPs may help understanding the etiology of T2D but also illustrate the GPCR pluridimensionality concept and extend the concept of bias signaling from ligand-bias to SNP-bias.

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Determination of receptor binding kinetics of M_1 muscarinic acetylcholine receptor (M_1 mAChR) antagonists using whole cell binding.

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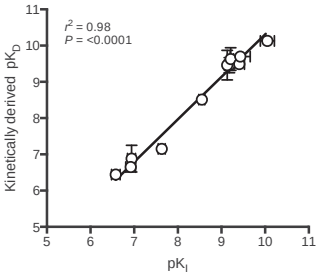
[1]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Parkville, VICTORIA



Introduction. The characterization of receptor binding kinetics of preclinical drug candidates is now becoming more readily appreciated as a key addition to the discovery process. Benefits of studying kinetics include increased understanding of prolonged duration of action[1], ability to differentiate between different pre-clinical candidates[2] and in the understanding of differential *in vivo* efficacies.
Aims. Using competition binding kinetics[3] and whole cell [^{3}H]-NMS binding we investigated the receptor binding kinetics of cold competitive antagonists acting at the M_1 receptor.
Results. Test compounds displayed a range of k_{off} values, varying from 0.73 ± 0.54 (n=4) to 0.006 ± 0.001 min^{-1} (n=3) resulting in mean $t_{1/2}$ values of 1.5 ± 1.1 to 129.3 ± 29.7 min. A near perfect correlation of 0.98, with a *P* value of <0.0001 determined using Deming linear regression, was obtained from classical competition binding studies (pK_i) and the kinetically derived pK_D values. A good correlation was identified between the pK_i values and both the Log k_{on} and Log k_{off} values, with r^2 values of 0.81 and 0.58, respectively.
Discussion. In this study we have demonstrated that the use of competition binding kinetics enables the kinetic parameters of unlabelled compounds to be identified. We identified a wide range of $t_{1/2}$ values of the compounds tested. In addition a good correlation between the pK_i and k_{on} and k_{off} values were determined. This study has shown that compounds with similar pharmacological parameters and closely related chemical structures can display differential binding kinetics, highlighting the necessity for more rigorous pharmacology profiling. Ultimately, this increased pharmacology profile may help explain any unusual effects often observed in the clinic.

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[3]Motulsky & Mahan (1983) *Mol Pharmacol* 25: 1–9

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Use of hemi-equilibrium to determine the dissociation kinetics of histamine H_3 receptor antagonists.

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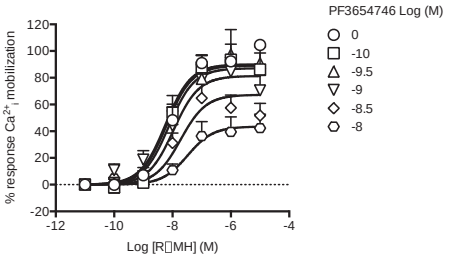
Introduction. Antagonism of the histamine H_3 receptor has been shown to be a potential benefit for patients of obesity, allergic rhinitis and cognitive disorders, including schizophrenia and Alzheimer's[1]. There are two major isoforms of the histamine H_3 receptor, hH_3-445 and hH_3-365, which have been shown to display differential pharmacology. However, no investigation into the kinetic parameters of histamine H_3 antagonists, at either isoform, has been performed.



Aims. As both radioligands available, [^{3}H]-N-α-methyl histamine and [^{125}I]-iodoproxyfan, display agonist activity, we utilized an intracellular calcium release assay run under hemi-equilibrium[2] conditions, in combination with a novel analytical approach, to determine the dissociation kinetics of a range of histamine H_3 antagonists.
Results. Test antagonists displayed a range of k_{off} values, resulting in $t_{1/2}$ values of 1.0 ± 0.0 to >70 min (the limit of quantification for slowly dissociating antagonists). In most cases there was little difference between the $t_{1/2}$ values obtained for each compound against both isoforms. However, both PF-03654746 and thioperamide displayed differential binding kinetics at the two isoforms; PF-03654746 rapidly dissociated from the hH_3-365 isoform with a $t_{1/2}$ of 5.5 ± 16.0 min compared to >70 min from the hH_3-445 isoform. Conversely, thioperamide dissociated rapidly from the hH_3-445 isoform with a $t_{1/2}$ of 3.8 ± 2.6 min compared to 67.0 ± 30.1 min from the hH_3-365 isoform.
Discussion. These data demonstrate the utility of functional assays ran under hemi-equilibrium conditions to determine the binding kinetics of unlabelled histamine H_3 antagonists. The difference in binding kinetics between the major isoforms may prove a novel mechanism in developing isoform specific compounds.

[1]Leurs et al (2005) *Nature Reviews Drug Discovery* 4(2): 107–120
[2]Kenakin T (2009) *Pharmacology Primer 3rd edition*

736

GPRC6a mediates cellular responses to L-amino acids, but not osteocalcin variants

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Introduction. By analysis of KO mice, the promiscuous class C GPCR, GPRC6a, has been shown to be involved in regulation of metabolism, inflammation and endocrine function. Such effects are described as mediated by L-amino acids, the bone-derived peptide osteocalcin or the male hormone, testosterone. However, verification of activity of osteocalcin and testosterone at GPRC6a *in vitro* has proved somewhat elusive.
Aims. This study characterized the pharmacology and signalling of recombinantly and endogenously expressed GPRC6a in response to all putative GPRC6a ligands, including recombinant and synthetic osteocalcin.
Methods. Receptor function was assessed in assays of Ca^{2+} mobilisation, cAMP and inositol phosphate accumulation, ERK1/2 phosphorylation (HEK293-mGPRC6a cells) and GLP-1 and insulin release (GLUTag and βTC-6 cells, respectively). GPRC6a expression was confirmed by FACS and/or qPCR.
Results. Co-expression of $G\alpha_q$ in HEK293-mGPRC6a cells enabled robust calcium mobilization and inositol phosphate accumulation in response to L-amino acids with a rank order of potency: L-ornithine > L-arginine = L-lysine. These responses were sensitive to application of the GPRC6a antagonist, NPS-2143. No agonist (or modulatory) activity could be detected for any form of osteocalcin or testosterone in any of the signalling assays. Furthermore, no ERK1/2 phosphorylation or cAMP accumulation could be detected in response to any putative GPRC6a ligand. Finally, stimulation of GPRC6a by basic L-amino acids in GLUTag and βTC-6 cells results in GLP-1 and glucose-dependent insulin secretion, respectively. No effect of osteocalcin variants or testosterone could be detected in either of these cellular assays.
Discussion. These data do not support a role for osteocalcin or testosterone as ligands for GPRC6a, suggesting that the reported *in vivo* effects requiring GPRC6a may be indirect, rather than *via* direct activation of the receptor.

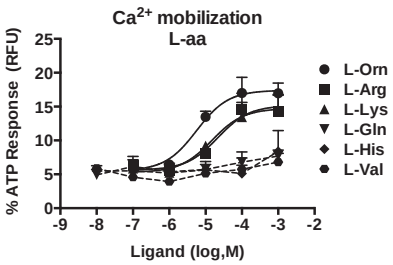

Figure 1. GPRC6a-mediated Ca^{2+} mobilization in response to L-amino acids in HEK293-mGPRC6a cells.

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Stimulation of glucose uptake by α_{1A}-adrenoeptors involves mTORC2, AMPK, and Rac1

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Introduction. It has been shown that stimulation of α_{1A}-adrenoceptors (α_{1A}-AR) causes glucose uptake in skeletal muscle involving phospholipase C and glucose transporter 4 (GLUT4) translocation to the plasma membrane (Hsu et al., 2004; Hutchinson et al., 2005). However the signalling pathways downstream of α_{1A}-AR activation linking to GLUT4 translocation remain to be identified.

Aims. To evaluate the signalling pathways activated by the α_{1A}-AR that are coupled to glucose uptake.

Methods. Signalling pathways mediating glucose uptake were investigated using selective kinase inhibitors, siRNA and detected by measuring ^{3}H 2-deoxyglucose uptake, western blots, α-screen assays and confocal microscopy.

Results. Noradrenaline (NA), and the selective α_{1A}-AR agonists A61603 and oxymetazoline, and the Ca^{2+} ionophore A23187 concentration-dependently increased glucose uptake in CHO-K1 cells stably expressing the α_{1A}-AR. The AMPK inhibitor Cmpd C and siRNA of the mTORC2 component rictor significantly inhibited α_{1A}-AR-mediated glucose uptake. The Rac1 inhibitor NSC23766 inhibited glucose uptake to NA, A61603, oxymetazoline, and A23187 by 85%, 80%, 71%, and 81% respectively. Western blot and α-screen assay showed that α_{1A}-AR agonists had no effect on Akt and Erk phosphorylation but increased mTOR and AMPK phosphorylation. Confocal analysis showed increased GLUT4 translocation following stimulation by NA, A61603, oxymetazoline, and A23187.

Discussion. α_{1A}-AR mediate distinct pathways that activate mTORC2 and AMPK, and Rac1 has a key role for glucose uptake potentially due to actin reorganization which is involved in the translocation of glucose transporters to the plasma membrane.

Hsu JH et al (2004) Planta Med. 70(12):1230-3
Hutchinson DS et al (2005) Endocrinology 146(2):901-12

Functional Selectivity of GPR43

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Introduction. G protein-coupled receptor 43 (GPR43) is the endogenous receptor for the short chain fatty acids (SCFA's) acetate, propionate and butyrate and an attractive therapeutic target for several inflammatory diseases (Bindels, Dewulf, & Delzenne, 2013). The orthosteric binding site of GPR43 is highly homologous to GPR41 (Schmidt et al., 2011) which makes ligand selectivity problematic. Synthetic allosteric ligands have been previously described which have greater selectivity and improved potency over the SCFA's. The functional selectivity of these ligands has not been comprehensively examined and as such biased signalling may have been overlooked.

Aims. To profile the signalling pathways of acetate and the allosteric ligands on GPR43 and quantify any biased signalling.

Methods. CHO-K1, stably expressing human GPR43 (DiscoveRX) and CHO-K1 (Cellbank) were used for all experiments. Sodium acetate (Chem supply) and the ligands 2-(4-chlorophenyl)-3-methyl-N-(thiazol-2-yl)butanamide (4CMTB) (Lee et al. 2008) and 4-((4-(2-chlorophenyl)thiazol-2-yl)amino)-4-oxo-3-phenylbutanoic acid (4CTAP) (Hoveyda et al., 2010) were synthesised in house with both the racemic, R and S enantiomers tested. Calcium responses were measured using Invitrogen Fluo-4 Direct™ assay (Life Technologies). cAMP was measured using a LANCE® Ultra cAMP assay (Perkin Elmer). Phosphorylation of ERK 1/2 was assessed using the Alphascreen® Surefire® pERK1/2 (Thr202/Tyr204) assay kit. Label-free assays were performed using the Enspire® optical label-free biosensor.

Results. Acetate was an agonist for calcium and ERK signalling with an EC_{50} value of ~150 µM. 4CMTB was an ago-allosteric modulator for ERK1/2 signalling. The D enantiomer for 4CMTB was the most potent with an EC_{50} of 20 nM, this being ~100 fold more potent that the L enantiomer. However, 4CMTB was only a positive allosteric modulator through calcium signalling. 4CTAP was shown to be an antagonist for acetate induced ERK1/2 and calcium signalling. Signalling via Gαs detected by cAMP showed an interesting biphasic response for all the ligands tested that warrants further testing. None of these ligands inhibited forskolin induced cAMP production via Gαi activation.

Discussion. We have shown that stereochemistry can change the potency of a ligand and in some cases its function. This data evidences the complex nature of GPR43 signalling and highlights the need for thorough testing of ligands via multiple signalling pathways to fully understand the potential of ligands as therapeutic options.

Bindels L B Dewulf E M & Delzenne N M (2013) Trends Pharmacol Sci *34*(4) 226–32
Hoveyda H Brantis C E Dutheuil G *et al.* (2010) PCT Int. Appl. WO2010066682
Lee T Schwandner R Swaminath G *et al.* (2008) Mol Pharmacol *74*(6) 1599–1609
Schmidt J Smith N J Christiansen E *et al.* (2011) *J Biol Chem* *286*(12) 10628–40

739

Stimulus bias of allosteric modulators at the metabotropic glutamate receptor subtype 5.

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Introduction. Allosteric receptor modulators offer the advantage of spatial and temporal fine-tuning of endogenous agonist activity, allowing for increased selectivity, reduced adverse effects and improved clinical outcomes. Some allosteric compounds can also differentially modulate distinct signalling pathways arising from the same receptor, a phenomenon referred to as stimulus bias or biased modulation. Emerging evidence for the glutamatergic theory of schizophrenia suggests the metabotropic glutamate receptor subtype 5 ($mGlu_5$) is a promising target. Current allosteric modulators of $mGlu_5$ have largely been classified based solely on their effects in modulating intracellular calcium responses of orthosteric agonists alone, resulting in a narrow cataloguing of compounds.
Aims. To assess $mGlu_5$ allosteric modulators across multiple endpoints of receptor activation, in order to determine their potential for engendering biased agonism and/or modulation.
Methods. Intracellular calcium mobilisation, IP_1 accumulation and PKC phosphorylation were assessed in stably transfected $mGlu_5$ HEK293 cells and mouse striatal and cortical neurons. Allosteric ligands were profiled alone and in the presence of orthosteric agonist.
Results. VU0403602 is a positive allosteric modulator and agonist of glutamate-mediated calcium mobilisation ($\log\beta=1.02\pm0.09$, $\log\tau_B=-0.126\pm0.06$), an agonist of IP_1 accumulation ($pEC_{50}=9.01\pm0.28$) and a neutral allosteric ligand of glutamate-mediated PKC phosphorylation. VU0360172 is a positive allosteric modulator of glutamate-mediated calcium mobilisation ($\log\beta=0.83\pm0.12$), a partial agonist of IP_1 accumulation ($pEC_{50}=7.84\pm0.12$), a negative modulator of glutamate-mediated IP_1 accumulation ($\log\beta=-0.12\pm0.038$), with a trend towards negative allosteric modulation of glutamate-mediated PKC phosphorylation.
Discussion. Our results highlight that stimulus bias is operative between pathways that were traditionally considered to be linearly linked. Stimulus bias may provide a potential explanation for the diverse *in vivo* effects of $mGlu_5$ allosteric modulators. Ultimately, we will lay the groundwork for rational drug design of pathway-targeted compounds in the treatment of an otherwise refractory CNS disorder.

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μ-Opioid receptor signaling mechanisms: quantifying bias and kinetics

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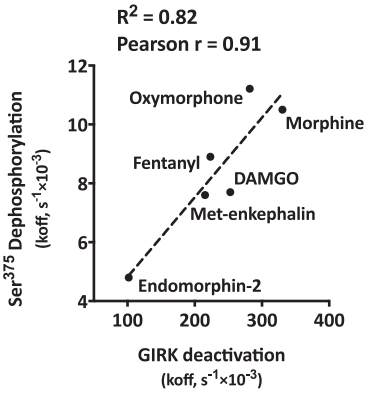

Introduction. Attempts to develop opioid analgesics with reduced ability to produce tolerance and dependence after chronic exposure, have recently focused on ligand-direct signaling or biased agonism, whereby different agonists at a G protein-coupled receptor differentially interact with distinct signaling effectors. Understanding of unique characteristics of each agonist-receptor pair including agonist efficacy, affinity and the kinetics are extremely important for discovery of novel biased analgesics that selectively activate the signaling pathways mediating therapeutic effects but not adverse side effects.
Aims. To determine biased agonism and kinetics of a range of MOPr ligands for different signaling pathways including GIRK activation, Ser^{375} phosphorylation, β-arr2 recruitment and internalization in AtT20-MOPr cells.
Methods. Electrophysiology for GIRK current recording, immuno-staining for Ser^{375} phosphorylation and internalization and BRET technique to determine β-arr2 translocation.
Results. The present study illustrated that the kinetics of MOPr for these signaling pathways were agonist dependent. There was a strong positive correlation for the agonist off-rate among all pathways. Furthermore, the off-rate kinetics were correlated with the agonist ability to induce receptor internalization.
Discussion. Slowly dissociating agonists such as endomorphins produce greater endocytosis relative to their initial signaling efficacy than rapidly dissociating agonists; therefore the internalization ability of an agonist can be characterized by duration of receptor occupancy that is intrinsic for each ligand-receptor pair.

Kenakin T (2013). *British journal of pharmacology* 168**:** 554-575

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Does RXFP1 form heteromers with GPCRs relevant in heart failure?

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Introduction. Relaxin is a 2 chain, 3 disulphide bonded member of the insulin/relaxin peptide superfamily that is the cognate ligand for the relaxin family peptide receptor 1 (RXFP1). Despite relaxin successfully completing a Phase III clinical trial for the treatment of acute heart failure, the mechanisms responsible for these effects are yet to be identified. Recent studies suggest that the anti-fibrotic actions of relaxin are dependent on the formation of heteromers of RXFP1 with the angiotensin receptor AT2.
Aim. This study investigates the formation of heteromers between RXFP1 and G protein-coupled receptors (GPCRs) that are known to be dysregulated in cardiac failure and whether heteromer formation is associated with functional consequences.
Methods. Interactions between RXFP1 and other GPCRs was examined in transiently transfected HEK-293 cells using bioluminescence resonance energy transfer (BRET). BRET Heteromer Investigation Technology (HIT) was used in transiently transfected HEK-293 cells and stably expressing HEK-RXFP1 cells to examine possible functional interactions.
Results. BRET saturation assays showed constitutive heteromerization between RXFP1 and angiotensin II receptor type 1 (AT_1R), β_2- and β_3-adrenoreceptors and endothelin type A (ET_AR) and B (ET_BR) receptors. BRET HIT studies in HEK cells transiently transfected with AT_1R, RXFP1 and G protein-coupled receptor kinase 2 (GRK2) showed a decreased signal between AT_1R and GRK2 following activation of RXFP1 with relaxin.
Understanding RXFP1 interaction with other GPCRs and their subsequent signalling profiles may identify roles for RXFP1 that may be relevant to heart failure.

Teerlink JR et al (2013) Lancet 381:29-39
Chow BS et al (2014) Kidney Int. 86:75-85

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Investigating a novel mechanism of activation of the relaxin family peptide 2 receptor (RXFP2)

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Introduction. The INSL3 (insulin-like peptide 3) receptor, RXFP2, is a unique GPCR as it contains a large extracellular domain, containing a single low density lipoprotein class a module (LDLa), and 10 leucine rich repeats (LRRs). The LDLa is critical for receptor activation, whereas the LRRs comprise the major binding site. The LDLa is linked to the LRRs through a linker region, with no structural homology to other proteins. Evidence has suggested that this linker has important functions in RXFP2. A deeper understanding as to how the linker in RXFP2 acts to couple the ligand binding LRRs to the LDLa is required to facilitate future drug discovery efforts targeting RXFP2.
Aims. To determine the role of the linker for RXFP2 function with mutagenesis, binding assays, cell signaling assays and with structural biology.
Methods. Transfected HEK293T cells expressing mutant (alanine substituted residues within the linker) receptors, generated using Quickchange site directed PCR, were assayed using a colorimetric CRE reporter assay, measuring effective receptor activation. Following this, a europium labelled INSL3 peptide was used to measure peptide binding affinities. Finally, the LDLa module attached to the linker was generated, and peptide interactions were studied using HSQC NMR.
Results. Mutations made in RXFP2 did not affect cell surface expression, nor peptide binding affinity as demonstrated through saturation binding. Functional analysis of these receptors as measured by changes in cAMP activation highlighted that EC50 values were not affected other than at residue D46A, however, decreases in Emax's were seen largely from mutations in linker residues D46, W50, and less from residues G45, G49 and F54.
Discussion. The results suggested that particular residues in the linker play an important role in the activation mechanism of RXFP2, but not in ligand binding. It is assumed that reduced activity demonstrated primarily by residues D46 and W50 are a result of altering secondary/tertiary structure in the linker, and thus preventing presentation of the module for adoption of active conformations, however this hypothesis needs further exploration.

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Interaction of agonists and antagonists with the transmembrane region of Protease Activated Receptor 2

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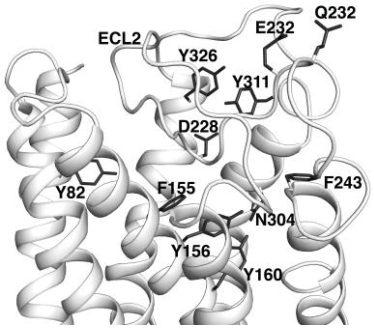

Introduction. Protease Activated Receptor 2 (PAR2) is a GPCR involved in metabolism, inflammation and cancers. It is self-activated by intramolecular binding of the new N-terminus exposed by serine proteases. Short synthetic peptides can also activate PAR2 and are routinely used as surrogates for proteases in PAR2 studies. The ECL2 of PAR2 has been reported to be important in receptor-ligand interactions for both native and synthetic agonists.
Aims. To identify the ligand binding site(s) on PAR2 in mediating activation, antagonism and intracellular signalling associated with PAR2.
Methods. 28 hPAR2 mutants were studied by site-directed mutagenesis, guided by computer-based ligand docking studies. Each cell line was treated with trypsin, synthetic agonists (2f-LIGRLO-NH_2, GB110), and antagonist (GB88) and tested for intracellular Ca^{2+} release. EC_{50}/IC_{50} generated by each mutant was compared to WT.
Results. Mutations clustered in the TM region were found to affect PAR2 interaction with the ligands (max potency reduction >100-fold). There was a high correlation between these effects and different PAR2 ligands suggesting similar binding locations. However, there was no significant correlation between the profiles observed for synthetic compounds versus those for trypsin, indicating different binding sites for these ligands.
Discussion. Understanding ligand interaction with PAR2 is fundamental to understanding receptor activation, signal transduction and rational drug design. This study illustrated the significance of key TM residues in PAR2 and has pinpointed the binding sites of synthetic agonists and an antagonist. These three ligands clearly have a different binding site from the protease-cleaved tethered ligand and highlights potential problems in biomedical studies to date of inferring that synthetic ligands can be used as surrogate agonists for endogenous proteases.
Suen JY et al (2012) Br J Pharmacol 165:1413-1423

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Comprehensive analysis of factors influencing quantification of biased signalling at the mu-opioid receptor

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Introduction. The dynamic structure of GPCRs allows different ligands to stabilise the receptor into unique active conformations, which can result in differential activation cell signalling pathways and, eventually, in distinct physiological outcomes. This phenomenon, known as “signalling bias” or “functional selectivity”, can be exploited to design new opioids that selectively activate signalling pathways that lead to analgesia whilst minimising side effects that are elicited by activation of other signalling pathways. However, bias is not an absolute quality, rather it is a dynamic and multidimensional phenomenon; it is always relative to a reference ligand, and it is dependent on both cellular protein complement as well as the spatiotemporal properties of the different signalling endpoints.
Aims. This work has investigated how the cellular background influences the direction of biased agonism of μ opioid receptor (MOR) agonists and the impact of signalling kinetics on the detection of bias.
Methods. We have quantified signalling bias at the MOR across multiple different signalling pathways in CHO-MOR overexpressing different signalling effectors, and quantified biased signalling at multiple time points.
Results. This work has revealed that the relative signalling bias of set of ligands is different in CHO cells and AtT20 cells . Additionally, this work has shown that the time points selected for measuring activation of different signalling effectors can significantly alter the bias observed across different pathways.
Discussion. Altogether this demonstrates the changeable nature of ligand bias, which presents a significant challenge when attempting to predict biased signalling *in vivo*. However these studies have also identified ligands that maintain unique signalling bias profiles in different cell lines, which may be predictive of distinct physiological outcomes. A greater understanding about biased signalling in native tissue and *in vivo*, will enable links between distinct signalling bias profiles and specific physiological responses to be established, providing essential new information for the design opioids with effective analgesia and less side effects.

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Small molecule inhibitors of invertebrate dopamine receptors as leads for new mode-of-action insecticides

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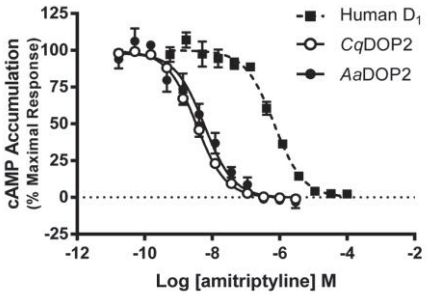
Introduction. New mode-of-action insecticides are required to control invertebrate vectors of neglected infectious diseases such as dengue and malaria. Invertebrate biogenic amine-binding GPCRs mediate essential neurological processes and are attractive targets for discovery of novel insecticides.

Aims. Study aims were to analyse the pharmacology of D1-like dopamine receptors from mosquito and tick vectors, identify receptor antagonists and evaluate *in vivo* effects.

Methods. Receptors were cloned, expressed and pharmacologically characterized in mammalian cells. Chemical library screening was conducted to identify receptor antagonists that were subsequently evaluated using *in vitro* cellular assays and *in vivo* whole-organism assays.



Results. Invertebrate receptors exhibited dopamine-stimulated cAMP responses *in vitro*. Multiple small molecule antagonists demonstrated high *in vitro* potency and significant mortality of mosquitoes *in vivo*. Several chemistries showed greater than 100-fold selectivity for invertebrate receptors versus the orthologous human D1-like receptor. Structure activity relationship studies were used to investigate receptor-ligand interactions, develop a preliminary pharmacophore and identify candidate insecticide leads.

Discussion. These studies provide proof of concept for a target-based approach to discover novel small molecule inhibitors of invertebrate dopamine receptors as leads for development of pest-selective, safer insecticides.

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The impact of internalization on compartmentalized trafficking and signalling of the delta-opioid receptor

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Introduction. Endocytosis of G protein-coupled receptors (GPCRs) is traditionally viewed as a regulatory mechanism that attenuates signalling by depleting receptors from the cell surface. However, recent work suggests that endocytosis of GPCRs may generate signals in defined subcellular compartments, with unique outcomes. The delta opioid receptor (DOPr) is a major therapeutic target for pain, but the importance of receptor endocytosis for signalling has not been examined.

Aim. To define the importance of DOPr trafficking for the generation of signals in defined subcellular compartments.

Methods. The human embryonic kidney (HEK) 293 cells expressing DOPr were studied. The ability of DOPr to activate G-proteins and to recruit β-arrestins (β-arrs) was determined using bioluminescence resonance energy transfer (BRET). Förster resonance energy transfer (FRET) biosensors targeted to the plasma membrane, cytosol or nucleus were used to assess the subcellular activation of ERK and PKC.

Results: The DOPr agonists SNC80, DADLE and ARM390 which have been postulated to have strongly, moderately and weakly internalising capabilities respectively (all 100 nM) all induced movement of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_{io}$ relative to $G\beta\gamma$. No changes were detected for either $G\alpha_s$ or $G\alpha_q$, suggesting that DOPr only activates $G\alpha_{i/o}$ G proteins. While all ligands activated G proteins, only SNC80 and DADLE (but not ARM390) induced recruitment of β-arrs to DOPr. The ligands also induced distinct spatiotemporal signalling profiles. SNC80 induced a transient increase in plasma membrane and cytosolic PKC phosphorylation. In contrast, DADLE caused a sustained increase in plasma membrane and cytosolic PKC activity, and there was no effect of ARM390. The distinct temporal profiles were also observed when ERK activity was measured: SNC80 induced transient cytosolic and nuclear ERK, DADLE induced sustained cytosolic and nuclear ERK, while ARM390 only induced sustained cytosolic ERK.

Discussion. Though preliminary, the data suggests that a range of protein interactions and spatiotemporal signalling profiles result upon stimulation. However, further studies will be required to determine whether the distinct spatiotemporal signalling profiles of the ligands are linked to differential β-arr recruitment and DOPr internalisation.

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Pharmacological Characterisation of Synthetic & Putative Endogenous Allosteric Modulators of the M_2 Muscarinic Acetylcholine Receptor (mAChR).

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Introduction. G protein–coupled receptors (GPCRs) are the major mediators of intercellular chemical communication. The recognition that virtually all GPCRs possess additional allosteric binding sites that can bind synthetic ligands, termed *allosteric modulators*, has revolutionised the field.
Aims. The widespread presence of allosteric sites across virtually all GPCRs suggests that there may be hitherto unappreciated roles for these sites in both health and disease, including interaction with endogenous modulators [1]. Certainly, the G protein itself is the best example of an endogenous allosteric modulator of GPCRs. Another striking example is the human eosinophil Major Basic Protein (MBP), a peptide constituting ~50% of the inflammatory peptides released following eosinophil degranulation, that binds allosterically to the M_2 mAChR [2]. We hypothesised that other endogenous proteins bearing similar structural features to MBP, such as polycationic, and arginine-rich peptides, may also interact allosterically with the M_2 mAChR [3,4].
Methods. The pharmacological properties of L-arginine (L-Arg), poly-L-arginine (PLA), dynoprhin-A (Dyn-A), and myelin basic protein (MyBP), were investigated, at the M_2 mAChR.
Results & Discussion. In [^{3}H]NMS radioligand binding assay, both the synthetic peptide, PLA, and the endogenous proteins, Dyn-A and MyBP appeared to bind allosterically with the M_2 mAChR. At the level of function, both in ERK1/2 phosphorylation and calcium mobilisation assays, all three putative modulators allosterically altered ACh-mediated responses. Collectively, these data highlight the potential role of an allosteric site on a GPCR in regulating physiology and/or pathology via endogenous allosteric ligands.

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New fluorescent antagonists for the histamine H_1-receptor

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Introduction. Molecular tools to better understand G protein-coupled receptors (GPCRs) are vital for drug design and optimization. GPCR drug discovery often focuses on characterizing the affinity and efficacy of ligands but rarely considers ligand kinetics. The dissociation kinetics of a fluorescent GPCR ligand can be directly monitored using confocal microscopy coupled to a perfusion system (May et al).
Aims. To develop small molecule-based fluorescent probes for the histamine H_1 receptor (H_1R), and demonstrate utility of these molecules as drug discovery tools, in particular in reference to receptor-ligand kinetics.
Methods. Fluorescent probes based on the H_1R antagonist mepyramine and the recently described antagonist VUF131816 (de Graaf et al) were synthesised. The affinities of these compounds for the H_1R were analyzed, and the H_1R dissociation rates of the fluorescent probes determined.
Results. All the fluorescent probes tested retained affinity at the H_1R compared to the parent pharmacophores. Confocal imaging showed that displaceable membrane binding, with low levels of non-specific intracellular accumulation, could be detected. Kinetic experiments revealed a very slow dissociation rate at the H_1R.
Discussion. Fluorescent probes are very powerful drug discovery and development tools, and can be used to interrogate a receptor-ligand interaction at the molecular level in a single, live cell. Fluorescent ligands with similar affinity can display different dissociation kinetics at the H_1R.
This work has received support from the EU/EFPIA Innovative Medicines Initiative Joint Undertaking, K4DD grant no 115366.

May et al P (2010) Mol. Pharmacol. 78, 511-523.
de Graaf et al (2011) J. Med. Chem. 54, 8195-8206.

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The Calcitonin gene-related peptide family of receptors exhibit G-protein coupling bias.

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Introduction. Calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and adrenomedullin 2 (AM2) are peptide hormones and are involved in vascular angiogenesis, vasodilation, heart disease and pain response. These peptides signal through the CGRP, AM_1 and AM_2 class B G protein-coupled receptors with different potencies (1). These receptors couple to $G\alpha_s$ and are obligate heterodimers of the calcitonin receptor-like receptor (CLR) and receptor activity-modifying proteins (RAMPs) 1-3 (2). The CGRP and AM_1 receptors when stimulated with CGRP or AM respectively can couple to $G\alpha_i$ (3). However, there is no complete picture of $G\alpha_i$ coupling to these receptors.
Aims. To investigate $G\alpha_i$ coupling to the CGRP, AM_1 and AM_2 receptors on binding of CGRP, AM and AM2.
Methods. We expressed the CGRP, AM_1 and AM_2 receptors in a yeast system with a $G\alpha_s$ and $G\alpha_i$ chimera adapted to couple to the yeast-mating pheromone-reporter system (4) upon treatment with CGRP, AM or AM2. We verified this coupling in HEK293S and Cos7 cells by pertussis toxin inhibition of $G\alpha_i$ coupling to the CGRP, AM_1 or AM_2 receptors and measurement of cAMP accumulation.
Results. The yeast reporter system indicated coupling of all three receptors to $G\alpha_i$ upon CGRP, AM and AM2 binding. In HEK293 cells $G\alpha_i$ coupled to the CGRP and AM_1 receptors upon CGRP stimulation. In Cos7 cells $G\alpha_i$ coupled to the CGRP receptor when stimulated with AM and the AM_1 receptor on stimulation with AM and AM2.
Discussion. The yeast system provides essential information on the ability of the CLR based receptors to couple to different G-proteins so directing further investigation of G-protein coupling in mammalian systems. Our results suggest that the CGRP, AM_1 and AM_2 receptors show $G\alpha_i$ coupling-bias dependent on the cell line.

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Probing agonist and antagonist interactions at the relaxin-3 receptor RXFP3

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Introduction. Relaxin-3 is a highly conserved neuropeptide involved in the modulation of stress, feeding and cognitive processes. Its receptor is the Class A G protein coupled receptor GPCR135, now known as RXFP3. Structural knowledge of relaxin-3/RXFP3 interactions will assist structure-based drug design of smaller blood brain barrier penetrating relaxin-3 mimetics to treat neurological disorders. The residues contributing to binding (R8, R12, R16, I15, F20) are on the relaxin-3 B-chain central helix whereas the C-terminal residues R26 and W27 are important for activation. We have developed an RXFP3 specific antagonist which retains the core binding residues but with the C-terminal activation residues replaced by a non-native Arginine residue. Importantly this Arginine contributes an additional high affinity binding interaction.
Aims. To identify the sites of agonist and antagonist binding in RXFP3 using mutagenesis and homology modelling.
Methods. Conserved glutamic (E) and aspartic (D) acid residues in the N-terminus and extracellular loops of RXFP3 were mutated to Alanine to charged interactions. This approach successfully identified interaction sites for R12, R16 and R26 and was used to generate an RXFP3/relaxin-3 binding model to further predict potential ligand interactions with the transmembrane domains of RXFP3 for activation. These residues were similarly mutated to Alanine and tested for receptor binding and activation using cAMP activity assays.
Results. E141A, D145A and E244A in extracellular loops 1 (EL1) and 2 (EL2) showed significant decreased agonist and antagonist binding compared to wild type RXFP3. Seven transmembrane mutations (W138A, T162A, V241A, L246A, K271A, F364A and T346A) resulted in markedly decreased agonist binding without significant reduction in antagonist binding compared to wild type RXFP3. Interestingly, one transmembrane mutation (W339A), which showed the same level of wild type RXFP3 binding, demonstrated complete loss of activity.
Discussion. Based on the model, E141 and D145 in EL1 and E244 in EL2 are important for agonist binding to R26, R16 and R12 respectively. The significant decrease in activity despite no change in binding affinity suggests that W339 might be involved in cation-π interaction or π- π stacking with W27.

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Heterodimerization of the Calcium--Sensing Receptor with T1 Taste Receptors

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Introduction. The calcium-sensing receptor (CaSR),and T1 taste receptors are all class C G-protein coupled receptors that are co-expressed in GI-associated endocrine tissue including insulin-secreting pancreatic islet beta cells. Although T1Rs form heterodimers of the type T1R1/2:T1R3, it is not known whether the CaSR forms heterodimers with any of the T1Rs and, if so, the consequent functional significance.
Aims. The study aimed to determine whether heterologous co-expression of the CaSR and one of the T1Rs in CHO cells results in heterodimer formation and whether any novel receptors arising have distinctive functional properties.
Methods. The methodology used was immunoprecipitation, using anti-FLAG beads (Sigma). IPone assays were conducted using CISBIO IPone kit.
Results. The results indicate that the CaSR forms heterodimers preferentially with T1R3 and to a lesser extent with T1R2. The calcium-sensing properties are comparable to that of the wild-type CaSR as reported by the IPone signalling pathway. Experiments aimed at defining the macronutrient-sensing properties of the novel receptors are in progress.
Discussion. Heterodimerization of the CaSR with T1R3 may be stabilized by a disulphide between conserved Cys residues, i.e., C129 and C129 in both receptors. The impacts of hetero-dimerization of the CaSR with the T1Rs on nutrient sensing and receptor-dependent biased signalling properties require investigation.

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Harnessing unnatural selection: engineering thermostabilised α_1-adrenoceptors for drug discovery

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Introduction. α_{1A}- and α_{1B}-Adrenoceptors (α_{1A}-AR & α_{1B}-AR) are G protein-coupled receptors (GPCRs) that play critical roles in regulating the cardiovascular and nervous systems. Recent evidence suggests α_{1A}-AR & α_{1B}-AR drive opposing responses to adrenaline or noradrenaline activation in modulating cardioprotection, neuroprotection and seizure suppression. However the lack of subtype selective ligands makes it difficult to probe the individual roles of α_{1A}-AR & α_{1B}-AR and validate them as targets for heart and neurological diseases. To facilitate structural biology and thus structure based drug design of more subtype selective compounds, we use directed evolution techniques such as Cellular High-throughput Encapsulation Solubilisation and Screening (CHESS) to engineer thermostabilised GPCRs. Stabilised GPCRs can be purified and applied to biophysical techniques such as X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) to aid structure-based drug design (SBDD).
Aims. To thermostabilise α_{1A} and α_{1B}-ARs using CHESS and enable structural investigation for drug discovery.
Methods. CHESS is a novel directed evolution technique which converts libraries of α_1-AR cells into microcapsules which retain the plasmid of encoding receptors after detergent solubilisation. Microcapsules containing stabilised α_1-AR were probed with fluorescent Prazosin (QAPB) and microcapsules containing stable mutants selected using Fluorescence activated Cell Sorting (FACS). 10 rounds of CHESS were conducted on both α_{1A}-AR & α_{1B}-AR.
Results. Several highly stable α_{1A}-AR & α_{1B}-AR mutants were engineered, which could be purified in detergents and retained the ability to bind to known ligands. These mutants carried only 10-14 mutations and their high expression levels and stability makes them perfect candidates for X-ray crystallography. Their stability over long time periods in purified form also enabled us to measure the binding of methoxamine and noradrenaline using Saturation Transfer Difference (STD) NMR, a method commonly used for fragment screening, but has never been applied to GPCRs.
Discussion. This project will broaden our fundamental structural understanding of α_1-ARs and validate our cutting edge technology for enabling the application of X-ray crystallography and NMR to GPCRs to facilitate SBDD.

The role of phosphorylation sites in rapid desensitization of the μ-opioid receptor

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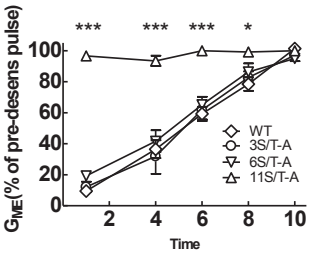

Introduction. Tolerance and addiction to opioids are serious clinical and social problems that result in part from the loss of μ-opioid receptor (MOPr) function when activated by different opioids.
Aims. The aim of this study was to determine the influence of C-terminal phosphorylation sites on rapid desensitization of MOPr.
Methods. Wild type MOPr, 3S/T-A, 6 S/T-A and 11S/T-A mutants were expressed stably in AtT20 cells. Using perforated patch-clamp recording we examined the effects of MOPr activation, desensitization and re-sensitization using activation of GIRK channels by a submaximal concentration of Met^5-Enkephalin (10 nM) to measure receptor activity and somatostatin (100 nM) coupling to native SSTR2 to determine heterologous desensitization.
Results. MOPr desensitization and resensitization produced by 5 min exposure to Met-Enkephalin (10 μM) at 37°C for 3S/T-A and 6S/T-A mutants did not differ from wild type but desensitization was abolished in the 11S/T-A mutant. However, upon exposure to morphine (10μM) at 37°C, all three mutants showed desensitization similar to wild type MOPr, which was found to be PKC dependent. Desensitization, when detected, was largely homologous for both met-enkephalin and morphine.
Discussion. Because 3S/T-A suppresses, and 6S/T-A abolishes MOR endocytosis, these findings suggest that homologous desensitization can occur independently of the phosphorylation and arrestin-dependent mechanisms that drive endocytosis. However, C-terminal phosphorylation sites are necessary for desensitization because mutation of all C-terminal sites (11S/T-A) abolishes desensitization with Met-Enkephalin.

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