



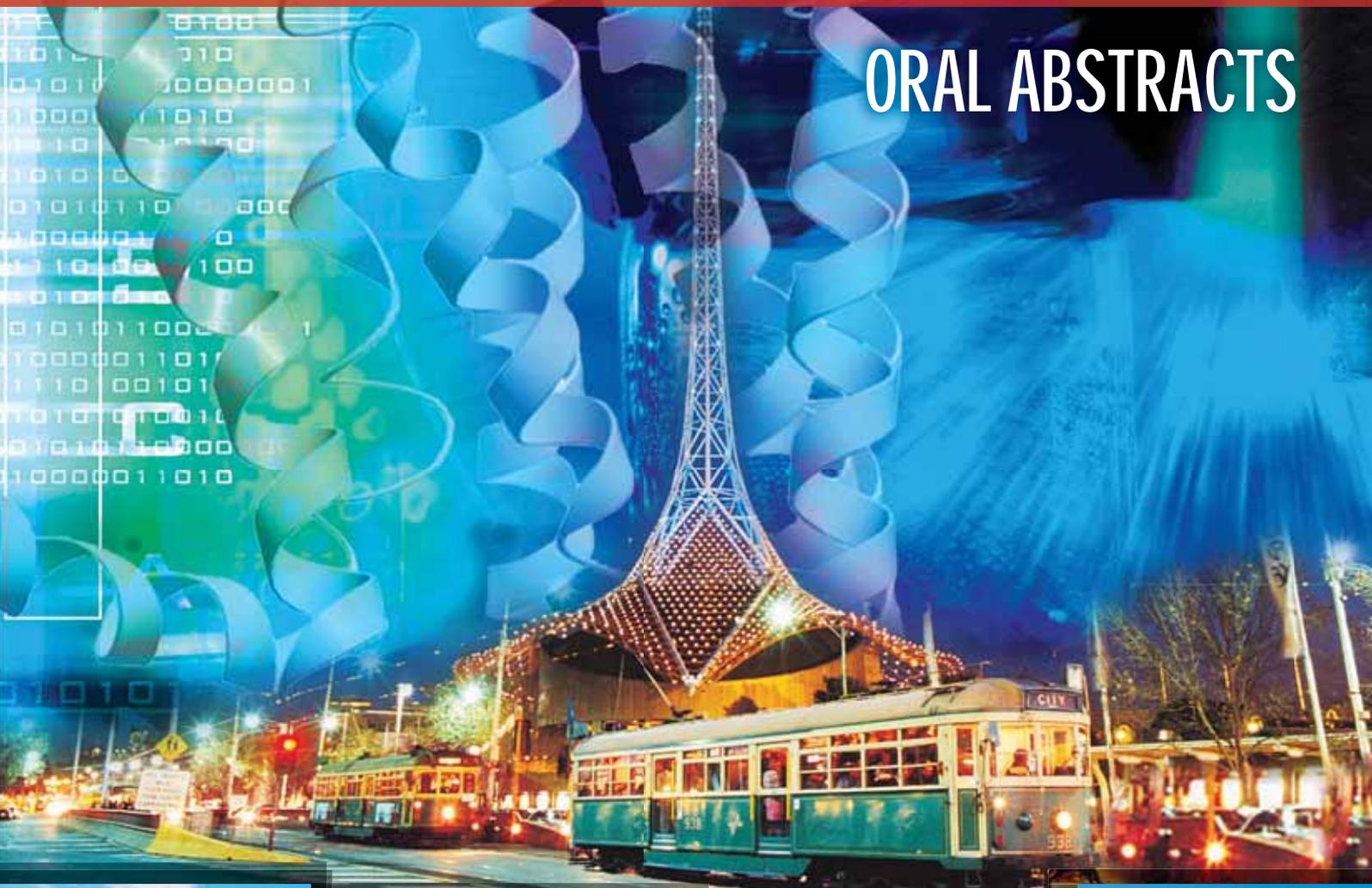
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ORAL ABSTRACTS



Therapeutic agents and drug targets: GPCRs and beyond, opportunities and challenges.

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About half of modern drugs target GPCRs, although the trend decreases since the completion of the human genome project and with the almost endless possibilities offered by biologics. Monoclonal antibodies targeting proteins/enzymes, are on the rise, if only because of perceived limited chemical space and lack of selectivity for low molecular weight (LMW) compounds. Although orthosteric binding pockets of GPCRs are conserved across receptors families (see glutamate receptors), the structural and functional knowledge of GPCRs is increasing dramatically: thus, the availability of GPCR-ligand crystals enables the design of more selective LMW ligands. Many GPCRs have allosteric sites which are much less conserved across / within receptor families, paving the way for more selective allosteric / bitopic modulators. Even the orthosteric space offers great opportunities, especially when taking into account G-Protein-dependent versus -independent receptor modulation, receptor interaction with different G-proteins, or RAMPs interacting with calcitonin (like) receptors, all resulting in multiple conformations of the same receptor ligand complex. Constitutive activity may vary with pathological state, this expands the field of application for both agonists and inverse agonists. The main challenge is to identify translational models relevant to human disease, since receptors do signal differently in different cellular backgrounds and pathologies (e.g. 5-HT_{2C}R editing and increased constitutive activity in spinal cord injury). Better understanding of a) pathology and b) structural biology of GPCRs and their partners, contributes to better drug design, avoids potential cross reactivities (e.g. agonists targeting 5-HT_{2A} or 5-HT_{2B}R may cause hallucinations or cardiac fibrosis/pulmonary hypertension, respectively) and enables precision medicine. Thus, GPCRs and their interacting partners as drug targets are on track. The molecular pharmacology and structural biology of GPCRs, their computational biology and chemistry, structure-based drug design, druggability, biased signaling and more, are at the core of this meeting.

Kobilka BK, Lefkowitz RJ. Nobel Prizes 2012: *Angewandte Chemie-International Edition* **2012**, 51, 11199. Kruse A et al. *Nature* **2012**, 482, 552-556. Wacker D et al. *Science* **2013**, 340, 615-619. Wu HX et al *Science* **2014**, 344, 58-64.

Translating G-protein coupled receptors from chemistry to clinic: Apelin and biased signalling in the cardiovascular system

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Introduction. Apelin (Pyr¹apelin13) binds a single G-protein coupled receptor and has an emerging role in human cardiovascular homeostasis. Apelin causes vasodilatation and it is the most potent inotrope discovered to date in isolated human heart. This signaling pathway is down-regulated in cardiovascular disease where short term systemic infusion of apelin to replace missing peptide produces beneficial vasodilatation and an increase in cardiac output. These results suggest synthetic agonists would be of therapeutic benefit. A limitation of many agonists acting at GPCRs is that, after signalling via G-proteins to produce a physiological action, the target receptor is internalised via the β -arrestin pathway, limiting the beneficial physiological action.

Aim. We hypothesize that a 'biased' agonist preferentially activating the G-protein pathways rather than β -arrestin, will reduce desensitization, produce a more robust response, continued vasodilatation and beneficial inotropy.

Results. Using computational chemistry, we have designed and identified the a 'biased' apelin agonist, MM07 and shown that it has comparable activity to the native peptide in G-protein mediated pathways *in vitro* but lower β -arrestin and internalization activity. MM07 caused rapid and significant peripheral arterial dilatation in the human forearm. The magnitude of the response was significantly greater than the endogenous peptide. Importantly, there was no evidence of desensitization when repeated infusions were given. In the human hand vein, MM07 significantly reversed an established noradrenaline pre-constriction. In anaesthetized rats intravenous boluses of MM07 caused a significant increase in cardiac output, without evidence of positive chronotropy with a magnitude greater than that the native peptide. Daily intra-peritoneal injects of MM07 prevented the development of pulmonary arterial hypertension in the monocrotaline rat model.

Discussion. The results suggest MM07 functions as biased agonist *in vitro* and is more effective *in vivo* than apelin in increasing cardiac output and vasodilatation. Biased apelin agonists may have a therapeutic advantage in the treatment of conditions such as pulmonary arterial hypertension.

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Orphan GPCRs in the development of cardiovascular disease

Nicola J Smith, Molecular Cardiology & Biophysics, Victor Chang Cardiac Research Institute

G protein-coupled receptors (GPCRs) are the most tractable drug targets in the genome yet a vast majority of them remain orphans, that is, they are yet to be paired to their endogenous ligand. Without a ligand, understanding even the most basic physiology and pharmacology of these receptors is challenging, resulting in the therapeutic potential of many GPCRs remaining untapped. Here I will present the work of our group examining the therapeutic potential of one orphan GPCR, GPR37L1, which appears to be a major regulator of blood pressure control. By using a variety of molecular and pharmacological approaches, we have been able to determine that GPR37L1 signaling is regulated by a ligand-independent mechanism involving the N-terminus of the receptor and we now have evidence that this novel signaling paradigm is present *in vivo*. Future studies will now be aimed at both deorphanizing GPR37L1 and determining how the novel mechanism of activation and inactivation is involved in blood pressure control.

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Targeting protease-activated receptors for prevention of arterial thrombosis: Current and future approaches

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Platelet-dependent arterial thrombosis is the most common cause of death in Australia. As a result, drugs that block platelet function are sought for improved anti-thrombotics. Thrombin is the most potent endogenous platelet activator. There are two thrombin receptors on human platelets, PAR1 and PAR4 – both of which are capable of inducing platelet activation by thrombin. We have previously shown that PAR-deficient mice are protected against a series of experimental thrombosis models, but do not exhibit spontaneous bleeding events. These proof-of-concept studies paved the way for the development of PAR antagonists as novel anti-thrombotic agents, leading to the recent FDA approval of the first PAR1 antagonist, vorapaxar. However, vorapaxar is being issued with a boxed warning excluding certain patient groups due to a significant bleeding risk in these groups, suggesting there is much to learn regarding thrombin signalling and platelet function in the setting of arterial thrombosis. Current antagonists target only PAR1 and the function of PAR4 is poorly understood. Given recent clinical setbacks with PAR1 antagonists, further defining the relative functions and overall importance of the two human platelet thrombin receptors in the setting of thrombosis has become of great significance.

Protecting the heart through opioid receptor activation

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Development of effective cardioprotective therapies to limit myocardial damage with ischaemia-reperfusion (I-R), and improve longer-term outcomes, remains an important yet unrealised clinical goal. The opioid receptor (OPR) system, including the delta-, kappa- and mu-OPRs and endogenous opioid ligands (endorphins, dynorphins, enkephalins), is a logical candidate for such clinical exploitation, mediating powerful myocardial protection via cardiac and central signalling pathways, and adaptive cytoprotective 'conditioning' responses to stress at cell through to organism levels. The delta- and kappa-OPRs are strongly implicated in mediating beneficial myocardial effects. Sustained opioid receptor activation (SLP) is highly efficacious in aged and diseased hearts, appearing to engage unique protein kinase A (PKA)/ β_2 -adrenoceptor (β_2 AR) and G_s -protein dependent signalling to induce profound long-lived protection against ischemic injury. Further distinguishing itself from classical 'conditioning' responses that are inhibited by aging and common diseases, protection arises independently of caveolae signalling domains and caveolin-3, and mitochondrial K_{ATP} channels. SLP thus exhibits highly desirable properties relevant to the development of still elusive cardioprotective therapy and warrants further investigation.

Formyl Peptide Receptors - Novel GPCR targets for the treatment of cardiovascular disease

Rebecca H Ritchie, Heart Failure Pharmacology, Baker IDI Heart and Diabetes Institute, Melbourne, VIC

Myocardial infarction (MI) and the resultant heart failure remain a major cause of death, both in Australia and across the globe. This is despite clinical advances such as thrombolysis and percutaneous revascularisation interventions. These treatments have focused on restoring blood flow to the ischaemic tissue to prevent tissue necrosis and preserve organ function in the very short-term (hours). Myocardial injury however continues to evolve over days and weeks post MI, with adverse cardiac remodelling and cardiac contractile dysfunction ultimately progressing to heart failure. Development of new therapeutic strategies for treating MI after the event (while the injury is still evolving), alone or concurrent with standard care, is thus urgently warranted, to reduce progression to heart failure and death in affected patients post MI. Targeting annexin-A1, an endogenous glucocorticoid-regulated anti-inflammatory mediator, may represent one such potential therapeutic approach. Annexin-A1 and its peptide mimetic Ac2-26 binds to and activates FPR1 and FPR2 subtypes of formyl peptide receptors, an interesting G protein-coupled receptor family with broad systemic distribution. FPR activation inhibits neutrophil activation, migration and infiltration, and likely also favours efferocytosis. Systemic inflammatory disorders to inhibit neutrophil activation, migration and infiltration. Until recently, studies on the cardioprotective actions of annexin-A1 have largely focused on its early anti-inflammatory effects (within hours) as a mechanism of preserving myocardial viability following MI. Our laboratory provided the first evidence of the direct protective annexin-A1 actions on myocardium, independent of inflammatory cells in cardiomyocytes and isolated hearts *in vitro*. More excitingly, our laboratory is also the first to demonstrate reductions in each of cardiac necrosis at 24h, cardiac inflammation at 48h, and cardiac fibrosis at 7 days after myocardial ischaemic insult, in mice *in vivo*. Preservation of cardiac contractile function is also observed, even several weeks. In contrast, annexin-A1 deficient mice exhibit an exaggerated response to each of these after MI. Annexin-A1 thus represents a novel modulator of myocardial viability and contractile function for rescuing the chronically ischaemic heart *in vivo*, and represents a valuable and novel target for improving outcome after MI.

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The blood-brain barrier: Is it more of a barrier to CNS access in Alzheimer's disease?

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Introduction. While there are multiple reports of the blood-brain barrier (BBB) being perturbed in Alzheimer's disease (AD), the impact of such changes on the BBB transport of drug-like molecules has received little attention. Furthermore, whether such modifications to the BBB are responsible for the reduced brain levels of the cognitive-enhancing docosahexaenoic acid (DHA) observed in AD remains to be investigated.

Aims. The aim of this study was to assess the rate of BBB transport of various drug-like molecules and DHA in mouse models of AD and identify possible mechanisms responsible for the observed alterations in BBB transport.

Methods. Radiolabelled marker drug-like molecules (with varying mechanisms of BBB transport) were transcardially perfused in 18 month male wild-type (WT) and 3xTg AD mice. Following perfusion, the cortex and hippocampus were dissected, analysed for radioactivity and brain-to-perfusate ratios determined. Similarly, the rate of BBB transport of ^{14}C -DHA in 8 month female WT and APP/PS1 mice was assessed. Microvessels from both mouse models were isolated and proteins such as collagen IV (basement membrane protein) and fatty acid binding protein 5 (FABP5, an intracellular carrier protein) were measured. The involvement of FABP5 in ^{14}C -DHA transport across the BBB was assessed *in vitro* (using hCMEC/D3 cells) and *in vivo* (using FABP5-deficient mice).

Results. In both 3xTg and APP/PS1 mice, the paracellular route of transport appeared intact using ^{14}C -sucrose as a BBB integrity marker. The BBB transport of passively-diffusing markers (^3H -diazepam and ^3H -propranolol) decreased 1.9-2.7 fold in 3xTg AD mice, which was associated with a 33.5% increase in cerebrovascular basement membrane thickness. The BBB transport of ^{14}C -DHA was reduced by 42.1% in APP/PS1 mice relative to WT mice, and this was associated with an 18% decrease in FABP5 expression. DHA bound to FABP5 in the nM range and BBB transport of ^{14}C -DHA was attenuated by up to 40.0% in models where FABP5 was genetically silenced.

Discussion: The BBB transport of lipophilic drugs appears to be decreased in AD, likely due to cerebrovascular basement membrane thickening. However, whether people with AD are at a lower risk of CNS exposure of drugs requires further investigation. Furthermore, attenuated BBB function of FABP5 may lead to the decreased DHA brain levels observed in AD, and may be an attractive target to enhance DHA neuroprotection in AD.

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Prescribing considerations for people with dementia

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Prescribing for people with dementia is complex because of the limited applicable evidence base and the extreme variability of people with dementia and of dementia itself. This paper uses the National Prescribing Service prescribing competencies framework to address prescribing considerations for people with dementia. Understanding the person and their clinical needs requires knowledge of the person's medical, functional and social issues, which are often multiple because dementia is predominantly a disease of old age. Understanding treatment options and how they support the person's clinical needs is challenging because, with the exception of the symptomatic treatments available for dementia itself, there is almost no evidence on the efficacy and safety of non-pharmacological or pharmacological therapies for people with dementia. Extrapolation of clinical trial data to older people with multiple comorbidities including dementia must consider pharmacokinetic and pharmacodynamic changes arising from physiological changes with ageing, dementia and other comorbidities, as well as drug interactions. Working in partnership with the person to develop and implement a treatment plan requires review of the person's therapeutic goals, which are likely to change as the dementia progresses, and may involve discussion with a person responsible or reference to an advance directive. Communicating the treatment plan clearly to other health professionals is critical, as patients with significant cognitive impairment are unlikely to be able to do so themselves, often require assistance with supply and administration of medicines, and frequently have many concurrent health care providers. Monitoring and reviewing the person's response to treatment is important as it is hard to predict response in the absence of evidence, may be difficult for the person with dementia to report adverse events, and therapeutic goals often change over time. Maintaining professional practice and communicating and collaborating effectively with the person and other health professionals are also highly relevant, in particular respecting the person with dementia and their autonomy.

National Prescribing Service Limited (2012). Competencies required to prescribe medicines.

http://www.nps.org.au/data/assets/pdf_file/0004/149719/Prescribing_Competencies_Framework.pdf. Accessed October 20th 2014.

Drug use in dementia: Patterns and outcomes

J Simon Bell^{1,2}, and Danijela Gnjidic³. Monash University¹, Parkville, VIC; Cognitive Decline Partnership Centre², Hornsby, NSW; Fac of Pharmacy, Univ of Sydney³, Sydney NSW.

Introduction. Dementia is characterised by cognitive and related functional decline which may confer greater susceptibility to adverse drug events (ADEs). Clinicians have an important role in assisting people with dementia and their carers to manage their medicines.

Aims. To critically evaluate the current evidence on patterns and outcomes of medicine use in people with dementia.

Methods. Published evidence in relation to the prevalence and clinical outcomes of medicine use in people with dementia was reviewed.

Results. Over- and under-treatment of comorbid conditions is common in people with dementia. Polypharmacy is highly prevalent, and may be partly attributable to the continuation of long-term medicines for which the benefits no longer outweigh the risks. Medicine selection is challenging due to the limited evidence base specific to people with dementia. Nevertheless, emerging evidence suggests a dose-response relationship between use of sedative medicines such as benzodiazepines and an increased risk of dementia. Moreover, cumulative exposure to medicines with anticholinergic and sedative effects is associated with increased risk of ADEs in people with dementia.

Conclusions. Future work is needed to implement evidence-based strategies to prevent and detect medicine-related problems in people with dementia.

Patterns of drug use among people with dementia: A Nordic example

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Introduction. Drug treatment in dementia is complicated by an increased sensitivity to centrally acting drugs and by communication problems due to cognitive, psychiatric and behavioural changes. There is a risk of overtreatment with psychotropic drugs and an undertreatment of somatic conditions. However, patterns of drug use among persons with dementia has until recently gained little attention.

Aims. To show patterns of drug use among people with dementia from a Nordic perspective.

Methods. The presentation will provide examples of register and population-based data in Sweden that have been used for analyses of drug use among dementia patients. 1) The Swedish Prescribed Drug Register represents one of the largest pharmacoepidemiological databases in the world. The register was introduced in July 2005 and contains individual-based information about all dispensed prescriptions to the whole population of Sweden (about 9.7 million inhabitants). This register can be record-linked through the personal identification number to the nationwide Swedish Patient Register to obtain hospital diagnoses of dementia. 2) The Swedish Dementia Registry is a national quality registry on dementia disorders that was launched in 2007. At present, about 42 000 dementia patients are included and 95% of all memory clinics in Sweden provide data. 3) The Swedish National Study of Aging and Care in Kungsholmen (SNAC-K) is an ongoing population-based longitudinal study that was launched in 2001. The SNAC-K cohort includes 60+ year-old persons (baseline n=3 363) who are examined individually by a nurse, a physician and a psychologist. Extensive socio-demographic, clinical and cognitive data are collected, including prescription and over-the-counter drugs, clinically validated dementia diagnoses, memory tests and blood samples.

Results. We have found that there are inequalities in drug treatment of dementia patients in Sweden. We have also shown that use of antipsychotics differs between dementia disorders and that treatment with cholinesterase inhibitors may decrease the need for antipsychotics and anxiolytics. Finally, our results indicate that dementia patients are undertreated for osteoporosis, but adequately treated for pain in Sweden.

Discussion. Drug treatment in dementia patients is a difficult and understudied research topic. More studies on large cohorts of dementia patients are needed to assess the outcomes of drug treatment with the ultimate goal of improved care for this vulnerable group of patients.

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Ability of older people with dementia or cognitive impairment to manage medicine regimens

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Impaired cognition has a significant impact on a person's ability to manage their medicines. The aim of this presentation is to provide methods for assessing their capacity to safely manage medicines, and strategies for supporting independent medicines management by people with dementia or cognitive impairment living in the community.

The literature indicates that as cognitive impairment progresses, the ability to plan, organise, and execute medicine management tasks is impaired, leading to increased risk of unintentional non-adherence, medication errors, preventable medication-related hospital admissions and dependence on family carers or community nursing services to assist with medicines management. Impaired functional capacity may not be detected by health professionals in routine clinical encounters. Assessment of people living with dementia' (or carers') ability to safely manage medicines is not undertaken routinely, and when it is there is variability in the methods used. Self-report and informant report may be helpful, but can be unreliable or prone to bias. Measures of cognitive function are useful, but may lack sensitivity and specificity. Direct observation, using a structured, standardised performance-based tool, may help to determine whether a person is able to manage their medicines and identify barriers to adherence such as inability to open medicine packaging.

A range of strategies have been used to support independent medicines management in people with cognitive impairment, but there is little high-quality research underpinning these strategies. Further studies are needed to develop and evaluate approaches to facilitate safe medicines management by older people with cognitive impairment and their carers.

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Reflections on teaching complex concepts

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G protein-coupled receptors (GPCRs) are the largest drug target family encoded by the human genome and thus a tremendous opportunity for future researchers. While many fundamental pharmacodynamic concepts remain as valid today as when first introduced into the field, they are often perceived by undergraduate students to be complex or dry. Pharmacodynamic concepts such as allosteric modulation and biased agonism are indeed complex and are not adequately addressed in contemporary pharmacological textbooks. My approach to teaching such concepts is to use visual aids as much as possible rather than words. Although quantitation is vital to pharmacology, I nonetheless keep equations to a minimum, as most students tend to have an aversion to mathematics. I freely acknowledge that I teach to the top 20% of students who are likely to respond to the material if the lecturer is really engaged and clearly knowledgeable about the subject matter. I also believe it is absolutely vital to reinforce the pharmacodynamic concepts I teach using real-world examples. Many of these concepts currently have a widespread impact on drug discovery but, again, are not covered in adequate detail in textbooks, which also lack concrete examples of their application. For instance, I use a study of a patient overdosing on benzodiazepines to illustrate safety issues associated with allosteric receptor mechanisms; I talk about the rebound effects of histamine H₂ antagonists upon cessation in treating gastric ulcers due to receptor upregulation mediated by inverse agonism, and I give examples of differential clinical efficacy of beta blockers in treating heart failure that may be due to biased agonism, rather than beta blockade. I have found that these methods, taken as a whole, can clarify complex pharmacodynamic concepts, particularly for health professional students.

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Teaching prescribing to undergraduate medical students

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Introduction. Prescribing errors occur in all health jurisdictions, however, they represent a significant patient safety concern in Australian hospitals. Evidence suggests that junior doctors are responsible for most prescribing errors. The Australian Commission on Safety and Quality in Healthcare has identified improving safety and quality of medication usage in Australia as one of its priorities; and the National Prescribing Service has developed a prescribing competency framework.

Aim. To develop and implement a pharmacology and therapeutics program for students in clinical years which increased their focus on more accurate prescribing.

Method. The Monash University MBBS course as delivered in the Gippsland region was examined to identify the student perceptions of their preparation for prescribing and medication management.

Results. During the first two clinical years in Gippsland students participate in the teaching program of applied pharmacology tutorials with case studies. In their final year, the focus is on medication review, prescribing on the national inpatient medication chart and prescribing discharge medications. Students offered constructive comments on the course, particularly requiring more education about interactions; and they identified pharmacists as the most useful clinical resource.

Discussion. Feedback from the students is assisting in modifying the course content and approach. The involvement of pharmacists in the teaching of this content enhances the interprofessional learning opportunities, enabling students to appreciate the role and expertise of other health professionals they will work with in patient care.

<http://www.safetyandquality.gov.au/our-work/medication-safety/>

<http://www.nps.org.au/health-professionals/cpd/prescribing-competencies-framework>

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Unrecognisable transparency for quality and productivity and smarter education

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Introduction. Universities have assumed a greater role than ever before in Australian life, leading to fresh and growing interest in understanding education processes and outcomes.

Aims. The presentation reviews recent innovations that have sought to improve the transparency of this facet of higher education.

Methods. It reviews several innovations into the assessment and evaluation of student engagement and learning outcomes.

Results. The analysis shows that enhancing transparency can improve quality and productivity.

Discussion. The presentation discusses emerging trends and portends implications for students, academics and institutions.

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The role of GPCR signalling via dual kinase receptor transactivation in cardiovascular disease

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Introduction: Growth factor stimulation of proteoglycan glycosaminoglycan (GAG) hyperelongation leading to increased LDL binding and retention is the initiating step in atherosclerosis. G protein coupled receptor (GPCR) signalling is traditionally mediated through transactivation-independent signalling pathways and the transactivation of protein tyrosine kinase receptors (PTKR). We have recently discovered that the thrombin-activated GPCR protease-activated receptors (PARs) can also transactivate serine/threonine kinase receptors (S/TKR).

Aim: Our aim was to assess the contribution of dual transactivation-dependent signalling to the action of thrombin on the synthesis of the chondroitin sulfate proteoglycan core protein biglycan and the mRNA expression of the enzymes mediating GAG elongation in human vascular smooth muscle cells.

Methods: GAG enzyme mRNA expression levels were assessed by real-time quantitative polymerase chain reactions. Phospho-Smad and phospho-Erk protein expression was quantified by Western blotting. Proteoglycan synthesis was assessed by radiolabeled sulfate and radiomet/cys incorporation and molecular size by SDS PAGE.

Results: Direct agonists of PAR-1, PTKR (EGFR) and a S/TKR (TGFBR1) stimulated mRNA expression of several GAG enzymes associated with GAG hyperelongation. Pharmacological antagonism studies revealed that the action of thrombin utilised both PTKR and S/TKR to elicit a stimulatory action on mRNA expression of GAG synthesizing enzymes. We revealed that while the PTKR mediated transactivation response was due to matrix metalloproteinase (MMP) activation and phosphorylation of Erk, the S/TKR transactivation pathway occurred through phosphorylation of the transcription factor Smad2, did not involve MMPs and was mediated by a cytoskeletal rearrangement-of the Rho-kinase/integrin pathway.

Discussion: This work shows that all of the signalling of the action of thrombin on proteoglycan and GAG gene expression occurs via transactivation-dependent pathways with no involvement of transactivation-independent pathways. We are seeking a common signalling point which may serve as a therapeutic target for the prevention of atherogenic changes in the structure of lipid-binding proteoglycans aimed at generating a product that would work with statins to reduce cardiovascular disease.

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Angiotensin II-mediated EGFR transactivation: identify new signalling mediators important for tissue remodelling in cardiovascular disease and cancer

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Introduction. G protein-coupled receptors (GPCRs) 'hijack' epidermal growth factor receptors (EGFR) to drive cell growth in a process termed EGFR transactivation.

Aims. Our interest is in investigating the mechanism whereby the activated angiotensin type I receptor (AT₁R) transactivates the EGFR. While candidates, such as matrix metalloproteases and EGF ligands have been identified, the molecular events underlying this process remain unresolved.

Methods and Results. To study this mechanism, we have developed a stable cellular model of EGFR transactivation by introducing the AT₁ receptor (AT₁R) into human mammary epithelial cells using retroviral delivery, and determined that the cells were stably expressing a functional form of the receptor. After stimulation of these cells with angiotensin II (AngII), we observe a robust activation of the EGFR, which is blocked by the EGFR antagonist tyrphostin AG1478. We have used this model to optimise a high-throughput short interfering RNA (siRNA) screening approach to identify genes that are involved in AT₁R/EGFR transactivation, and have subsequently performed a primary screen with the Dharmacon siGENOME kinome siRNA library before embarking on a genome-wide screen. From our kinome analysis, we have identified a number of genes of interest (TRIO, BMX, CHKA), which we have tested in secondary and tertiary siRNA screens to further validate these candidates.

Discussion. We are in the process of further characterising the role of these hits in AT₁R/EGFR transactivation. The outcomes from these studies will provide us with more information about the specific factors involved in AT₁R/EGFR transactivation, and to discover potential targets for the prognosis and treatment of cardiovascular disease.

Endosomal platforms for signaling pain

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Introduction. G protein-coupled receptors at the plasma membrane control most biological processes and are a major drug target. Activated receptors traffic to endosomes, but whether endosomal receptors generate signals that underlie complex pathophysiological processes and are viable therapeutic targets is unexplored. Herein we report that the substance P neurokinin 1 receptor in endosomes generates sustained signals that underlie pain transmission.

Aims. We sought to determine the importance of neurokinin 1 receptor endocytosis for signal transduction, neuronal activation, and pain transmission, and to define whether the endosomal receptor is a therapeutic target for pain.

Methods. We used resonance energy transfer approaches in HEK cells to study receptor trafficking and its association with key regulatory proteins, and to define the importance of trafficking for the generation of signals in defined subcellular compartments. We studied neurokinin 1 receptor trafficking in spinal neurons *in vivo* after activation of nociceptors, and assessed mechanical hyperalgesia. We assessed activation of spinal neurons in slice preparations by patch clamp recordings.

Results. The endosomal neurokinin 1 receptor signaled by $G\alpha_q$ -mediated processes to activate extracellular signal regulated kinases in the nucleus and protein kinase C and cAMP in the cytosol. Clathrin and dynamin inhibitors blocked substance P-evoked receptor endocytosis and excitability of spinal neurons and prevented hyperalgesia. The neurokinin 1 receptor antagonist spantide, when conjugated to cholestanol to promote endosomal targeting, selectively inhibited endosomal signaling and caused sustained analgesia.

Discussion. Our results reveal a critical role for endosomal signaling of G protein-coupled receptors in the complex pathophysiology of pain, and demonstrate the utility of endosomally-targeted antagonists.

Delineating the dynamics of mu-opioid receptor signalling and regulation

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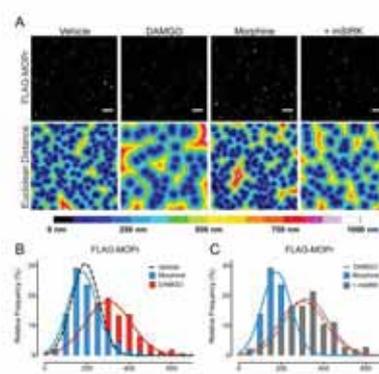
Introduction. Decades of research have focused on the differential regulation and internalisation of the mu-opioid receptor (MOPr) by morphine compared to the prototypical agonist DAMGO, in an effort to design more effective analgesics. To date, there are no studies examining compartmentalised signalling of MOPr in live cells, no information on MOPr spatiotemporal signalling profiles, and no knowledge of whether this is regulated by receptor trafficking. Whether MOPr resides within biochemically-defined lipid-rich plasma membrane (PM) regions is controversial, and the lack of a unifying view is likely due to the invasive nature of methods used to isolate these domains.

Aims. To investigate the mechanisms controlling the spatiotemporal signalling of the MOPr

Methods. We have used BRET to measure MOPr endocytic trafficking and have correlated this with signalling measured from bulk compartments in single cells using FRET biosensors.

Results. The regulation of MOPr trafficking and signal compartmentalisation are interdependent. MOPr initially resides within a unique PM microdomain. Morphine stimulation of MOPr initiates a PM-localised $G\alpha_i/o$ - $G\beta\gamma$ -PKC activation that results in sequestration of MOPr and a sustained signalling profile (PM-PKC phosphorylation and cytosolic ERK phosphorylation). In contrast, DAMGO does not activate PKC allowing MOPr translocation within the PM and a transient signalling profile: $G\alpha_i/o$ -mediated cytosolic ERK phosphorylation, β Arrestin recruitment, receptor internalisation, and transient β Arrestin-mediated nuclear ERK phosphorylation.

Discussion. Our results suggest that MOPr-mediated signalling is highly compartmentalised and ligand-dependent and that distinct MOPr membrane localisations lead to diverse spatiotemporal signalling.



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Molecular insights into metabotropic glutamate receptor allosteric modulation

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Introduction. Dysfunction of glutamatergic neurotransmission has been implicated in numerous neuropsychiatric and neurological disorders. The metabotropic glutamate (mGlu) receptors, are G protein-coupled receptors (GPCRs) that play a modulatory role in the CNS. In particular, metabotropic glutamate receptor subtype 5 (mGlu5) is expressed throughout the CNS, and is a promising therapeutic target for multiple CNS disorders including: schizophrenia, depression and Alzheimer's disease. In order to selectively target mGlu5, our efforts are directed towards small molecule allosteric modulators that interact with sites distinct from the endogenous ligand-binding site. Allosteric modulators have the capacity to maintain spatial and temporal aspects of neurotransmission. Further, they have the potential to engender unique receptor conformations, as such allosteric modulators may differentially influence distinct functional outcomes coupled to receptor activation, a phenomenon referred to as 'stimulus-bias'.

Aims. Research is focussed on two major aims: 1) to understand the full spectrum of functional consequences of mGlu5 allosteric modulation; 2) to understand the structural basis of mGlu5 allosteric modulation.

Results. We have validated operational models of agonism and allosterism to rigorously quantify the myriad of pharmacological effects elicited by mGlu allosteric modulators in radioligand binding and high-throughput second messenger assays. Select mGlu allosteric modulators have been further characterized in primary astrocyte cultures and preclinical rodent models of disease. SAR analysis, site-directed mutagenesis and computational modeling have been integrated to probe the structural basis of allosteric modulation.

Discussion. A greater understanding of the functional consequences of mGlu5 allosteric modulation beyond intracellular Ca^{2+} mobilisation is needed, especially for pathways thought to be relevant *in vivo* for modulation of glutamatergic neurotransmission.

Crystal structures are static and alone cannot provide insights into the dynamic processes that underlie receptor activation and allosteric interactions. We will continue to combine detailed structure-function information with SAR and computational modelling to facilitate rational structure-based drug discovery efforts to identify novel biased allosteric modulators of mGlu₅ and other family C GPCRs.

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Optimising medication use in people with dementia: Evidence and opportunities

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Older people with dementia have a high prevalence of geriatric syndromes, including delirium, falls, frailty, incontinence and insomnia. While the aetiology is usually multifactorial, medications are a potentially avoidable risk factor. Both over- and under-treatment with medications is common. Polypharmacy may occur because people continue to use long-term preventative medications for which the benefits no longer outweigh the risks. Medication selection for people with dementia is a complex process that involves both prescribing medications that are beneficial and 'deprescribing' medications that are potentially harmful. Pharmacological risk assessment tools may assist clinicians to identify people at risk of medication-related problems. However, most explicit or criterion-based tools do not consider peoples' treatment priorities or goals of care. Conducting active learning sessions for nurses and aged care workers may help to minimise harmful medication use, maintain health-related quality of life and reduce hospitalisation. Evidence-based strategies such as Home Medicines Reviews and Residential Medication Management Reviews are effective at improving the quality use of medicines. There are further opportunities for clinicians, consumers and carers to work together to optimise medication use by people with dementia.

Novel inhibitors of glycine transporters

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Introduction. Chronic pain is a significant global health, economic and social problem (Gaskin et al, 2012). Currently used analgesics provide adequate pain relief in only a small proportion of chronic pain patients. Recent studies have revealed that glycinergic transmission in the spinal cord is diminished in neuropathic pain (Ahmadi et al, 2002; Harvey et al, 2004; von Hehn et al, 2012). Two subtypes of glycine transporters, GlyT1 and GlyT2, work to regulate extracellular glycine concentrations. GlyT1 is expressed throughout the CNS, while GlyT2 shows much more restricted expression patterns and is predominantly expressed by glycinergic terminals in the spinal cord and brain stem. Drugs that can enhance dysfunctional glycinergic transmission in neuropathic pain, and in particular GlyT2 inhibitors, are widely recognised as potential chronic pain therapeutics.

Aim. To identify and characterise the activity of a series of bioactive lipids at glycine transporters.

Methods. Transporters were expressed in *Xenopus laevis* oocytes and activity was monitored using two-electrode voltage clamp.

Results. Subtle differences in lipid-based compounds produces selective GlyT inhibitors (Pearlman et al, 2003; Wiles et al, 2006). We have previously revealed that the free fatty acid, arachidonic acid, selectively inhibits GlyT1, while the related acyl-amino acid, N-arachidonyl glycine (NAGly), is a selective GlyT2 inhibitor. We have identified additional glycine- and carnitine-conjugated lipids that show similar properties to NAGly. The most potent of the compounds are Oleoyl-L-Carnitine (OLCarn), N-Oleoyl-Glycine (NOGly) (Carland et al, 2013) and a ω -3 monounsaturated C16-glycine derivative. OLCarn is the most potent GlyT2 inhibitor, with an IC₅₀ of 340 nM, 15-fold more potent than NAGly. However, it is slowly reversible. The rate of reversibility can be enhanced with the use of the lipid-extracting agent β -cyclodextrin (Carland et al, 2013) or by mutations of residues in transmembrane domain 7 of GlyT2 that face the hydrophobic core of the bilayer.

Discussion. Our group has identified a series of bioactive lipids that inhibit the glycine transporter GlyT2. These compounds show promise as analgesics. They represent a novel class of drugs. Further, our observations suggest that the interaction between these bioactive lipid inhibitors and GlyT2 is likely to be mediated by the lipid-protein interface. This finding opens up the possibility of developing a new class of GlyT2 inhibitors.

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Targeting synaptic adaptations in chronic pain

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Introduction. Chronic pain can be difficult to manage with current therapeutics. A large body of evidence in animal models and humans suggests that chronic pain involves persistent pathological adaptations. These include changes to excitatory synaptic plasticity and inhibitory neurotransmission. Some of these adaptations in neuropathic pain are potential new therapeutic targets. Here we investigate two systems that undergo adaptations during the development of neuropathic pain resulting in changes to pain signalling. These include adenosine signalling through A1 receptors in nociceptive pathways and inhibitory neurotransmission mediated through glycinergic circuits in the spinal cord.

Aims. The aims of this study were to find pharmacological strategies to reduce pain signals by targeting the changes that occur in adenosine and glycinergic signalling in chronic pain.

Methods. In this study we used a partial sciatic nerve ligation (PNL) model of chronic pain in adult rats. Synaptic currents were measured in whole-cell voltage-clamp from laminae I or II cells. Tungsten electrodes placed in the dorsal roots or inner laminae were used to elicit eEPSCs and eIPSCs respectively.

Results. In the spinal cord and periphery, adenosine inhibits excessive neuronal activity through the A1R and activation of this receptor produces antinociceptive properties. We hypothesized that this increase in endogenous adenosine in chronic pain states would increase the effect of an A1R allosteric enhancer. This was supported by our results that show the A1R allosteric enhancer, VCP171, is more effective at reducing excitatory synaptic currents in spinal cord slices from neuropathic animals. Much of the fast inhibitory neurotransmission in the spinal cord is mediated by glycine, which when removed, results in the pathological symptoms of neuropathic pain. We have found that glycinergic neurotransmission is reduced, and in some cells abolished, in the superficial laminae of the dorsal horn of animals with neuropathic pain, and in these animals GlyT2 inhibitors act to increase glycinergic neurotransmission by prolonging the duration of the glycinergic synaptic current.

Discussion. The adenosine and glycine systems are attractive targets for pain therapeutics for neuropathic pain because of adaptive changes in these pain signalling mechanisms.

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UDP-glycosyltransferase 8 (UGT8) galactosidates bile acids and modulates FXR signalling.

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Introduction. The UDP glycosyltransferases (UGTs) catalyze the addition of sugar residues to small lipophilic chemicals including drugs, toxins, metabolic byproducts, hormones, and signaling molecules, rendering them more polar and more readily excreted and/or functionally inactive. The 19 members of the UGT1 and UGT2 families use UDP-glucuronic acid; we previously showed that the two members of the UGT3A family use UDP-N-acetylglucosamine, UDP-glucose and UDP-xylose. The sole member of the last UGT family, UGT8, uses UDP-galactose. To date the only known substrate of UGT8 is ceramide, which it galactosidates in the synthesis of brain sphingolipids; thus UGT8 is considered biosynthetic and not involved in metabolism of endo- or xenobiotics.

Aims. To determine the role of UGT8 in metabolism of endo- and xenobiotics.

Methods. Molecular cloning, mutagenesis and heterologous protein expression. Substrate screening using TLC and UPLC-MS based glycosylation assays. Kinetic analysis. Expression analysis using RT-PCR. Analysis of signalling pathways using luciferase reporter assays and RT-PCR. Molecular modelling.

Results. Human and mouse UGT8 cDNAs were expressed in HEK293T and Caco2 cells. We screened known UGT substrates for activity with UGT8 and discovered that UGT8 efficiently galactosidates natural bile acids (BA) and drug-like BA-analogues. The best substrate was 6 α -ethyl-chenodeoxycholic acid (6-ECDC, INT747), a potent and selective FXR agonist. UGT8 expression in cells reduced the response of FXR to 6-ECDC as indicated by luciferase reporter assays and analysis of endogenous FXR targets. We found that UGT8 is expressed in kidney and gastrointestinal tract but not liver, and endogenous UGT8 activity was measured in human kidney microsomes. Mutagenesis and modeling identified a residue in the UGT8 signature sequence involved in galactose selectivity.

Discussion. We show for the first time that UGT8 is involved in metabolism of BA and may be an important player in metabolic homeostasis and disease via control of BA ligands for FXR and TGR5. BA galactosides are uncharged and are predicted to have different properties to glucuronides including poor interaction with transporters involved in enterohepatic recycling. 6-ECDC is in clinical trials for NASH and PBC and galactosidation in intestine and kidney by UGT8 is likely to play an important role in the disposition and elimination of this emerging new drug.

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Population pharmacokinetic modelling of colistin methanesulphonate and formed colistin in patients receiving intermittent haemodialysis

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Introduction. Critically-ill patients with impaired renal function are commonly infected by multidrug-resistant Gram-negative bacteria. Colistin, administered as its inactive prodrug colistin methanesulphonate (CMS), is increasingly used as last-line therapy in these patients. The PK of CMS and colistin in intermittent haemodialysis (HD) has not been studied using amounts in dialysate plus systemic, pre- and post-membrane plasma concentrations.

Aims. To quantitatively describe and predict the time course of CMS and formed colistin simultaneously in plasma and dialysate of HD patients.

Methods. Ten HD patients received a single iv CMS dose (150 mg colistin base activity) over 30 min. Serial blood samples were collected over 50 h, multiple dialysate and pre- and post-membrane samples during HD (1.5 to 5.5 h after start of the infusion, to optimally characterise the impact of HD) and cumulative 24 h urine where applicable. Concentrations were determined by HPLC. Population modelling was performed in S-ADAPT.

Results. A model with two disposition compartments for CMS, one for colistin, and first-order formation of colistin well described the data. For CMS, total body clearance (excluding HD clearance) was 2.66 L/h (23%) [population mean (between subject variability)], HD clearance 4.26 L/h (26%), and terminal half-life 11.9 h (35%). For colistin, total clearance/fm (excluding HD clearance; fm, fraction of CMS metabolised to colistin) was 5.97 L/h (33%), HD clearance 3.99 L/h (44%), and terminal half-life 24.8 h (27%). The average CMS amount recovered in dialysate was 30.6% (range 19.4 to 49.9%) of the dose, whereas < 8.6% of the dose was recovered as colistin in each patient.

Discussion. Our detailed analysis provides new insights on the mode of CMS and colistin removal by HD and highlights the importance of optimal timing of HD in relation to CMS administration. Performing HD while CMS concentrations are high results in substantially decreased colistin concentrations and thereby an increased risk of therapeutic failures. Therefore HD needs to be scheduled at the end of a dosing interval.

Extremes of body size in the dosing of high-dose methotrexate chemotherapy for non-Hodgkin lymphoma

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Introduction. Arbitrarily dose capping chemotherapy based on body surface (BSA) in obese individuals with haematological malignancies is common practice due to clinician concerns regarding possible drug overexposure resulting in harmful side effects.

Aims. To investigate the PK plausibility of capped BSA dosing in obese and overweight adult non-Hodgkin lymphoma (NHL) patients being treated with high-dose methotrexate (HD MTX).

Methods. A HD MTX population PK model was developed (using non-linear mixed effects modelling) from retrospectively collected observations of 108 adults at a tertiary referral metropolitan hospital. Inclusion criteria were receiving iv HD MTX as part of the Hyper-CVAD chemotherapy protocol for aggressive NHL during 2002 to 2012. This model was used to simulate MTX plasma concentrations and overall exposure in a virtual population of 1000 individuals, randomly sampled with stratification for body size from the National Health and Nutrition Examination databases. Simulations were conducted with MTX dosing (200mg/m² over 2 h followed by 800mg/m² over 22 h) based on i) actual BSA, and ii) dose capped at 2m² in individuals with a BSA >2m².

Results. The model developed from the observed data was a two-compartmental model incorporating renal function and body size as covariates influencing CL and V of the central compartment respectively. Simulated MTX plasma concentrations in uncapped obese (n=129) and overweight (n=286) individuals in the virtual population did not differ significantly from normal weight patients (n=370) (p=0.68 and p=0.79 respectively), with 95% of the simulated concentrations falling below the toxicity levels at 48 and 72 h. Dose capped obese individuals displayed a reduced mean MTX exposure compared to dose capped overweight individuals (276µmol*h/L versus 286µmol*h/L). Renal function demonstrated a clear impact on the PK in all individuals irrespective of body size.

Discussion. When dosing is based on actual BSA, PK changes with HD MTX in NHL appear to be minimal in obese and overweight individuals compared to individuals of normal body weight. Hence, capping BSA dosing in obesity may be unnecessary and possibly result in suboptimal therapy. Instead, dosing adjustments in extremes of body size should appropriately consider renal function to avoid toxicity.

Prostacyclin activates the IP-receptor to promote migratory behaviour in breast cancer cells that over-express cyclooxygenase-2

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Introduction. Metastasis is the major cause of cancer death. Cyclooxygenase-2 (COX-2) is over-expressed in many cancers, which implicates downstream prostaglandins (PGs) in metastatic progression (Wang and DuBois, 2010). PGE₂ production is correlated with metastatic disease (Pan *et al.*, 2008) but potential roles of other PGs are unclear.

Aim. To elucidate the roles of individual PGs and underlying mechanisms on the migration of breast cancer cells that over-express COX-2.

Methods. *In vitro* migration of MDA-MB-468 breast cancer cells that stably expressed COX-2 (MDA-COX-2) was evaluated in 3D-matrigel droplets. PG formation was determined by LC-tandem MS. Roles of PGs in migration were tested directly, using PG-synthase-selective inhibitors and using prostanoid receptor antagonists and siRNAs.

Results. Arachidonic acid (20 µmol/L) increased MDA-COX-2 cell migration relative to vector-transfected cells (112±8 vs 78±3 cells, P<0.01). Inhibition of prostacyclin (PGI₂) and PGE₂ synthases and antagonism of the IP and EP4 prostanoid receptors decreased cell migration. PGE₂ (0.1 µmol/L) acted via the EP4 receptor and the stable PGI₂ analogue cicaprost (1 nmol/L) acted via the IP receptor to enhance migration directly (96±4 vs 73±6 cells/40 hr, P<0.05 and 111±2 vs 85±5 cells/40 hr, P<0.05, respectively). Inhibitors of other PG synthases and other prostanoid receptor antagonists were inactive.

Discussion. These findings implicate the PGI₂-IP and PGE₂-EP4 axes in the pro-metastatic activity of MDA-COX-2 cells. Both pathways should be considered in the development of novel anti-metastatic strategies.

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Pan MR et al (2008) J Biol Chem 283:11155-11163

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Innate immune signalling genetics associated with cognitive dysfunction and adverse opioid effects in cancer pain patients on transdermal fentanyl.

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Introduction: Transdermal fentanyl (opioid analgesic) targets patients with stable opioid requirements, especially cancer patients. Innate immune signalling is implicated in cancer pain, opioid analgesia, cognitive dysfunction and other adverse opioid effects. We hypothesised that genetic variants increasing innate pro-inflammatory signalling cause poorer pain control and increased adverse symptoms in cancer pain patients on transdermal fentanyl.

Aim: To investigate if genetic variants in innate immune pathways implicated in pain processing and opioid activity influence the serum fentanyl concentration-response relationships in cancer pain patients on transdermal fentanyl.

Methods: Cancer pain patients (468) from the European Pharmacogenetic Opioid Study on transdermal fentanyl were genotyped for 21 SNPs in 15 genes: *CASPI*, *BDNF*, *CRP*, *LY96*, *IL6*, *IL1B*, *TGFB1*, *TNF*, *IL10*, *IL2*, *TLR2*, *TLR4*, *MYD88*, *IL6R* and *OPRM1*. Lasso and backward stepwise generalised linear regression were used to identify non-genetic (serum fentanyl concentration, clinical, patient) and SNP predictors, respectively, of pain intensity (average Brief Pain Inventory < 4), cognitive dysfunction (Mini-Mental State Examination < 24) and adverse events (nausea, tiredness or constipation). Cross-validation error and randomised permutation were used to determine optimal SNP models and their significance (P). Generalised multiple dimension reduction was used to identify putative epistatic SNP interactions. Model predictive value was summarised by area under ROC curve (AUROC).

Results: Innate immune SNPs did not predict pain intensity (P > 0.8). Serum fentanyl concentration (weakly), old age, low Karnofsky functional status and *MYD88* rs6853 wild-type (P = 0.03) positively predicted cognitive dysfunction (AUROC = 0.73). In addition to breakthrough opioid use and depression, a putative epistatic interaction between *IL6*, *IL6R* and *IL10* SNPs predicted self-report of nausea, tiredness and/or constipation (AUROC = 0.78).

Discussion: Results support the involvement of innate immune signalling in cognitive dysfunction and opioid-related adverse effects in cancer patients, with risk partly genetically determined. These findings require external replication, and it remains to be determined whether innate immune genetics modulate the effects of the cancer itself (including chemotherapy), opioids, or both, in the development and maintenance of these adverse symptoms.

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A multistate model for the role of multiple medication use and Drug Burden Index in frailty state transitions and death: the CHAMP study

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Introduction. Frailty is recognized as an important geriatric syndrome and is associated with mortality. Multiple medication use and the use of sedative and anticholinergic medications are associated with incident frailty.

Aims. To investigate the potential roles of multiple medication use and Drug Burden Index (DBI) on frailty transitions and death in community-dwelling older men.

Methods. Data were sourced from the Concord Health in Ageing Men Project (CHAMP); an ongoing cohort study of community-dwelling men 70 years and over in Sydney. Self-reported questionnaires and clinic visits were administered at baseline, two and five years. Participants were followed every four months to record aged care admission and death. Frailty was assessed using Fried's frailty phenotype. The total number of regular and as-needed medications and DBI (a measure of exposure to sedative and anticholinergic medications) were calculated. Multistate modelling was used to characterize the transitions across three frailty states (robust, pre-frail and frail) and progression to death. The number of medications and DBI were incorporated into the transitions (separately), adjusting for comorbidity, age, cognitive impairment, education level and living status.

Results. Each additional medication was associated with a 4% greater risk of transitioning from the robust to pre-frail states (95% confidence interval [CI]: 0.996 to 1.09) and 22% greater risk of death from the robust state (95% CI: 1.06, 1.41). Each one unit increase in DBI (e.g. exposure to the minimum dose of two anticholinergic or sedative medications) was associated with a 73% greater risk of transitioning from the robust to pre-frail states (95% CI: 1.30 to 2.31) and a 2.75 fold greater risk of dying from the robust state (95% CI: 1.60 to 4.75).

Conclusions. These findings suggest that multiple drug use and DBI may be important and potentially avoidable contributing factors to the transition from the robust to pre-frail states, and from the robust state to death.

Prevalence of potential statin-drug interactions in frail and robust older inpatients

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Introduction. Statins are common preventative medications used in older people. A significant proportion of older people are exposed to polypharmacy, placing them at an increased risk of statin-drug interactions.

Aim. To examine the prevalence of potential and clinically relevant statin-drug interactions in frail and robust older inpatients.

Methods. A cross-sectional study of patients aged ≥ 65 years prescribed a statin and admitted to a tertiary referral teaching hospital in Sydney was conducted. Data on sociodemographics, comorbidities, and medication use were collected from medical records. Potential statin-drug interactions were defined if listed in four international drug information sources, and clinically relevant statin-drug interactions were defined as those interactions with the highest severity rating in at least three of four international drug information sources. Frailty was assessed using the Reported Edmonton Frail Scale. Descriptive statistics were used to summarise participant characteristics and prevalence of statin-drug interactions.

Results. To date, a total of 116 patients were recruited (median age 79, interquartile range [IQR] 15), with 40 frail (34%) and 76 (66%) robust patients. The median number of medications was not significantly different in frail (median 9, IQR 5) and robust (median 8, IQR 4.5) patients ($p=0.05$). Potential statin-drug interactions were identified in 11.2% ($n=13$) of the total population, 12.5% ($n=5$) of frail patients and 10.5% ($n=8$) of robust patients. The most commonly prescribed potential statin-drug interaction combinations involved the cardiovascular drugs amiodarone and simvastatin ($n=3$), diltiazem and atorvastatin ($n=2$) and gemfibrozil and rosuvastatin ($n=2$). Clinically relevant statin-drug interactions were identified in 9.5% ($n=11$) of the total population, 10% ($n=4$) of frail patients and 9.2% ($n=7$) of robust patients, with amiodarone and simvastatin ($n=3$) being the most common.

Discussion. Potential statin-drug interactions, particularly those involving cardiovascular drugs are common in older inpatients. Future studies should evaluate the clinical outcomes of statin-drug interactions in older people.

Psychotropic drug utilisation in older people in New Zealand from 2005 to 2013

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Introduction. Psychotropic drug utilisation has increased world-wide among older people (aged 65 years or older), in relation to utilisation of other drugs.

Aims. The objective of this population-level study was to describe and characterise the national utilisation of psychotropic drugs in older people in New Zealand from 2005 to 2013.

Methods. Repeated cross-sectional analysis of population-level dispensing data was conducted from 1 January 2005 to 31 December 2013. Data on utilisation of psychotropic drugs were extracted and categorised in accordance with the World Health Organization Collaborating Centre for Drug Statistics classification system.

Utilisation was measured in terms of the defined daily dose (DDD) per 1,000 older people per day (TOPD).

Results. Overall, utilisation of psychotropic drugs showed a 22.5% increase between 2005 and 2013. Utilisation increased for antidepressants (81.9-110.4 DDD/TOPD), antipsychotics (6.8-8.7 DDD/TOPD), hypnotics and sedatives (59.4-65.5 DDD/TOPD); whilst utilisation of anxiolytics decreased (11.4-10.7 DDD/TOPD). Utilisation of atypical antipsychotics increased (4.6-6.8 DDD/TOPD), with the highest percentage change in DDD/TOPD being contributed by olanzapine. Utilisation of tetracyclic antidepressants and venlafaxine utilisation grew rapidly by 1.5 and 4.5 times, respectively, between 2005 and 2013. Utilisation of zopiclone was greater than that of other hypnotics in 2013.

Discussion. Psychotropic drug utilisation in older people increased by one fifth between 2005 and 2013. Increased utilisation of selective serotonin reuptake inhibitors and atypical antipsychotics appeared to be a compensatory substitution for the decline in the utilisation of tricyclic antidepressants and typical antipsychotics, respectively.

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Structure-activity analysis of biased agonism at the adenosine A₃ receptor

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Introduction. The adenosine A₃ receptor (A₃AR) receptor is a novel therapeutic target for a range of conditions, including cancer, inflammation and ischemia (Jacobson & Gao, 2006). As a result, there has been extensive investigation into the structure-activity relationship (SAR) of the A₃AR, yielding insights into ligand affinity, efficacy and subtype selectivity. However, there has been no investigation into the SAR of biased agonism at the A₃AR, a phenomenon that has growing implications for future drug discovery (Kenakin et al., 2012).

Aims. To investigate the bias profile of a series of (N)-methanocarba substituted A₃AR agonists with sequentially extended C2 substituents in comparison to prototypical A₃AR agonists with a ribose ring and no C2 extension.

Methods. Fluorescence approaches were used to determine ERK1/2 and AKT phosphorylation, calcium mobilization and cAMP accumulation in CHO cells stably expressing the human A₃AR. A propidium iodide-based assay assessed A₃AR agonist-mediated cytoprotection of CHO cells after 24-hour serum starvation. Biased agonism was quantified as described previously (Kenakin et al., 2012).

Results. All A₃AR agonists mediated a robust increase in ERK1/2 and AKT1/2/3 phosphorylation, stimulation of calcium mobilization, inhibition of cAMP accumulation and promotion of cell survival (n=3-6). In contrast to the prototypical A₃AR agonists, the structurally distinct ligands exhibited significant bias towards cell survival (p<0.05, one-way ANOVA, Tukey Post Hoc test). Analyses demonstrated a significant positive correlation between the size of the C2 substituent (in angstroms) and the calculated bias factors (r²=0.85, p<0.05).

Discussion. The positive association between the size of the C2 substituent and bias towards cell survival suggests that increasing C2 size results in ligand-receptor interactions that preferentially stabilize conformations that promote cytoprotective signaling.

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Kenakin T et al (2012) *ACS Chem Neurosci* 3:193-203

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Structure of the M₄ Muscarinic Acetylcholine Receptor and Insights into Its Allosteric Modulation

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Introduction. Muscarinic acetylcholine receptors (mAChRs) have long been an important target for drug discovery, but drug development at the five mAChRs has been hampered by a high degree of sequence conservation within the orthosteric ligand-binding site, making it very difficult to develop subtype selective agonists or antagonists that target this site. In recent years, there has been substantial progress in the discovery of more selective mAChR ligands that act via interaction with allosteric sites.

Aims. To better understand the structural basis of selectivity and allostery at the M₄ mAChR, an important CNS drug target, we aimed to solve the structure of the M₄ receptor bound to the antagonist, tiotropium.

Results. Overall, the tiotropium-bound M₄ mAChR structure shares many similarities with the inactive M₂ and M₃ receptor structures, however we found some notable differences in the orthosteric ligand-binding site, as well as a molecule of the crystallization precipitant, polyethylene glycol, bound to the allosteric site. In an effort to further dissect the molecular mechanism of allostery at the M₄ receptor, we utilized our inactive-state M₄ structure together with an active-state M₄ mAChR model (based on the recently solved active-state M₂ mAChR structure) to rationalize the effects of targeted mutations on the interaction between a positive allosteric modulator and acetylcholine.

Discussion. We have identified an allosteric "hotwire" that links the allosteric and orthosteric sites and is composed primarily of the interfaces between TMs 2, 3 and 7. Overall, our findings indicate that it is possible to combine crystal structure data with mutagenesis data to uncover new insights into GPCR allosteric modulation.

Ryanodine receptors: a novel target for cigarette smoking-related lung disease

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Introduction. Chronic Obstructive Pulmonary Disease (COPD) is a major incurable global health burden and will become the third largest cause of death in the world by 2020. Cigarette smoking (CS) is the major cause of COPD and accounts for more than 95% of cases in industrialized countries. Given that current treatment for COPD is relatively ineffective, there is a vital need for novel therapeutic targets to oppose the effects of CS.

Aims. To characterise airway reactivity to methacholine (MCh) and serotonin (5HT) in naïve mice and following short and long-term CS exposure.

Methods. Male Balb/C mice (6-8 weeks) were exposed to 9 cigarettes/day for 4 days (short-term protocol) or 6 cigarettes/day, 5 days/week for 8 weeks (long-term protocol). Sham mice were exposed to room air. Mice were then culled and 150 µm thick lung slices prepared for phase-contrast microscopy analysis of airway contraction. Separate lungs were harvested for Q-PCR analysis and immunohistochemistry of receptors involved in airway contraction.

Results. Monophasic contraction to 5HT became biphasic and transient after both short- and long-term CS exposure, while contraction to MCh was unaltered. Expression of contractile agonist-specific receptors was unaltered, however all isoforms of the ryanodine receptor (RyR1, 2, and 3) were downregulated. Immunohistochemistry confirmed decreased RyR3 expression in airway smooth muscle after CS. Dantrolene, a ryanodine receptor antagonist, shifted the 5HT concentration-response curve to the right, but had no effect on MCh contraction.

Discussion. This is the first study to show that CS exposure selectively alters small airway contraction to 5HT and causes downregulation of ryanodine receptors involved in airway contraction. Targeting the contribution of ryanodine receptors to altered airway reactivity may provide a novel treatment strategy for COPD.

A novel single chain H2 relaxin analogue causes biased signalling at the Relaxin Family Peptide Receptor 1

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Introduction. Relaxin Family Peptide Receptor 1 (RXFP1) and its cognate peptide ligand H2 relaxin have established roles in reproductive physiology and also have anti-fibrotic, wound healing and vasodilator properties. Although H2 relaxin has successfully completed a phase III clinical trial (RELAX-AHF) for its vasodilatory benefits in acute heart failure (Teerlink et al, 2013), its rapid-acting antifibrotic actions (Samuel et al, 2009) may also have been involved. The usefulness of relaxin is limited by a complex synthesis and insolubility. This study examines a novel single chain relaxin analogue (SCRA) with significantly improved synthetic yield and solubility.

Aims. To examine the signalling profile of the SCRA at RXFP1 and compare it to the native H2 relaxin peptide.

Methods. High-throughput assays were used to measure p38MAPK, pERK1/2 and cAMP accumulation in HEK293 cells stably expressing RXFP1. Expression of collagen-degrading matrix metalloproteinase (MMP)-2 was assessed in rat renal myofibroblasts, while cardiac fibrosis was measured in isoproterenol (ISO)-injured mice.

Results. In HEK-RXFP1 cells, the SCRA was a partial agonist with ~60-70% of the efficacy and ~10-fold lower potency than H2 relaxin at p38MAPK and ERK1/2. However, SCRA activated cAMP accumulation at only the highest concentrations tested (10⁻⁶M: ~15% relaxin max; 10⁻⁵M: ~65% relaxin max). SCRA increased expression of MMP-2 *in vitro* and prevented cardiac fibrosis *in vivo* to the same extent as native H2 relaxin, in rat renal myofibroblasts and ISO-injured mice, respectively.

Discussion. Determination of the signalling profile of H2 relaxin and the SCRA at RXFP1 is important for our understanding of the physiological roles of the relaxin system and for their development as novel therapeutic agents. The SCRA was biased in favour of ERK1/2 phosphorylation vs cAMP accumulation suggesting that this peptide would display beneficial anti-fibrotic properties without side effects associated with generation of cAMP.

Teerlink et al (2013) Lancet. 381:29-39

Samuel CS et al (2009) Curr Opin Nephrol Hypertens. 18: 9-14

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miR-126 regulation in a mouse model of laser-induced choroidal neovascularisation

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Introduction. Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly. In the “wet” AMD, abnormal blood vessels (known as choroidal neovascularisation or CNV) grow from the choroid into sub-retinal space, causing vision loss. CNV is currently most widely treated with VEGF inhibitors. However, current treatments have shown to be ineffective in a subset of patients. miR-126, one of the most abundant miRNAs in endothelial cells, has recently been implicated in modulating angiogenic factors in vascular development.

Aim. To determine the role of miR-126 on angiogenesis in the laser-induced CNV mouse model.

Methods. CNV was induced by laser photocoagulation in C57BL/6 mice. CNV was confirmed using fundus photography and choroidal flat mount after laser treatment. Mice eyes were harvested at day 14 for Western blot analysis of protein and qPCR analysis of miRNA and mRNA. The CNV mice were then randomly divided into three groups (n=10/group) to receive either no injection, 3uL of either vehicle alone or vehicle+miR-126 mimic via intravitreal injection. Treatment effect was assessed by fundus photography, fluorescein angiography and confirmed by choroidal flat mount. Retinal tissue was harvested at day 14 for miRNA, mRNA and protein analysis.

Results. Significantly decreased expression of miR-126 was observed by qPCR analysis in CNV mice compared to untreated mice (n=4, p<0.05). Vascular endothelial growth receptor A (VEGF-A), kinase insert domain receptor (KDR) and sprouty-related EVH1 domain-containing protein 1 (SPRED-1) levels were upregulated with CNV in both mRNA and protein analysis. Restoration of miR-126 via transfection significantly reduced SPRED-1 and VEGF-A (a potent stimulator of angiogenesis) mRNA and protein levels (n=4, p<0.05), as measured by qPCR and western blot analysis. Also, the CNV size was reduced, demonstrated by fluorescein angiography and flat mount after miR-126 restoration.

Discussion. miR-126 has been shown to be a negative modulator of angiogenesis in the eye. The restoration of miR-126 was able to overcome the increased VEGF-A and SPRED-1 levels in the retina of CNV mice, reducing CNV. However, the downstream signalling of miR-126 needs to be further explored and characterised. The **therapeutic**

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The role of the extracellular vestibule in β_2 adrenoceptor orthosteric and allosteric ligand interactions.

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Introduction. Molecular dynamics studies suggest that β_2 adrenoceptor (β_2 AR) ligands first associate with the extracellular vestibule (area surrounding extracellular loop ECL2 and ECL3 and the top of transmembrane helix TM5 and TM7) before traversing down a narrow pathway and adopt their final position in the orthosteric pocket (Dror *et al.*, 2011). Using mutagenesis, we demonstrate the importance of this vestibule not only for orthosteric but also allosteric interactions at the β_2 AR.

Aims. To characterise the role of the extracellular vestibule in β_2 AR ligand binding and receptor activation.

Methods. Human β_2 AR mutants were constructed using DpnI-mediated site-directed mutagenesis. COS-1 membrane preparations were used to determine the affinity of isoproterenol and the dissociation rate of [³H]dihydroalprenolol (DHA) at the mutant receptors. Bioluminescence resonance energy transfer (BRET) sensor for cyclic AMP, CAMYEL, was used to quantify receptor activation in stable CHO cells.

Results. All mutants generated result in increased dissociation rate of [³H]DHA (K_{off} (min^{-1}), wild type 0.038 ± 0.003 , D192K/K305D 0.042 ± 0.004 , D192A 0.053 ± 0.005 , K305A 0.085 ± 0.011 , K305D 0.100 ± 0.005 , F193I 0.080 ± 0.015 , F193A 0.236 ± 0.062 , H296A 0.067 ± 0.021 , Y308A 0.148 ± 0.015 , n=3-4). Most notably, F193A^{45,52} and Y308A^{7,35} result in a 6 and 4 fold increase in [³H]DHA dissociation rate respectively and produce a significant decrease in the affinity of isoproterenol (pK_i , wild type 6.5 ± 0.1 , F193A 5.4 ± 0.2 , Y308A 5.5 ± 0.2 , n=3-7, p<0.05). Interestingly, Y308A^{7,35} also decreases the modulatory effects of β_2 AR allosteric modulator THRX100361. BRET functional studies to characterise the effects of these mutations on receptor activation are currently being performed (wild type isoproterenol pEC_{50} 6.2 ± 0.1 , n=6).

Discussion. We now provide pharmacological evidence supporting the role of the β_2 AR extracellular vestibule in both orthosteric and allosteric ligand interactions, in particular the aromatic amino acids lining the pathway into the orthosteric pocket.

Dror R *et al.* (2011). PNAS 108: 13118-13123.

Beta2-adrenoceptor signalling promotes invasion of human breast cancer cells

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Introduction. Activation of β -adrenoceptor (β -AR) signalling by chronic stress or with the use of exogenous agonists, induces breast cancer metastasis *in vivo*, an effect which is blocked by β -AR antagonists (Sloan et al, 2010). This suggests that β -blockers may have potential in adjuvant therapy to slow breast cancer progression.

Aims. To identify the signalling pathways involved in β -AR potentiation of breast cancer cell invasion.

Methods. MDA-MB-231HM human breast cancer cells were treated with non-selective and selective β_2 -AR agonists and antagonists and population based signalling assays were performed to monitor changes to calcium mobilisation, pERK1/2 and cAMP levels in the cells. Invasion assays were used to investigate the functional effects of β -AR signalling pathways.

Results. Salbutamol, salmeterol, formoterol, adrenaline and noradrenaline all induced cAMP production and inhibited phosphorylation of ERK1/2 in MDA-MB-231HM cells. Formoterol, adrenaline and noradrenaline also increased calcium mobilisation, however the partial β_2 -AR agonists salbutamol and salmeterol had no effect. To confirm specific activation of β_2 -AR, and not β_1 -AR in these cells, the formoterol-induced change in cAMP and pERK1/2 was further characterised by Schild analysis using non-selective, β_1 -AR selective and β_2 -AR selective antagonists (propranolol, CGP 20712A and ICI 118551, respectively). Concentration-response curves were shifted to the right with increasing concentrations of propranolol (pA_2 cAMP 10.31 ± 0.75 , pERK 10.25 ± 0.15) and ICI 118551 (pA_2 cAMP 9.45 ± 0.04 , pERK 10.11 ± 0.07) but not CGP 20712A (pA_2 cAMP 6.31 ± 0.54 , pERK 5.91 ± 0.71). Activation of β_2 -AR increased the invasiveness of MDA-MB-231HM cells, suggesting a cellular mechanism for the effects of β_2 -AR signalling on tumour cell metastasis.

Discussion. This study has identified that in MDA-MB-231HM cells, non-selective and β_2 -AR selective agonists increase cAMP production and inhibit phosphorylation of ERK1/2 in a β_2 -AR dependent manner. Some agonists can also induce calcium mobilisation. Therefore, it is likely that activation of cAMP and/or inhibition of pERK1/2 signalling may have an important role in promoting breast cancer invasion.

Sloan E et al (2010) *Cancer Res* 70: 7042-7052.

Novel positive allosteric modulators of the delta opioid receptor

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Introduction. Opioid receptors are the prototypical targets for the treatment of acute and chronic pain, yet the therapeutic efficacy of orthosteric ligands is severely limited by the development of adverse side effects. Recently, compounds targeting the allosteric site of opioid receptors have been suggested as an alternative approach with improved pharmacological properties.

Aims. The aim of this work was to characterise novel allosteric modulators of mu- and delta-opioid receptors.

Methods. Ligands were screened in cAMP, ERK1/2 phosphorylation and arrestin recruitment assays for modulator and agonist activity in CHO FlpIn cell lines stably expressing wild type mu- (MOR) or delta-opioid (DOR) receptors. We then employed molecular modelling and site-directed mutagenesis to identify residues within the DOR that govern modulator binding and transmission of cooperativity of DOR positive allosteric modulators.

Results. Positive allosteric modulators (PAMs) and silent allosteric modulators (SAMs) were fully characterised in hMOR and hDOR-CHO cells. Two structurally similar DOR PAMs were docked into the hDOR crystal structure in Schrodinger to identify potential binding site residues for these compounds. PAM activity in pERK1/2 AlphaScreen was then screened in mutant DORs stably expressed in CHO cells. Mutation of lysine 214 (K5.39) to alanine increased efficacy of the orthosteric agonist SNC80 ($\Delta \log \tau_A = 1.75 \pm 0.16$; $P < 0.0001$; $n = 4$), and of compounds CSC070960 ($\Delta \log \tau_B = 1.10 \pm 0.41$; $P < 0.001$; $n = 4$) and CSC070973 ($\Delta \log \tau_B = 1.48 \pm 0.21$; $P < 0.0001$; $n = 4$), without significant changes to functional affinity or cooperativity. We also identified residues in extracellular loop 2 and transmembrane domain 2 important for efficacy of CSC070973 but not CSC070960.

Discussion. These results, and future mutagenesis and structure-based activity studies, will allow a deeper understanding of the allosteric pocket at the opioid receptor family, and will help increase the diversity of tool compounds to explore the therapeutic potential of these receptors.

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From drug metabolism and pharmacogenetics to diagnostics and drug discovery

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Understanding immunotoxic side effects of hydralazine led to research into arylamine N-acetyltransferases. (NATs). Polymorphism in human NAT2 was the major genetic factor leading to an adverse reaction only in slow acetylators. The active site of NAT was demonstrated to be shared almost universally across all N-acetyltransferase enzymes which have now been identified in a wide range of species.

Humans have two functional adjacent polymorphic gene loci and through comparison with mice, one locus, *NAT1* in humans encodes a protein, NAT1 with an endogenous metabolic role. *NAT1* is expressed in early embryos and is overexpressed in some breast cancers. It is a candidate target as a diagnostic marker and for anti-tumour therapy. NAT1 probes which change colour on binding to human NAT1 are being developed through a programme of structure directed medicinal chemistry.

Comparative studies show NAT in *Mycobacterium tuberculosis* is encoded in an operon whose gene products catabolise cholesterol and are essential for intracellular survival of *M. tuberculosis*. NAT and HsaD, a C-C bond hydrolase from the same operon, are anti-tubercular targets. We are exploring novel inhibitors of each of these enzymes using a fragment based approach.

Minchin, R. F. and Butcher, N. J. *Pharmacological Reviews*. 2012, **64**: 147-165, 2012.

Sim, E., Abuhammad, A. and Ryan, A. *Br J Pharmacol*. 2014 **171**:2705-25. doi: 10.1111/bph.12598.

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Fragment screening of GPCRs

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Fragment-based screening has emerged as a powerful technique for drug discovery of in the last 15 years, but, until recently, its utility for GPCRs has been limited by their instability and the consequent lack of biophysical and structural information. The StaR technology developed by Heptares, in which the introduction of a small number of mutations locks the GPCR in a single active or inactive state and stabilises the receptor, has enabled this barrier to be overcome. Locking the receptor in a specific pharmacological form allows the generation of a reagent ideally suited for screening for molecules matching that pharmacology. The increased stability resulting from the StaR approach also permits screening in harsher conditions than may otherwise be feasible.

Using the StaR approach, fragment screens have been performed on several GPCRs, which, in combination with structural studies, have enabled the identification of lead and candidate molecules. Details of the technical challenges involved will be presented, along with a perspective on the outlook for fragment-based drug discovery for GPCRs.

Nanoscale Biophotonics, a window into the body

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The Australian Research Council Centre for Nanoscale BioPhotonics (CNBP) brings together physicists, chemists and biologists focused on a grand challenge – controlling nanoscale interactions between light and matter, to probe the complex and dynamic nanoenvironments within living organisms. The emerging convergence of nanoscience and photonics offers the opportunity of using light to interrogate nanoscale domains, providing unprecedentedly localised measurements. This will allow biological scientists to understand how single cells react to and communicate with their surroundings. This science will underpin a new generation of devices capable of probing the response of cells within individuals to environmental conditions or pharmacological treatment, creating innovative and powerful new sensing platforms.

When this technology is applied to the assessment of GPCR pharmacology a whole new range of experimentation is enabled. For example, the manipulation of ligand availability using nanoscale photo-sensitive gating, such that the ligand is made available at a single cell resolution, for a defined period of time, with simultaneous quantification of the cellular signalling events and phenotypic changes. Such power comes from the combined use of functionalised surface chemistry on optical fibres that are constructed with nanoscale features.

These platform technologies promise to provide *in vitro* to *in vivo* translation capacity, with the advent of devices like “Cells-On-A-Tip” fibres or “COAT” fibres. Such technologies will enable the same measurements that were performed *in vitro*, to be conducted *in vivo*, in the organ of choice, following systemic drug delivery and monitored real time over an inter-dosing interval. These advances will provide high throughput cost effective platforms, which will allow for a much better understanding of multicellular systems pharmacology.

The use of BRET to study receptor complexes

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Introduction. Depending on the context, receptors function in complexes with ligands, effectors, trafficking and scaffolding proteins, and potentially other receptors. Bioluminescence Resonance Energy Transfer (BRET) is a technology that has been used to monitor proximity of proteins of interest, and G protein-coupled receptor complexes in particular, for the last 15 years. Over this period, there have been a number of developments in terms of the components of the BRET assay system, but also how the technology has been applied.

Aims. To establish and utilise novel approaches to monitor receptor complexes in live cells and in real-time, including the development of NanoBRET using NanoLuc engineered from the luciferase found in deep sea shrimp, *Oplophorus*.

Methods. BRET is an approach that enables proximity between proteins of interest to be monitored in real-time and in live cells (Pflieger and Eidne, 2006; Pflieger et al., 2006). It involves non-radiative energy transfer from a donor luciferase enzyme to a spectrally-suitable energy acceptor, which then emits light of a characteristic wavelength. The resonance energy transferred is inversely proportional to distance (to the sixth power) and therefore acceptor emission implies donor-acceptor proximity of less than about 5 nm.

Results & Discussion. This presentation will highlight recent advances and results that exemplify novel uses of BRET to study a range of receptor complexes. This will illustrate that BRET is now a very powerful method for investigating most aspects of receptor function, albeit in model cell systems.

Pflieger K and Eidne K (2006) Nature Methods 3:165-174

Pflieger K et al. (2006) Nature Protocols 1:337-345

The emerging importance of receptor kinetics in drug discovery: what goes (k)on must come (k)off

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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 action¹, ability to differentiate between different pre-clinical candidates² and in the understanding of differential *in vivo* efficacies. At present these effects are dependent on the suitability of a radioligand and therefore limits which receptors and molecules can be investigated.

Aims. This presentation will highlight firstly the techniques employed to characterize unlabelled compounds kinetic profile, either by using traditional radioligand binding³, or use of functional assays run under hemi-equilibrium⁴ conditions in combination with a novel analytical approach. Secondly, with reference to various examples, I will demonstrate how these techniques have been utilized to help further understand the differential pharmacology sometimes observed in the clinic.

¹Slack RJ et al (2011) *Br J Pharmacol* 164(6): 1627–1641

²Mould R et al (2014) *Br J Pharmacol* 171(2): 351–363,

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

⁴Kenakin T (2009) *Pharmacology Primer 3rd edition*

Protective actions of ghrelin on cerebral vascular function and the brain post-stroke

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Introduction. The ghrelin-related peptides, acylated ghrelin (AG) and des-acylated ghrelin (DAG), are best known as neuroendocrine hormones. However, recent evidence suggests these peptides may have other biological functions.

Aims. In this study we tested whether AG and DAG exert protective effects on: 1) cerebral arteries by examining their effects on nitric oxide (NO) bioactivity and superoxide (O₂⁻) production; and 2) brain injury and oedema after transient ischaemic stroke.

Methods. NO bioactivity and superoxide production were assessed in mouse cerebral arteries using a perfusion myograph and chemiluminescence, respectively. Transient ischaemic stroke was induced in mice by middle cerebral artery occlusion (MCAo) for 0.5 h followed by reperfusion (23.5 h).

Results. Exogenous AG had no effect on the tone of cerebral arteries from wild-type (WT) mice or O₂⁻ production. By contrast, DAG elicited powerful NO vasodilator responses (EC₅₀ <10 pmol/L) and suppressed O₂⁻ production. Vasodilator responses to DAG were sustained in the presence of YIL-781 (an antagonist of the classical ghrelin receptor, GHSR), and in arteries from *Ghsr*^{-/-}. Next, we found evidence that endogenous production of DAG normally regulates NO bioactivity and O₂⁻ levels in the cerebral circulation. Specifically, we found that NO bioactivity was markedly reduced in *Ghrelin*^{-/-} vs. WT mice and superoxide levels were elevated. Treatment of WT mice with DAG at the time of reperfusion reduced infarct and oedema volumes 24 h after MCAo. Moreover, *ghrelin*^{-/-} mice had larger infarct and oedema volumes than WT mice, and cerebral vascular O₂⁻ production was markedly elevated.

Discussion. This study provides the first evidence of protective actions of exogenous and endogenous DAG on the cerebral circulation and the brain after stroke, and it provides evidence of an important role for, and the existence of a novel ghrelin receptor in cerebral arteries.

Role of insulin-regulated aminopeptidase (IRAP) in mediating ischemic damage in the brain

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Stroke is a neurovascular disease which is the leading cause of adult disability and the second greatest cause of death in Australia. A focus of our research group has been to investigate the role of a protein called insulin-regulated aminopeptidase (IRAP) in ischemic stroke. IRAP is an aminopeptidase which cleaves of small peptide substrates including oxytocin, vasopressin, met- and leu-enkephalins and is also involved in the regulation of GLUT4 trafficking. Under normal physiological state, IRAP is expressed predominantly in neurons in the brain. However, following brain injury, its expression is significantly upregulated in activated astrocytes and microglia. We observed a dramatic neuroprotective phenotype in the global IRAP knockout mice against cerebral ischemic damage. In a mouse model of focal ischemic stroke induced by transient middle cerebral artery occlusion, the IRAP KO mice displayed a significant reduction in infarct volume in the striatum and neocortex compared to wildtype controls, with a corresponding improvement in neurological performance at 24 h post-ischemia. We also obtained further proof-of-concept that pharmacological inhibition of IRAP also provided neuroprotective effects in a conscious rat model of stroke. We demonstrated that treatment with the novel small molecule non-peptide IRAP inhibitor, HFI-419 post-stroke, significantly reduced infarct volume and edema and attenuated neurological and motor deficits in the endothelin-induced focal ischemia in the spontaneously hypertensive rat. Our studies provide compelling evidence for the involvement of IRAP in the various cellular events leading to cell death in the infarct core and induction and progression of cell death in the penumbra region. Secondly, these studies provide a rationale for the development of IRAP inhibitors as new therapeutic candidates for the arrest of brain damage and programmed cell death following ischemic stroke.

Using Stem cells and targeting astrocytes to repair the injured brain after stroke

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Despite attempts to prevent brain injury following ischaemic stroke, most sufferers end up with long term deficits. To this end we have been developing therapies that can restore functional deficits after damage has ensued.

Aims: To investigate the effects of undifferentiated vs pre-differentiated human NPC transplants into the rat brain 7 days post-stroke; and in separate studies explore the use of Rho kinase inhibitor Fasudil (HA-1077) to maintain trophic astrocyte support, reducing glial scarring and accelerate functional recovery.

Methods: We isolated and characterized human neural progenitor cells (NPCs) from the subventricular zone and directed their differentiation towards neural lineage cells (GABAergic interneurons). Using the rat endothelin-1 model of stroke for long term recovery (Abeyasinghe et al, 2014), GABAergic neurons, undifferentiated NPCs or media alone were transplanted at 7 days into both damaged and non-damaged brain regions (n=10/group) and further recovered for 28 days. In separate studies the effects of Fasudil (10mg/kg, i.p. daily) treatment commencing 3 days after stroke was investigated and compared to vehicle (saline) controls (n=9/group) for 28 days.

Results: Accelerated improvements in motor function (cylinder test and neurological deficit scores) were detected 7 days post-transplant in rats treated with pre-differentiated GABAergic cells in comparison to media alone and undifferentiated hNPC treated groups. Histopathology indicated that pre-differentiated cells maintained their GABAergic neural phenotype, showed evidence of synaptogenesis, up-regulated expression of both GABA and calcium signalling proteins associated with neurotransmission and increased neurogenic activity within the SVZ. In contrast, undifferentiated SVZ-hNPCs predominantly differentiated into GFAP-positive astrocytes and incorporated into the glial scar. In separate studies stroke rats treated daily with Fasudil showed early improvements in deficits (cylinder test and neurological deficit scores) with reduced glial scar formation compared to vehicle controls.

Discussion: This is the first study to show enhanced exogenous repopulation of a neuronal phenotype after stroke using techniques aimed at differentiation prior to transplant. Accelerated functional recovery can also be achieved by targeting astrocytes to maintain trophic glial morphology with reduced glial scar formation.

Abeyasinghe et al (2014) PLoS One. 9(5):e97007.

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Hypoxia-inducible factor – 1 (HIF-1) as a novel target for brain repair

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Hypoxia-inducible factor-1 (HIF-1) is the key transcription factor regulating the expression of many hypoxia-responsive genes, including erythropoietin and vascular endothelial growth factor (VEGF). Such hypoxia-inducible changes allow the body to adapt to a lower oxygen environment. Under normoxic conditions, HIF-1 α protein is constantly being degraded due to HIF-1 prolyl hydroxylase enzymes (PHDs) which hydroxylate proline residues on HIF-1 α causing degradation of HIF-1 α and consequently, constitutive levels of HIF-1 α protein are low. Previous studies have shown that mild hypoxia and drugs that can inhibit PHD activity can increase expression of HIF-1 and subsequent target gene expression in the brain. Over the past few years, we have employed this strategy in a number of in vitro and in vivo models of ischemic brain injury in rats, to examine whether mild hypoxia or compounds which can increase HIF-1 (PHD Inhibitors) can protect the brain against injury. Our studies have shown that preconditioning with mild hypoxia can protect against hypoxic-ischemic (HI) injury in newborn rats, and that this protection is mediated by HIF-1 and its target genes. Similarly, we found that a single, subcutaneous (s.c) injection of the PHD inhibitors desferrioxamine (DFX) or ethy-3,4-dihydroxybenzoate ((EDHB) both at 200mg/kg, s.c.) can protect against HI injury. Mechanistic studies have found neuroprotective actions of DFX and EDHB are mediated by astrocytes which increase levels of HIF-1 and VEGF. Our more recent studies, have focused on using post injury treatment (or postconditioning) with mild hypoxia, starting 1 day after HI and endothelin-1 induced cerebral ischemia in newborn and adult rats, respectively. We have shown that postconditioning with mild hypoxia can reduce the extent of damage and promote functional recovery in these two in vivo models models of ischemic brain injury, and we are currently exploring the cellular and molecular mechanisms involved. Our findings indicate that modulation of HIF-1 and its target gene expression after HI brain injury is an effective strategy to promote brain repair.

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Trough concentrations of infliximab and adalimumab, and anti-drug antibodies, correlate with drug response in inflammatory bowel diseaseMurray L Barclay^{1,2}, Esther Helms¹, Barry Hock², Michael Schultz³, Lisa K Stamp². Dept of Clinical Pharmacology, Christchurch Hospital¹, Christchurch, NEW ZEALAND; Depts of Medicine and Haematology, University of Otago Christchurch², and Dept of Medicine, University of Otago Dunedin³, NEW ZEALAND.

Introduction. The TNF α antagonists including infliximab (IFX) and adalimumab (ADA) are the most effective treatments for inflammatory bowel disease (IBD) but more than half of patients either fail to respond, or lose response, by 12 months of treatment. Failed treatment may be due to low drug concentrations and/or presence of anti-drug antibodies.

Aims. We aimed to measure trough concentrations of IFX and ADA, and antidrug antibodies, in patients with IBD, and to correlate concentrations with disease activity.

Methods. Subjects were gastroenterology outpatients and inpatients at Christchurch Hospital with IBD on IFX or ADA. Blood samples were collected at least 12 weeks after treatment initiation, within 24 hours prior to a dose. Disease activity indices for CD and UC were recorded at the time of sampling and at treatment initiation. Concentrations of drugs and anti-drug antibodies were measured by enzyme-linked immunosorbent assay (ELISA).

Results. Sixty-one patients were studied, including 33 on IFX (24 CD, 7 UC, 1 indeterminate colitis) and 28 on ADA (all CD). Median (range) IFX and ADA concentrations were 7.9 (0-58) and 4.9 mg/L (0-59). Twenty-five patients had concentrations below 5 mg/L, a suggested threshold for drug activity. Crohn's Disease Activity Index (CDAI) was significantly higher in patients with drug concentrations <5 versus >5mg/L (median 166 vs 89, p=0.007). ROC analysis suggested a threshold value of 5-7mg/L is appropriate. Of 10 patients with drug concentrations <1mg/L, 5 had detectable anti-drug antibodies (3 IFX and 2 ADA antibodies).

Discussion. It is now possible to measure drug concentrations of IFX and ADA, and anti-drug antibodies to both drugs, in New Zealand. These results confirm published data showing that trough concentrations are correlated with disease control in IBD and that drug concentration and anti-drug antibody monitoring may aid in dosing decisions.

The ideal anticoagulant is a myth

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Introduction. Anticoagulants are important therapeutic agents for the treatment of various cardiovascular diseases. Inappropriate dosing carries the risk of therapeutic failure or excessive bleeding. Newer anticoagulants have been heralded as “game changers” [1] with the assertion that they have more predictable effects and do not require anticoagulant monitoring [1]. The ideal anticoagulant has been described as one that has a wide therapeutic range, is administered orally, has a predictable anticoagulant effect and does not need to be monitored [2].

Aims. To determine whether the ideal anticoagulant could exist.

Methods. Theoretical anticoagulants were considered that inhibited clot formation by (1) inhibiting vitamin K epoxide reductase, (2) competitively antagonising Xa, (3) competitively antagonising IIa, (4) binding the VIIa:TF complex. Simulations were performed using a previously developed systems model of coagulation [3] with normal variability incorporated on coagulation factors (I, II, V, VII, VIII, XI, XII and anti-thrombin). All theoretical compounds were administered orally with assumed bioavailability of 100% and had a between-subject variability of 40% on clearance (similar to currently approved anticoagulants). No variability was considered on affinity or any other PKPD parameters. A clotting time test, similar in spirit to INR, with a therapeutic range of 2 to 3.5 was used to assess clinical response.

Results. The values of INR equivalents across the four theoretical anticoagulants ranged (on average) from 1.3 to 5.4. The widest variability occurred for the VKOR inhibitor (1.2 to 5.9) and the smallest range for the prototypic VIIa:TF inhibitor (1.3 to 4.8). None of the anticoagulants achieved ideal status.

Discussion. The expectation should be that all anticoagulants used in therapeutic doses will require monitoring and that monitoring is not a function of the drug itself but rather the complex system on which the drug acts.

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2. Falavolo et al (2011) Clin Chem Lab Med 49:755-757
3. Wajima et al (2007) Clin Pharmacol Ther 86:290-289.

Neprilysin inhibitors preserve renal function in heart failure – meta-analysis of randomised controlled trials

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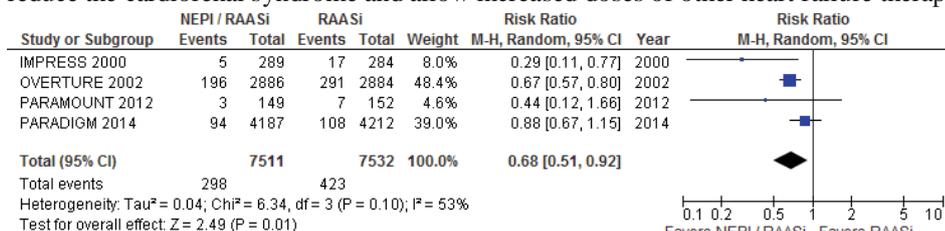
Introduction. Heart failure therapy can be limited by deterioration of renal function with renin-angiotensin-aldosterone system inhibition (RAASi). Recently, the superiority of the angiotensin receptor neprilysin inhibitor LCZ696 over enalapril alone was demonstrated. Neprilysin inhibitors (NEPi) may have direct beneficial renal effects that contribute to this superiority.

Aims. To determine the renal effects of NEP-RAASi in heart failure.

Methods. Meta-analysis of randomised controlled trials (RCTs) involving NEPi with either angiotensin converting enzyme inhibitors (ACEi) or angiotensin receptor blockers (ARB), reporting on decline in renal function as defined per individual study. Risk ratios (RR) were calculated using the Mantel-Haenszel random effects model.

Results. Four major studies were included (IMPRESS, OVERTURE, PARAMOUNT and PARADIGM-HF) with 15,043 participants. Mean follow up time was 51 weeks. Compared with ACEi or ARB alone, combined NEP-RAASi resulted in a reduction in risk of decline in renal function (RR 0.68, 95% confidence interval 0.51-0.92, $p=0.01$) (FIGURE)

Discussion. NEP-RAASi preserve renal function in heart failure compared with ACEi or ARB alone. This can reduce the cardiorenal syndrome and allow increased doses of other heart failure therapies.



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Insights into the pathophysiology of hyperuricaemia

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Introduction. Hyperuricaemia (HU, serum urate concentration ≥ 0.42 mmol/L) is the greatest risk factor for gout. As HU is increasingly being associated with metabolic syndrome disorders, the need to better understand the aetiology of HU is becoming ever more apparent.

Aims. Our aim is to understand the mechanisms and risk factors contributing to HU.

Methods. Hyperuricaemics (n=400) and normouricaemics (serum urate concentration < 0.42 mmol/L, n=344) were screened for the following factors: age, sex, ethnicity, rs2231142 genotype for ABCG2, FCU, metabolic syndrome disorders and estimated GFR (eGFR). Continuous and categorical factors were analysed together using multiple linear regression and stepwise binary logistic regression. Continuous variables were additionally entered into a separate 'categorical only' model and analysed in categories based on clinical thresholds. Model evaluation was conducted using classification tables and the area under the receiver operator curve (AUROC).

Results. The mixed categorical/continuous variable model was the marginally better model (AUROC 0.90, 81% classification accuracy) and found the greatest predictors as: ABCG2, FCU, male sex, BMI, serum triglyceride concentrations, eGFR and grams of alcohol/week. The 'categorical only' model (AUROC 0.87, 78% classification accuracy) had the same predictors as the mixed model with the exception of grams of alcohol/week and serum triglyceride concentrations. The greatest predictors were homozygosity for rs2231142 (odds ratio [OR]: of 10.4, 95% confidence interval [CI]: 2.4-46.0), male sex (OR: 10.5, 95% CI: 6.0-18.6) an FCU < 5 (OR: 3.6, 95% CI: 2.2-5.8) a BMI ≥ 30 (OR 26.4, 95% CI 2.1-228) and an eGFR between 15-29 mL/min/1.73m² (OR: 26.6, 95% CI: 3-230).

Discussion. This novel regression model identifies how renal (eGFR, FCU) and gut clearance (ABCG2) mechanisms independently influence HU. Optimising renal and gut clearance of urate together presents a new opportunity for treating hyperuricaemia.

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The influence of imatinib on disease progression in patients with gastro-intestinal stromal tumour (GIST).

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Introduction. Imatinib is a tyrosine kinase inhibitor used in Australia as adjuvant therapy for gastro-intestinal stromal tumour (GIST). Trough concentrations of imatinib may correlate with response (1).

Aims. To determine the relationship between imatinib plasma concentrations and CT scan determined disease progression.

Methods. Plasma concentrations were available in 43 GIST patients (metastatic or unresectable, positive CD117 staining). Trough and occasional peak concentrations were collected every 3 months along with CT scan (duration 3-58 months, 2-38 samples per patient). A PK model was developed in NONMEM (Version7.2). A logit PD model was developed to evaluate the relationship between imatinib exposure (Cumulative AUC) and disease progression at 2 years. Covariates such as age, base line tumour length and body mass index were also evaluated in the PD model.

Results. The PK data was best described by a once compartment model with first order absorption. The mean (between subject variability, %) of CL/F was 12.4 L/h (31.8), V/F 339 L (12.3) and Ka 1.5 h⁻¹ (fixed). A between occasion variability was identified on CL/F to be 21.5%. Lean body mass was a key covariate on CL/F and V/F. Cumulative AUC (range 1780 – 46539 mg.h/L) had a small but significant (P < 0.05) influence on GIST progression. No other covariates improved the PD model. A typical patient with the mean population CL taking a standard 400 mg dose of imatinib will have a 46% chance of disease progression at 2 years.

Discussion. The PK of imatinib in this group is similar to previous studies. This analysis provides some evidence that imatinib plasma concentration does influence the probability of disease progression in patients with GIST. A time to event analysis is being undertaken to evaluate this further. Given the high probability of disease progression at the standard dose there is a reasonable justification for dose escalation in imatinib therapy for GIST.

Von Mehren M and Widmer N (2011). *Cancer Treat Rev* 37:291-299.

Piperacillin pharmacokinetics in intensive care unit patients with anuria and acute kidney injury receiving sustained low efficiency diafiltration

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Introduction. Sustained low efficiency diafiltration (SLED-*f*) is being increasingly used in intensive care units (ICU). However, PK information is not available to guide piperacillin dosing during SLED-*f*.

Aims. To describe PK of piperacillin in ICU patients with anuria and acute kidney injury receiving SLED-*f*.

Methods. This was an observational PK study in ICU patients receiving SLED-*f* for 6 h. Blood samples were collected at pre- and post-filter ports within the SLED-*f* circuit and arterial catheter when not receiving SLED-*f*. Sampling occurred during two dosing intervals; one during SLED-*f* and another without SLED-*f*. Piperacillin concentrations were measured using a validated MSMS method. Non-compartmental PK analysis was performed.

Results. Six patients were recruited. Sampling occurred with and without SLED-*f* for five patients and in one patient sampling occurred only during SLED-*f*. The median (IQR) age, weight, APACHE II score and SOFA score were, 58 (11) years, 80 (25) kg, 34 (10), and 9 (3) respectively. Observed median (IQR) C_{min} , CL_{total} (L/h), V (L) and $t_{1/2}$ (h) with and without SLED-*f* were 37.5 (26.3), 66.1 (32.0); 5.9 (1.9), 2.0 (3.5); 26.9 (5.7), 23.9 (11.6); 2.5 (1.5), 7.5 (4.2) respectively. The median percentage piperacillin extracted by the dialyser was 58% during the 6 h session.

Discussion. SLED-*f* effectively eliminated piperacillin in this study similar to that reported in patients with normal renal function (58% vs. 50%). Observed median C_{min} was higher than MIC clinical breakpoints for *enterobacteriaceae* spp. and *p. aeruginosa*. However, there were considerable inter-individual differences with observed C_{min} in two of the five patients was <16mg/L on SLED-*f*. Median V was higher than that reported in healthy volunteers (27 vs 15L) and similar to that reported in patients with sepsis (27 vs. 25L) consistent with fluid overload in acute kidney injury. Twice daily piperacillin dosing is required in some patients with anuria and acute kidney injury receiving SLED-*f* to avoid under dosing.

Using peer-assisted learning in an Honours program to enhance student engagement and performance

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Introduction. The coursework component of the Pharmacology Honours program at Monash University comprises the critical analysis of a scientific paper under exam conditions. Traditionally, teacher-led tutorials were utilised to highlight pharmacological techniques, experimental design and data analysis. However, although students were given opportunities to undertake practice exams, overall performance in the assessment task has been relatively poor.

Aims. The current study aimed to revise the learning activities associated with the literature critique exam to encourage student engagement and improve performance.

Methods. In 2013, a tutorial program was implemented which involved peer learning groups such that groups of 4-5 students critiqued components of a scientific paper, presented their findings to the class and lead class discussion. In 2014, to further align the learning activity (group work) with the assessment task (individual work), the exam format was changed to incorporate a peer-assisted learning component. Students were given the opportunity to discuss the scientific paper with their peers for 30 minutes before being given the exam questions which were then completed under usual exam conditions. The 2014 exam paper was identical to the 2013 paper. Evaluation of the effectiveness of this change in tutorial and exam format, was via comparison of the exam results and a student feedback survey at the end of the unit.

Results. Students entering the Honours program in 2012-2014 had a similar background knowledge in pharmacology with the average mark in the foundation 3rd year pharmacology unit ranging from $73.2 \pm 10\%$ (n=14, 2014) to $78.0 \pm 8.1\%$ (n=19, 2012). Following the 2013 revision of the tutorial format from 'teacher-led' to 'peer-assisted', there was no change in the exam marks (2013: $65.7 \pm 9.9\%$; 2012: $63.4 \pm 10.2\%$). However, the majority of students strongly agreed that the peer-group format of the tutorials enhanced their learning. The introduction of the peer group discussion at the start of 2014 exam appeared to increase exam performance ($74.0 \pm 7.3\%$).

Discussion: Our results suggest that the inclusion of peer-assisted learning in both the learning activities and assessment task may increase the level of engagement of students and improve their critical analysis skills.

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Learning styles and blended learning strategies for teaching pharmacology

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Introduction. Blended learning is the combination of online teaching modalities and face-to-face teaching (Halverson et al, 2014). In recent times, as the technical capacity for online education has expanded, the diversity of resources now available provides teaching strategies which enhance student engagement and learning experience (Suda et al, 2014). However few studies into online resources and student learning styles have been reported.

Aims. To determine the engagement of students with different learning styles with blended learning resources and teaching strategies in an undergraduate pharmacology course.

Methods. Consenting students enrolled in the third year pharmacology course in the School of Medical Sciences in 2011 completed a Visual, Auditory and Kinaesthetic (VAK) learning survey at the beginning of semester and an audit of learning activities and resources on conclusion of the semester.

Results. Ninety-nine students consented to participate and comprised of visual learners (57%), kinaesthetic learners (18%), visual/kinaesthetic (11%) and visual/auditory/kinaesthetic (7%) learners. Teaching activities and resources were evaluated with respect to student engagement and effectiveness for learning and no differences were observed between the cohorts of student with different learning styles. However, comparison of assessment outcomes and student learning styles demonstrated lower achievement in the kinaesthetic group compared to visual and visual/auditory/kinaesthetic learners ($P < 0.05$).

Discussion. Students, irrespective of their learning styles, were engaged similarly with the learning and teaching strategies for the pharmacology course, and the resources provided to the class did not favour any one learning style. However, in this cohort of students, assessment outcomes for students who identified themselves as kinaesthetic learners were poorer when compared to students with other learning styles.

Halverson et al (2014) *Internet and Higher Ed.* 20: 20-34; Oflaz M & Turunc T (2012) *Procedia Soc and Behav Sci* 46:1333-1338; Suda KJ et al (2014) *Cur Pharmacy Teach and Learn* 6: 367-372.

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Antagonist actions at the N40D (A118G) polymorphism of the human μ opioid receptor *in vitro*

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Introduction. Opioid antagonists can be effective therapeutic agents in treating alcohol dependence. Intriguingly, the efficacy of naltrexone is enhanced in individuals with the A118G SNP in the μ -opioid receptor (MOPr). This SNP results in a change of Asn to Asp at position 40 in the extracellular domain of MOPr. The reason for the increase in clinical efficacy is unclear, but may reflect differences in antagonist interactions with the D40 allele of MOPr.

Aims: To determine the functional effects of the clinically relevant opioid antagonists naloxone, naltrexone and its metabolite 6β -naltrexol at wild type (N40) and D40-MOPr.

Methods. AtT-20 cells stably expressing human N40-MOPr or D40-MOPr were used. Endogenous K channel activation was monitored using a membrane potential-sensitive dye in a Flexstation 3. Concentration-response curves to the opioid morphine or DAMGO were constructed in the presence of at least 3 concentrations of antagonist ($n=5$ for each). Schild analysis (GraphPad Prism) was performed to obtain values for pA_2 and K_b .

Results. Naloxone, naltrexone and 6β -naltrexol had no appreciable agonist activity at D40 MOPr. The pA_2 for naloxone vs morphine was 8.65 ± 0.07 at N40 and 8.63 ± 0.05 at D40 (Schild Slope 1.2 ± 0.1 , 1.3 ± 0.1 and K_b 2.0 ± 0.2 nM, 2.4 ± 0.3 nM for N40 and D40 respectively). The pA_2 for naltrexone against DAMGO was 8.55 ± 0.11 at N40 and 8.63 ± 0.07 at D40. The Schild slopes for naltrexone were 1.8 ± 0.2 for N40 and 1.6 ± 0.1 for D40, suggesting the assay was not at equilibrium in these conditions, precluding determination of a K_b . This was also the case with 6β -naltrexol for which the pA_2 for against DAMGO was 8.62 ± 0.1 at N40 and 8.68 ± 0.1 at D40 (Schild slopes 1.4 ± 0.2 and 1.5 ± 0.2 respectively).

Discussion: The difference in efficacy observed between carriers of the wild type and A118G opioid receptor SNP when treating alcohol dependence with naltrexone cannot be explained by obvious differences in antagonist activity measured by this assay. However, it may be that the antagonist ligands display signaling bias and differentially affect other MOPr-mediated signaling pathways. Alternatively, the enhanced effects of naltrexone may reflect alterations in opioid-mediated reward pathways resulting from expression of the D40 polymorphism.

Clinical use of an aminoglycoside ward-based Therapeutic Drug Monitoring (TDM) service in a tertiary hospital

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Introduction. Therapeutic Drug Monitoring (TDM) has substantially improved the safety and efficacy of aminoglycoside prescribing. Although a computerized Area Under the Curve (AUC) based method seems to provide additional advantages with dose prediction little information is available on its applicability in the clinical setting.

Aims. To assess the feasibility and impact of an AUC-based gentamicin TDM service at Flinders Medical Centre.

Methods. Hospitalized patients admitted between 01/08/2012 and 31/12/12 were identified and reviewed by a TDM team of clinical pharmacologists and pharmacists. Written recommendations were provided to the clinical team.

Results. 46 patients (age 57 years \pm 18), 37 of whom were surgical admissions (80.4%), were included in the study. Of these, 25 patients (54.3%) were recommended to have dose alteration: dose increase (n=18) dose reductions (n=2) and dose interval change (n=2). The clinical team followed recommendations in 43 patients (93.5%) Average length of stay in hospital was 11 (medical) and 8 (surgical) days respectively. There was no evidence of nephrotoxicity in the study group.

Discussion. The introduction of a computerized ward-based aminoglycoside TDM service has made a significant impact on gentamicin dosing modifications and acceptance by clinical teams. In order to better estimate the impact on objective outcome measures, e.g. overall length of stay in hospital, total number of treatment days, incidence of nephrotoxicity and achievement of Cmax and AUC targets, comparison with cohorts of patients admitted before the introduction of the service is now required.

Has the availability of new oral anticoagulants (NOACs) changed the way older inpatients are selected for oral anticoagulation?

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Introduction. The bleeding risk with warfarin appears to increase with age, and may be predicted by the level of frailty. In May 2014, the new oral anticoagulants (NOACs) were made available for restricted indications on a State-wide South Australian formulary, increasing their availability in South Australian hospitals.

Aims. We explored the characteristics of inpatients over the age of 60 recently initiated or about to commence oral anticoagulation (OAC) to investigate if NOAC availability changed prescribing practice in this group of people.

Method. Participants were identified by medical and pharmacy staff, and by review of dispensing and INR reports. Participants were categorised according to frailty, stroke and bleeding risks. Data was analysed by chi-squared tests.

Results. Data on 102 participants (all warfarin) pre-NOAC introduction (PRE) and 74 participants (46 warfarin & 28 NOAC) post-NOAC introduction (POST) were analysed. The POST group were older and had significantly frailer patients initiated on warfarin than in the PRE group, but there was no significant difference in frailty between PRE and POST groups overall. More participants initiated on warfarin in the POST group had higher CHADS2 scores than in the PRE group. There was no difference between the groups for gender, CHADS2 and HAS-BLED scores. In participants with atrial fibrillation (AF) in the POST group, just under half of the subjects were prescribed warfarin. There were very few participants prescribed NOACs for indications other than AF.

Discussion. Since NOACs were made available on the formulary, older inpatients prescribed OACs were older but didn't significantly differ with regards to frailty, stroke or bleeding risk. Warfarin patients in the post-NOAC period were frailer and had higher stroke risk. Patients with non-AF indications for OACs were more likely to be prescribed warfarin than a NOAC. This may reflect initiating prescribers' preference for warfarin in more complex patients. Whether frail elderly patients who wouldn't have received OACs before because of their bleeding risk are doing so post-NOAC introduction is unknown. The appropriateness of this change in practice needs further study.

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An audit of the use of hydroxychloroquine for the treatment of systemic lupus erythematosus

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Introduction. The antimalarial hydroxychloroquine (HCQ) has been used in the therapy of systemic lupus erythematosus (SLE) and other connective tissue disorders since the early 1950s,¹ and now it is used as standard therapy in Western countries.² HCQ has been demonstrated to have an overall protective effect on survival in people diagnosed with SLE, and recent reviews confirm it as an effective treatment with a low risk of adverse effects.¹

Aims. To audit the use of HCQ in patients with SLE at a tertiary Brisbane hospital.

Methods. Patients with SLE who were reviewed (outpatient or inpatient) at the Royal Brisbane Hospital (RBH) between March 2013 to March 2014 were identified. Charts were reviewed to confirm a diagnosis of SLE as well as their current treatment regimen, including dose of HCQ and/or steroid or other immunosuppressant agent. Patient demographics including age, height and weight were also collected where this data was available. Pathology tests including inflammatory markers and auto-antibodies were also obtained if these were performed. Patients with multiple visits during this time period had their last review used for analysis.

Results. 60 patients (48 females) with an average age of 50 years (17-80) were identified during the audit period. 45 (75%) were taking HCQ at the time of the review. 33 (55%) were taking steroids with an average daily dose of 8.4mg (2-30mg). Of the 15 patients not taking HCQ, 8 had a valid reason including prior adverse effects.

Discussion. HCQ is not only effective in the treatment of SLE but also in preventing and improving infections, thrombosis, hyperglycaemia and hyperlipidaemia, as well as improving the prognosis for survival.² The use of HCQ for SLE is standard of care with some advocating that all patients with SLE receive this therapy unless contraindicated. This audit confirms that most patients with SLE that attend RBH are currently on HCQ at appropriate doses, however there are still some patients that may benefit from its addition.

1. Carmichael SJ (2013) Intern Med J 43:547-53.
2. Momose Y (2013) J Dermatol 40:94-7.

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A case report of yttrium 90 microsphere dosimetry to estimate hepatic tolerance of subsequent lutetium 177-DOTA-octreotate therapy for metastatic neuroendocrine tumour.

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Introduction. Peptide receptor radionuclide therapy (PRRT) may be limited by hepatic, renal and bone marrow toxicity. Accurate, individualised dosimetry of vital organs and tumour is warranted to limit radiation toxicity and maximise efficacy. A 59 year old female with symptomatic metastatic small bowel neuroendocrine tumour was referred for PRRT with the radiolabelled somatostatin analogue lutetium-177 DOTA-octreotate (LuTATE), nine months following hepatic selective internal radiation therapy (SIRT) using yttrium-90 (⁹⁰Y) resin microspheres.

Aims. Dosimetry was used to assess the radiation risk to normal liver from LuTATE therapy following SIRT therapy, with a reference cumulative tolerance threshold biologically effective dose (BED) of 54 Gy_{BED} for a 5% risk in 5 years of developing radiation-induced hepatic disease.

Methods. Hepatic dosimetry was retrospectively estimated from a technetium-99m macroaggregated albumin single-photon emission computed tomography (SPECT) study acquired prior to SIRT. The SPECT images were normalised to reflect an assumed ⁹⁰Y microspheres dose distribution and analysed with Monte Carlo simulation. Liver parenchymal dose distribution was estimated based on the administered ⁹⁰Y microspheres dose of 1800 megabecquerel (MBq). As it was not feasible to calculate patient specific dosimetry for LuTATE prior to the first cycle of therapy, hepatic radiation dose was estimated from literature as 0.27 ± 0.05 mGy/MBq. LuTATE dosimetry is planned at the time of therapy.

Results. For ⁹⁰Y SIRT, the estimated mean dose to healthy liver was 12.8Gy_{BED}. Maximum healthy liver dose was estimated at 13Gy_{BED} for a planned four doses of LuTATE. Summing the estimated SIRT and LuTATE doses would result in an approximate total dose of 26 Gy_{BED} (well below the 54 Gy_{BED} tolerance threshold dose).

Discussion. From an hepatic radiation risk perspective, the patient was considered eligible to receive LuTATE despite previous SIRT. The actual radiation dose delivered to normal liver and metastases can be estimated following completion of patient-specific LuTATE dosimetry and all cycles of planned therapy.

Implementation of a formalised medication review protocol applied to older general medical patients.

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Introduction. Older patients are often frail, suffer several chronic diseases and receive multiple medications. Those on 8 or more medications are known to be at risk of adverse drug events and are potentially on medications that will not benefit them. In contrast, most older patients favour taking fewer drugs and avoiding any drug that is likely to cause side effects, irrespective of its efficacy. It has been shown that the total benefit of multiple drugs tends to be less than the sum of the projected benefits of individual drugs. This goes against the current therapeutic momentum to prescribe more medications on the basis of disease-specific clinical guidelines. Interventions which aim to optimise prescribing appropriateness in older patients have shown mixed results. Hospitalisation of older patients to general medicine units presents an opportunity for a whole-patient reappraisal of current medication. Finch, et al, 2013 showed that in a group of patients from a geriatric database, a medication review algorithm identified approximately 20% of medications as potentially inappropriate and a further 39% as uncertain.

Aims. The primary aim is to determine the number of potentially inappropriate medications that clinicians cease or wean (or intend to do so) after applying a formalised medication review protocol as part of routine care. Secondary aims were identification of: 1) frequency of drugs and drug classes assessed as being inappropriate; 2) patient characteristics associated with higher prevalence of inappropriate medications; 3) constraints in applying and interpreting the protocol; and 4) assessing acceptability of protocol-mediated recommendations for changes in prescribing from the perspective of treating clinicians.

Methods. Physicians and registrars of 6 general medical units applied a 5 step review protocol to medications of patients admitted for more than 24 hours who were aged 65 years or older and prescribed 8 or more regular medications. The protocol used current evidence to assess and verify 1) current medications; 2) identify patients at risk; 3) evaluate medicine utility; 4) target those with least benefit; 5) deprescribe under supervision.

Results and discussion. A formalised protocol for medication review in older general medicine patients with polypharmacy is effective at identifying medications that should be deprescribed and supporting prescribing physicians in executing this task.

Finch A, et al (2013) ASCEPT 2013 ASM. P. 45

Warfarin-associated spontaneous haemarthrosis following use of complementary and alternative medicines

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Introduction. Spontaneous haemarthrosis associated with warfarin is an uncommon event compared to other bleeding events on this drug. Risk factors include advanced age, previous trauma to the joint, within the first year of therapy, and poorly controlled international normalised ratio (INR, Hylek et al 2010). The effects of complementary and alternative medicines in combination with warfarin are described, although poorly defined (Samuels 2005)

Aims. To present a case of warfarin-associated spontaneous haemorrhage following use of a herbal chilblain cream and discuss potential mechanisms and consequences.

Methods. We present a novel case of an 88 year old man who developed a spontaneous haemarthrosis whilst on warfarin despite a well managed INR and a total knee joint replacement many years prior. Other biochemical markers were consistent with warfarin use alone. This was following concurrent use of Weleda Frost cream to treat chilblains. This contains multiple agents known to affect platelet function and alter other coagulation parameters and have been associated with bleeding on anticoagulants. These include horse radish, cod liver oil, capsicum, and arnica. The patient has recovered after withdrawal of the herbal cream despite maintaining an INR of 2.0. The event has been reported to the New Zealand Pharmacovigilance Centre and the manufacturer has been contacted.

Results. This potential interaction scores +3 on the Naranjo probability score suggesting a possible adverse drug reaction. Multiple mechanisms for these herbal medications affecting the coagulation system have been described and are reviewed.

Discussion. This is the first reported case of a warfarin-associated spontaneous haemarthrosis following transdermal absorption of herbal preparations. Greater definition of mechanisms and awareness of potential clinical pitfalls when using these complementary medications is required amongst clinicians to promote safer use of anticoagulants.

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Assessing patient knowledge and education techniques in order to improve safety with warfarin therapy: A review of the literature

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Introduction. Adequate patient knowledge and education have been identified as vital factors associated with safe warfarin use.

Aims. To assess the level of patient knowledge and understanding of warfarin in both hospital and community managed patients and also examine various warfarin education programs for their effectiveness.

Methods. A systematic literature search of the OVID Medline, Embase and CINAHL Plus databases was performed. The studies that assessed patient knowledge and warfarin education programs were analysed and synthesised.

Results. A total of 29 relevant peer-reviewed articles were identified. These included 14 articles that assessed patient's warfarin knowledge and understanding, 13 articles that assessed the effectiveness of a single warfarin education program and two articles that compared warfarin education programs. The majority of studies were conducted in a hospital setting.

Discussion. Patient knowledge of warfarin was found to be sub-optimal and associated with reduced anticoagulation control. A variety of educational programs were implemented and found to be successful in improving patient knowledge and understanding of warfarin. This review has highlighted the need to further assess patient knowledge in community-based settings and provide targeted education to facilitate improved management of patients who are prescribed warfarin.

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Combining human bone marrow-derived mesenchymal stem cells with an anti-fibrotic more effectively reverses airway fibrosis in an experimental model of chronic allergic airways disease

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Introduction. Airway remodelling (AWR) is a key pathology of asthma for which there is no effective cure. While stem cell based-therapies have been successful in treating acute asthma, they have had limited efficacy in a chronic disease setting characterized by fibrosis.

Aims. Hence, this study determined if the presence of an anti-fibrotic (serelaxin/SRLX) would aid human bone marrow-derived mesenchymal stem cells (BMSCs) in reversing AWR in a chronic allergic airways disease (AAD) murine model, which mimics several features of human asthma.

Methods. 6-8 week-old female Balb/c mice were subjected to a 9-week model of ovalbumin (OVA)-induced chronic AAD, before sub-groups of mice (n=6/group) were either untreated (OVA alone) or treated with BMSCs alone (intranasally (i.n)-administered with 1×10^6 cells/week under isofluorane anesthetic), SRLX alone (i.n-administered daily at 0.8mg/ml) or a combination of both from weeks 9-11. Control mice (n=6) received saline instead of OVA.

Results. OVA-treated mice underwent increased airway inflammation (AI), epithelial damage, TGF- β 1 expression, myofibroblast differentiation, sub-epithelial collagen deposition (fibrosis) (all assessed by morphometry of various histological stains), total lung collagen deposition (hydroxyproline assay) and airway hyperresponsiveness (invasive plethysmography) compared to their saline-treated counterparts (all $p < 0.001$ vs saline group). The presence of SRLX significantly aided the ability of BMSCs (in combination-treated mice) to reverse OVA-induced subepithelial and total lung collagen deposition (both $p < 0.05$ vs either treatment alone). Furthermore, combination-treated mice had higher collagen-degrading matrix metalloproteinase (MMP)-9 levels (gelatin zymography) compared to OVA and OVA+SRLX-treated mice ($p < 0.01$ vs both groups).

Discussion. These findings suggest that the presence of an anti-fibrotic improves the therapeutic efficacy of BMSCs in a chronic AAD setting.

Bronchodilator responsiveness is impaired in a mouse model of COPD exacerbation

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Introduction. Influenza virus infections are a common cause of chronic obstructive pulmonary disease (COPD) exacerbations (AECOPD). These AECOPD cause an accelerated decline in lung function, hospitalisation and even death. The majority of COPD patients have small airway obstruction, which contributes to the overall breathlessness and decreased efficacy of current medication. The recent development of the lung slice technique to assess small airway contraction and relaxation *in situ* has the potential to provide insights relevant to the disease setting.

Aims. To characterise small airway reactivity in a mouse model of AECOPD.

Methods. Male Balb/C mice (6-8 weeks) were exposed to cigarette smoke (CS) generated from 9 cigarettes/day (or air for sham) for 4 days. On day 5, mice were infected with $1 \times 10^{4.5}$ PFU of the influenza A virus Mem71 (flu, H3N1). Mice were culled 7 days post-infection and 150 μ m thick lung slices prepared for phase-contrast microscopy analysis of airway contraction and relaxation. Separate lungs were harvested for Q-PCR analysis of bronchodilator receptor expression.

Results. There was no change in contraction to methacholine (MCh) or serotonin (5HT) between sham+PBS, sham+flu, CS+PBS or CS+flu treated mice. However, following submaximal pre-contraction with MCh or 5HT, bronchodilator responses to salbutamol (SALB) and isoprenaline (ISO) were impaired in CS-exposed mice compared to sham-exposed mice. Moreover, mice exposed to CS and infected with flu had a decreased maximum bronchodilator response compared to CS-exposed mice (10 μ M SALB % relaxation; sham+PBS: 31 ± 5 , sham+flu: 12 ± 5 , CS+PBS: 44 ± 8 , CS+flu: -32 ± 19 , $n=4$ /group). In addition, there was no change in the β_2 -adrenoceptor expression.

Discussion. These data indicate that bronchodilator responsiveness is impaired in a mouse model of influenza A virus-induced COPD exacerbation. Moreover, the lung slice technique is a means by which to assess novel dilators and/or mechanisms of impaired bronchodilator responsiveness.

TRPV4 mediates serotonin-induced oedema in airway but not non-airway tissues

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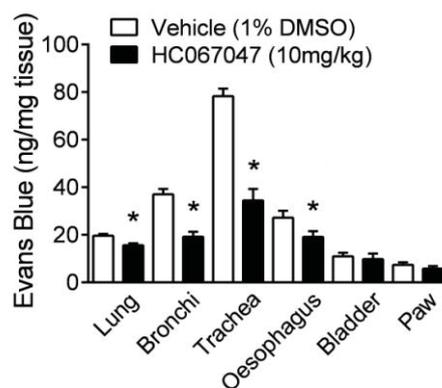
Introduction. Fluid leakage from the circulation to the surrounding tissues is tightly controlled by endothelial and epithelial barriers. Dysfunction of these barriers leads to oedema. High circulating serotonin (5-HT) levels are known to cause pulmonary oedema, a potentially fatal condition associated with respiratory disease. However, the mechanism downstream of 5-HT receptor stimulation leading to oedema is not well understood.

Aims. To investigate a role for the Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel in 5-HT-induced oedema.

Methods. C57Bl/6J mice were injected i.v. with Evans Blue dye before i.v. treatment with a selective TRPV4 agonist (GSK1016790A) or 5-HT. Tissues were collected, incubated overnight and tissue content of the dye determined by absorbance assay. Pre-treatment with a selective TRPV4 antagonist (HC067047, i.p.) was used to determine a role for TRPV4 in the oedematous tissues.

Results. GSK1016790A caused extravasation of dye in to the lung parenchyma, bronchi, trachea, oesophagus and stomach, which was inhibited by HC067047. 5-HT caused extravasation of dye in to the lung parenchyma, bronchi, trachea, oesophagus, bladder and paw. HC067047 inhibited 5-HT-induced oedema in the corresponding tissues that showed TRPV4-mediated responses (lung, bronchi, trachea and oesophagus), but not the bladder or paw (Figure).

Discussion. TRPV4 mediates 5-HT-induced oedema of the airways and oesophagus. However, both 5-HT and GSK1016790A also independently cause oedema in other tissues, implying the existence of alternative signalling mechanisms in oedema associated with activation of these receptors.



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Microenvironmental determinants of glucocorticoid responsiveness in airway smooth muscle

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Introduction. Glucocorticoids have a broad spectrum of anti-inflammatory actions which underpins their widespread use in airway inflammatory diseases. Nevertheless, there is diminished efficacy in patients with severe asthma and COPD. Efforts to understand these limitations in efficacy have focused on the impact of the biochemical microenvironment. However, the influence of the biomechanical microenvironment on glucocorticoid activity has largely been ignored.

Aims. To measure glucocorticoid responsiveness in airway smooth muscle (ASM) cultured in different settings to explore the effect of different extracellular matrices, 2D vs 3D cell culture and mechanical loading.

Methods. Human ASM cultures were starved 24h prior to seeding in various settings including: (i) (plastic) non-coated culture plates; (ii) monomeric(gelatin)/fibrillar (RTC) collagen coated culture plates; (iii) collagen gels and (iv) 3D multicellular spheroids (generated using 5×10^4 cells). Briefly, culture plates were coated with collagen prior to growing a monolayer of cells, or ASM was resuspended in a mix of DMEM and rat tail collagen and gels were cast in a 24-well plate. Lastly, cells were added to polyHEMA-coated, 96-well round-bottomed plates then subjected to centrifugation at 1000g to facilitate the generation of spheroids. Cells were incubated with 10nM Dexamethasone (Dex) either at t=0h (time of seeding) or t=24h for 4h. RNA was extracted and transcript levels were evaluated by quantitative RT-PCR.

Results. Expression of GILZ (an exemplar glucocorticoid-regulated gene) increased 10-fold on plastic and fibrillar collagen-coated plates, whereas there was an 18-fold increase on gelatin-coated plates at t=4h (n=4). However, ASM cultured in collagen gels showed minimal Dex-induced increase (2-fold, n=11). Further culture for 24h of these gels resulted in a marked (10-fold) increase in Dex-induced GILZ expression (n=11). ASM cultured as spheroids at t=4h have comparable GILZ expression level (n=4) to that of ASM cultured on plastic. Similar patterns of expression were also seen with another glucocorticoid-induced gene, MKP-1 (MAP Kinase Phosphatase-1).

Discussion. These observations suggest that the mechanical stiffness of environment surrounding ASM may play a role in determining the levels of glucocorticoid-induced genes.

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Expression and distribution of the delta opioid receptor is altered in acute colitis

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Introduction. Opioids regulate intestinal motility and secretion and are major suppressors of inflammatory signalling. Previous studies have demonstrated a role for the mu opioid receptor in controlling colitis-associated tissue damage and a role for immune-derived opioids in reducing disease severity and afferent signalling. Although recent studies highlight the role of endogenous enkephalins in inflammatory bowel disease, the relative contribution of the delta opioid receptor (DOR) to colitis development is presently unknown.

Aims. To characterize inflammation-associated changes in DOR distribution in the colon and to assess the contribution of DOR to proinflammatory signalling.

Methods. Acute colitis was induced in DOReGFP knockin mice and C57BL/6 mice (3% DSS, 5d). Disease activity and tissue damage were assessed. DOR distribution was determined by microscopy and the subcellular distribution of DOR and innervation density were analysed from captured images.

Results. DOReGFP was localized to the cell surface of myenteric neurons in control tissues. There was a significant increase in intracellular DOR and loss of cell surface-associated receptor in the acutely inflamed colon, indicative of endocytosis ($P < 0.001$, n=9 mice). Colitis was associated with an increase in the density of DOR-positive nerve fibres in the *muscularis externa* and mucosa relative to untreated controls. There was also a significant increase in the relative number of HuC/D positive neurons that expressed DOR and in the overlap between DOR and nitric oxide synthase. Treatment of mice with the DOR antagonist naltrindole (5mg/kg, i.p. daily) significantly increased relative weight loss and colon shortening (n=9 mice, $p=0.003$). No significant difference in histological damage score was demonstrated.

Discussion. We have characterised the contribution of DOR to disease severity in an acute model of colitis. The endocytosis of DOR in inflamed tissues suggests that there is a sustained elevation in the release of endogenous DOR agonists in disease. The inhibition of DOR results in augmented disease activity, but not of histological damage. Follow-up studies will examine the effects of enkephalinase inhibitors on colitic damage.

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A polar compound present in stinging nettle leaf extract inhibits the purinergic component of contractility in the rat prostate gland.

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Introduction. Benign prostatic hyperplasia (BPH), is a chronic progressive condition of the prostate and the major cause of lower urinary tract symptoms (LUTS) in males. BPH is associated with an increase in both prostate size and prostatic smooth muscle tone. The commercially available plant extract, stinging nettle (*Urtica dioica*), is increasingly used as an adjunct or alternative to conventional medications for the treatment and symptomatic relief of BPH. The root extract is commonly used to reduce prostate size; however symptom relief is greater with those medications that reduce contractility of the gland. Although not used commercially for BPH, leaf extract has been consumed in traditional medicine for the treatment of nocturia (Sezik et al, 2001).

Aims. To investigate both root and leaf stinging nettle extracts on the contractility of the rat prostate gland, to determine the pharmacological mechanism of action, and to identify the responsible bioactive.

Methods. Liquid-liquid partitioning, flash reversed-phase column chromatography and preparative HPLC were employed to separate and fractionate the stinging nettle leaf extract (500 mg/ml in 25% ethanol). Analytical HPLC, NMR, and LC-MS were used to characterise the bioactive fractions. Isolated organ bath studies were conducted to bioassay the effect of the extract and fractions on $\alpha\beta$ methylene adenosine 5'-triphosphate (ATP) (3 nmol/L – 10 μ mol/L) induced contraction of the isolated rat prostate gland.

Results. Whole leaf extract, the aqueous phase, and subsequently, four isolated fractions, attenuated $\alpha\beta$ methylene ATP induced contraction (n=6; p<0.001) of the isolated rat prostate gland.

Discussion. Attenuation of $\alpha\beta$ methylene-ATP induced contraction implies the extract acts as an antagonist at the P2X₁-purinoceptor ligand-gated ion channel. NMR and LC-MS data suggest the bioactive fractions contain phenolic compounds, such as cinnamic acids similar to those previously reported (Pinelli et al, 2008).

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Pinelli P et al (2008) J Agric Food Chem 56:9127-9132

The effectiveness of current and emerging pharmacotherapy in a new model of human prostatic smooth muscle tone

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Introduction. An increase in prostatic smooth muscle tone in the transition zone (TZ) of the human prostate gland is a major component associated in Benign Prostatic Hyperplasia (BPH), exacerbating lower urinary tract symptoms which severely affect the quality of life of men. Currently, there are many setbacks associated with standards of care in the management of BPH, including the unpredictable effectiveness of current treatment strategies.

Aims. In this study, we developed a new model of human prostatic smooth muscle tone, and tested the effectiveness of the gold standard treatment, tamsulosin (alpha-blocker), and an emerging treatment, sildenafil (phosphodiesterase 5 inhibitor) and tamsulosin/sildenafil in combination, on directly reducing human prostatic smooth muscle tone.

Methods. Resected benign TZ specimens were collected from the prostate gland of consenting patients undergoing radical prostatectomy. Subsequent recordings were made using conventional tension recording experiments.

Results. The application of tamsulosin (0.1nM) resulted in a reduction in amplitude of spontaneous contractions by 27%. The application of sildenafil (10 μ M) resulted in a reduction in frequency of spontaneous contractions by 40%, relative to control (n \geq 8, Student's paired t-test, P < 0.05). The combination of tamsulosin following sildenafil resulted in a reduction in amplitude by 87%. The combination of sildenafil following tamsulosin resulted in a reduction in amplitude and frequency by 70% and 65%, respectively (n=4, Student's paired t-test, P < 0.05).

Discussion. Tamsulosin and sildenafil reduce different aspects of prostatic smooth muscle tone in the human prostate gland. The combination of tamsulosin/sildenafil has a synergistic effect in comparison to either drug alone, suggesting that treatment strategies using combination therapies of alpha-blockers and phosphodiesterase 5 inhibitors may be more efficacious than either drug alone, in the management of BPH.

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Developing kidney damage models using induced pluripotent stem cells from patients with inherited kidney disease

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Introduction. Alport syndrome (AS) is an inherited kidney disease resulting from mutations in the *COL4A5* gene affecting kidney podocytes. *In vitro* kidney-disease modelling based on iPS cell-derived podocytes will provide a powerful tool for investigating the intracellular signalling defects underlying Alport syndrome as well as a platform for therapeutic compound screening.

Aims. To develop a modelling system using podocytes generated from human induced pluripotent stem (iPS) cells and to use that system to investigate the effects of the calcium sensing receptor on podocyte survival.

Methods. Skin biopsy-derived fibroblasts from patients with AS or kidney mesangial cells from healthy individuals (NHMC) were reprogrammed using retroviral transduction or Sendai virus and subsequently differentiated towards kidney podocytes under known culture conditions. Podocytes were incubated with Sytoblue and Sytox red (both at 1 μ M) for a death/detachment assays, or were loaded with Fura-2AM (10 μ M) for intracellular calcium measurement.

Results. iPS cell clones showing the highest efficiency of differentiation into podocytes, assessed by expression of podocin, podocalyxin and nephrin transcript and protein were used for subsequent functional studies. A 24 hour incubation with the positive allosteric modulator of the calcium sensing receptor, cinacalcet, elicited concentration-dependent death of both AS and NHMC podocytes (pIC₅₀ values were 5.7 \pm 0.2 and 5.8 \pm 0.2, respectively). In NHMC cells the pro-death/detachment effect of cinacalcet (10 μ M) was reversed by prior incubation with forskolin (10 μ M), but not by the PKC inhibitor Gö6973 (100nM) or the focal adhesion kinase inhibitor, PF562,271 (10nM). In AS cells the pro-death/detachment effect of cinacalcet (10 μ M) was blocked by PF562,271, but not by Gö6973 or forskolin. In calcium imaging assays, cinacalcet (10 μ M) was better at elevating intracellular calcium in AS than in NHMC podocytes (P<0.001, Student's paired t-test).

Discussion. Compared to NHMC podocytes, AS patient-derived podocytes show differences in CaSR signalling. These differences could be a contributing factor to the eventual end stage renal failure observed in patients with AS.

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GPCR and beta-arrestins regulating gamma-secretases in Alzheimer disease

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Alzheimer disease is a major health problem in our aging societies. Only limited treatment options are available, but current drug development focusses on two membrane bound proteases, β -secretase and γ -secretases. These enzymes cleave the Amyloid Precursor Protein and generate a spectrum of small hydrophobic peptides, called amyloid peptides or A β peptides. These peptides have a strong tendency to aggregate and cause amyloid plaques and neurotoxicity. Blocking or modulating the production of these peptides is likely to prevent or stop Alzheimer disease. However mechanistic side effects have seriously limited the effectiveness of these approaches and further basic work is needed to understand in better detail the physiological roles of these intriguing enzymes(1).

In an unbiased genome wide screen of modulators of these enzymes, an orphan G-linked protein receptor (GPR3) was identified which modulated gamma-secretase (2). We have shown that GPR3 regulation of γ -secretase activity preserves the cleavage of other substrates, i.e Notch, and therefore provides a potentially safer drug target than direct inhibition of the protease. We have also discovered that β -arrestins are involved in the regulation of the γ -secretase activity(3). We will provide the beginning of a mechanistic understanding how this pathway regulates this enzymatic activity.

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2. A. Thathiah et al., The orphan G protein-coupled receptor 3 modulates amyloid-beta peptide generation in neurons. *Science* **323**, 946 (Feb 13, 2009).
3. A. Thathiah et al., beta-arrestin 2 regulates Abeta generation and gamma-secretase activity in Alzheimer's disease. *Nat Med* **19**, 43 (Jan, 2013).

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Neuropeptide receptors as targets for relapse-prevention

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Relapse and hazardous drinking represent the most difficult clinical problems in treating patients with alcohol use disorders. Increasing our understanding of the brain circuits and chemicals that regulate alcohol intake and relapse offers the potential for more targeted therapeutic approaches to assist in relapse prevention. We have provided evidence for a role of numerous neuropeptides in cue and/or stress induced reward-seeking. This presentation will highlight recent studies on 3 neuropeptide systems, which can act independently and via circuit-level interactions to regulate relapse-like behaviour. Specifically, orexin, corticotropin releasing factor (CRF) and relaxin-3 all act, and appear to also interact, within circuits mediating cue and/or stress-induced relapse-like behaviour. For example, orexin1 receptors in the ventral tegmental area and prelimbic cortex regulate cue-induced reinstatement of alcohol-seeking in rats; relaxin-3 acts upon RXFP3 receptors in the bed nucleus of the stria terminalis to regulate stress-induced reinstatement of alcohol-seeking in rats.

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Role of metabotropic glutamate receptor 5 in neurodegenerative diseases

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Introduction: Glutamate is the major excitatory neurotransmitter in the brain and is essential for a wide range of functions including learning and memory, as well as cell development. The dysregulation of glutamatergic signaling is neurotoxic, causing cell death through the over-activation of glutamate receptors and elevated release of intracellular Ca^{2+} . This dysregulation of glutamate has been implicated in the neuronal death and pathogenesis of a number of neurodegenerative diseases including Alzheimer's disease (AD) and Huntington's disease (HD).

Aims: The aim of this study was to examine the potential role of metabotropic glutamate receptor 5 (mGluR5) as a mediator of neuronal dysfunction associated with both AD and HD and assess its potential as a therapeutic target for the treatment of these diseases.

Methods: We crossed mGluR5 knockout mice with either APP^{swe}/PS1 Δ E9 mouse model of AD or a Q111 knockin HD mice. We assessed alterations in plaque formation, A β oligomer formation, locomotor activity and learning in these mice.

Results: We find that the genetic deletion of mGluR5 reduces cognitive decline and reduces the deposition of A β oligomers, a neurotoxic protein associated with cognitive decline in AD, in the APP^{swe}/PS1 Δ E9 mouse model of AD. Additionally, we show that the genetic deletion of mGluR5 improves locomotion and motor activity, as well as reducing aggregated huntingtin pathology in the Q111 mouse model of HD.

Discussion: We find that A β activation of mGluR5 appears to initiate a positive feedback loop resulting in increased A β formation and AD pathology in APP^{swe}/PS1 Δ E9 mice via mechanism that is regulated by FMRP. In addition, a loss of mGluR5 expression improves rotarod performance and decreases the number of huntingtin intranuclear inclusions in mutant HD mice. Our data suggest that mutant huntingtin protein and mGluR5 exhibit a functional interaction that may be important for HD-mediated alterations in locomotor behaviour and the development of intranuclear inclusions. We conclude that mGluR5 represents an excellent target for the treatment of symptoms and pathology associated with both AD and HD.

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Novel approaches to the modulation of opioid receptor signaling: Implications for antinociception

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Introduction. Morphine and related opioids produce their analgesic effects by activating the opioid mu-receptor (MOPr) a member of the G protein coupled receptor (GPCR) family. MOPr couples to members of the Galphai/o family of G proteins. Signalling downstream of GPCRs, including MOPr, is terminated by regulators of G protein signalling (RGS) proteins, a family of > 20 accessory proteins that act as GTPase activating proteins or GAPs. Thus, RGS proteins are potential targets for the management of pain.

Aims. To test the hypothesis that endogenous RGS protein activity differentially regulates opioid signalling and this has consequences for opioid-mediated antinociception.

Results. Endogenous RGS proteins reduce the ability of opioid receptors to couple to the inhibition of adenylate cyclase and activation of the MAP kinase pathway in accordance with their role as GAPs, but counterintuitively, RGS proteins are required for the opioid-mediated release of intracellular calcium, suggesting an initiation of bias. In addition, RGS proteins show specificity for opioid receptors. Thus, RGS4 is selective as a GAP for G proteins associated with the opioid delta receptor (DOPr) over MOPr and the nociceptin receptor (NOPr). Conversely RGS19 is selective for MOPr associated G proteins over DOPr and NOPr. This specificity is maintained in vivo since RGS4 modulates DOPr-mediated antinociception but not MOPr-mediated antinociception. In mice expressing G proteins that are insensitive to the GAP activity of all RGS proteins (Galphao-RGSi) there is a naloxone-sensitive increase in baseline latency in heat antinociception assays compared to wild-type littermates, suggesting RGS proteins negatively modulate endogenous opioid peptide activity. Similarly in the mutant mice the potency of the MOPr agonist morphine, but not methadone, to block thermal nociception in the hot-plate test is increased; this is paralleled by an increase in the ability of morphine to inhibit GABA neurotransmission in the periaqueductal grey. In contrast, both morphine and methadone show reduced antinociceptive potency in the tail withdrawal test in the Galphao-RGSi mice compared to their wild-type littermate controls.

Discussion. RGS-modulation of opioid receptor signalling is manifested in vivo as changes in antinociception, but the effects are dependent on both the opioid used and the antinociceptive test employed. Some reasons underlying these anomalies will be discussed.

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Controlling signalling of class B G protein-coupled receptors

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Class B G protein-coupled receptors (GPCRs) respond to paracrine or endocrine peptide hormones and are involved in the physiology or pathophysiology of bone homeostasis, glucose regulation, satiety & gastro-intestinal function as well as pain transmission. As a result these receptors are targets for existing drugs that treat osteoporosis, hypercalcaemia, Paget's disease and type II diabetes and are being actively pursued as targets for migraine, depression & anxiety, irritable bowel syndrome / Crohn's disease and pancreatic diagnostics. Like most GPCRs, class B receptors are pleiotropically coupled and are subject to ligand-dependent differences in signaling and regulation, both via orthosteric peptide ligands and by allosteric, non-peptidic, small molecules. Using the glucagon-like peptide-1 and calcitonin receptors as models, we have been investigating how both orthosteric and allosteric ligands change the quality of signaling via these receptors, alone and in combination, and the mechanistic basis for these effects. This work is providing insights into how distinct regions of the extracellular surface of the receptor engage with ligands to propagate conformational changes through key polar interaction networks in the receptor core and how this can selectively modify interactions with effector proteins.

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Modulation of class B GPCR pharmacology by RAMPs: insights from adrenomedullin and amylin receptorsDebbie L Hay¹. School of Biological Sciences, The University of Auckland¹, Auckland, New Zealand.

Adrenomedullin is a cardioprotective 52 amino acid peptide and amylin is a 37 amino acid endocrine hormone, with important roles in glycaemic control and food intake. Both peptides bind to class B G protein-coupled receptors, which are typified by a substantial extracellular domain (ECD) and an extended orthosteric peptide binding site that encompasses this ECD and the transmembrane bundle of the receptor. For high affinity binding of adrenomedullin and amylin, a shared family of accessory proteins is also required. Thus, the receptors for adrenomedullin comprise the class B calcitonin-like receptor with receptor activity-modifying proteins (RAMPs), whereas RAMPs pair with the calcitonin receptor to form amylin receptors. This intriguing heteromeric mechanism for regulating GPCR function extends beyond these two receptors, with many more functional consequences of RAMPs now identified. Our current understanding of the relative contributions of the RAMP and GPCR to ligand pharmacology will be summarised. Collectively, these data will provide insight into the mechanism of RAMP-dependent receptor pharmacology, which appears to mostly be allosteric in nature. This has important implications for therapeutic targeting of RAMP-complexed GPCRs in cardio-metabolic or other disorders.

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A function for bitter taste receptors in human heartWalter G Thomas¹, Simon R Foster¹, Danielle Edwards¹, Brooke Purdue¹, and Peter Molenaar^{2,3}. School of Biomedical Sciences, University of Queensland¹, Brisbane, QLD; School of Biomedical Sciences, QUT², Brisbane, QLD; School of Medicine, University of Queensland³, Brisbane, QLD.

Introduction. The sensation of taste is mediated by G protein-coupled receptors in the tongue; whereas recent studies have shown that taste receptors are expressed in a variety of tissues, including the heart (Foster et al, 2014). Our hypothesis is that taste receptors contribute to cardiac physiology.

Aims. To present the current data relating to expression of taste receptors in the cardiovascular system and our ongoing attempts to delineate the function of these receptors *in vitro* and *ex vivo* in rat and human cardiac models.

Methods. Bitter taste ligands were tested on their cognate bitter taste receptors in mouse heart isolated from 8 week old male C57BL/6 mice, and perfused in Langendorff mode. Coronary flow, aortic pressure and left ventricular pressure were recorded during infusion of putative taste GPCR ligands identified in a compound screen. Human right atrial appendages were surgically removed from patients undergoing coronary artery bypass grafts or right atrial valve replacement at the Prince Charles Hospital. Intact right atrial trabeculae were dissected, mounted onto tissue electrode blocks and electrically paced in a 50 mL organ bath. A screen of bitter ligands with known TAS2R targets was performed and receptor-dependent ligand responses were validated in a heterologous expression system using a fluorescent imaging plate reader (FLIPR).

Results. In both mouse and human cardiac, specific bitter taste ligands were identified that exhibited concentration dependent effects on cardiac physiology (aortic pressure, systolic pressure in mice; robust cardiodepressive effects on the right atrial tissue). In addition, we confirmed that these bitter ligands activated their cognate TAS2Rs *in vitro*.

Conclusion. Our studies represent the first demonstration of profound bitter ligand-induced effects on cardiac function in rodent and human tissues, suggesting a functional role in calcium mobility within cardiac tissues, as well as potentially interfering with the diastolic phase during contraction. This project highlights a new area of cardiovascular biology and identifies new ligands and receptors that may be utilised to modulate cardiac function.

Foster SR, et al. (2014). *Pharmacology & Therapeutics* 8142:41-61.

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New GPCR targets for fibrosis: Relaxin family peptide receptor 1 (RXFP1)-angiotensin II (AT) receptor heterodimers

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Fibrosis is a hallmark of chronic organ disease and failure, for which there is currently no effective cure. Serelaxin (recombinant human gene-2 relaxin) has emerged as an effective anti-fibrotic therapy, primarily through its ability to disrupt transforming growth factor (TGF)- β 1 signal transduction and promote the activity of collagen-degrading matrix metalloproteinases (MMPs). Recent studies in TGF- β 1-stimulated human renal myofibroblasts (key matrix-producing cells) and myofibroblasts isolated from injured rat kidneys have demonstrated that serelaxin disrupts the pro-fibrotic actions of TGF- β 1 by signalling through its cognate receptor, Relaxin family peptide receptor 1 (RXFP1). This activates extracellular signal-regulated kinase phosphorylation (pERK)1/2 and a neuronal nitric oxide (NO) synthase (nNOS)-NO-cyclic guanosine monophosphate (cGMP)-dependent pathway to abrogate phosphorylation of the intracellular protein Smad2 (pSmad2). Serelaxin was additionally found to promote MMP-13, MMP-2 and MMP-9 activity through inducible NOS (iNOS)-mediated NO. These anti-fibrotic effects of serelaxin were completely blocked by the angiotensin II type 2 receptor (AT₂R) antagonist, PD123319 *in vitro* and *in vivo*, or when serelaxin was administered to AT₂R-knockout mice with experimental renal fibrosis. Subsequent bioluminescence resonance energy transfer (BRET) studies revealed that heterodimer complexes were constitutively formed between RXFP1 and AT₂Rs, and that while serelaxin did not directly bind to AT₂Rs, it did signal through RXFP1-AT₂R dimers to stimulate pERK1/2. These findings highlight a previously unrecognized interaction that may be targeted to control fibrosis progression.

Chow B et al (2014) *Kidney Int* 86:75-85.

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Applying pharmacology principles to target selection and target safety assessment

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Understanding whether the emerging safety profile for new drug candidates in drug development is driven by modulation of the pharmacological target of interest or by off-target activities at other molecular structures is a key part of early drug safety assessment. Historically drug safety regulatory guidelines have focused on Compound Safety assessments of individual chemical entities prior to initiation of clinical trials. However, early safety assessment of a future drug target arguably has a larger impact and requires a different philosophical approach. Target safety should be assessed in the exploratory phase of drug discovery using established principles of pharmacology. Combining this with an in-depth knowledge of the intended patient population to understand the impact of alterations in target expression in disease states and patient comorbidities can have a major impact on the future development path for new drug candidates.

Development of new in silico methods for assessing the pro-arrhythmic risk of drugs

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Introduction. In 2005, the American, European and Japanese drug regulatory agencies introduced guidelines for (i) preclinical and (ii) clinical assessment for the pro-arrhythmic risk of drugs. Pre-clinical studies were focused on in vitro assays for drug binding to hERG K⁺ channels and the clinical studies focused on changes in QT interval on the surface ECG, i.e. the thorough QT study. Since the introduction of these guidelines, no new drug has come on to the market that has subsequently had to be withdrawn due to pro-arrhythmia. Whilst it is widely acknowledged that the current regime is very sensitive it is also not specific and there is a widespread belief that many potentially useful drugs have not been brought to the market because they failed these tests. In late 2013, the Food and Drug Administration (FDA) issued a white paper outlining a new approach to assessing pro-arrhythmic risk of drugs. The expectation is that a comprehensive in vitro pro-arrhythmia assay (CIPA), which will involve testing drugs on multiple cardiac ion channels in in vitro assays and integrating this information using in silico modeling to predict effects on arrhythmia risk, will obviate the need for thorough QT studies. There would however still be an ECG assessment component undertaken as part of phase I safety trials.

Aims. To discuss the new CIPA initiative with an emphasis on the role of *in silico* modeling in integrating results from *in vitro* studies to predict pro-arrhythmic risk. I will also illustrate the role of modeling based on work from my laboratory.

Methods. Whole heart models based on the Cancer and Heart Soft Tissue Environment (CHASTE) framework using the O'Hara Rudy model of the human ventricular myocyte (Sadrieh et al., 2014) were modified to include molecularly accurate models of hERG drug binding. We then use partial least squares analysis of random perturbations to ion channel function to assess how altered activity of multiple channels can interact to alter the emergent ECG outputs.

Results. The effect of a simple reduction in I_{Kr} activity on the cardiac ECG (i.e. incorporation of simple IC₅₀ measurements for hERG drug block) can be significantly modified by altering other ion channel conductances. Furthermore, drugs that have the same IC₅₀ values but different kinetics of binding result in different levels of QT prolongation. Similarly, drugs with the same overall affinity but different relative affinities for open versus inactivated states have different effects on QT interval prolongation.

Discussion. Our results highlight the value of incorporating multi-channel block as well as kinetics and state-dependence of hERG drug binding in predicting the effect of drugs on ECG parameters. Multi-scale modeling also highlights that some results cannot be simply predicted from simulations in isolated cells. It remains to be seen, however, whether such multi-scale modeling efforts can improve the stratification of risk for pro-arrhythmia of drugs characterised at an *in vitro* level, as opposed to QT prolongation per se.

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Development of cardiac failure models as translational pharmacology models for diabetic cardiomyopathy and diastolic heart failure

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Heart failure is a major cause of death, for which there remains no cure, regardless of its aetiology. Traditionally, development of potential pharmacotherapies has focussed on the symptoms (perhaps rather than the underlying causes), and on systolic heart failure (i.e. where cardiac contractile function is impaired). Acute therapies have included strategies to boost contractile function (e.g. with inotropes) and/or unload the heart (e.g. with vasodilators), not all of which have been particularly exemplary for patient survival. Over the longer-term, ACE inhibitors are associated with beneficial effects on mortality and certainly delay disease progression, but there remains no pharmacotherapy for rescuing cardiac contractile function back to normal levels. Importantly, two heart failure aetiologies that are clearly distinct from traditional systolic heart failure now emerging at worrying rates likely require different therapeutic strategies to those currently available. Both diastolic heart failure (Heart Failure with Preserved Ejection Fraction, HFPEF) and diabetic cardiomyopathy are associated with poor prognosis. For example, diabetes is now recognised to not only escalate risk of heart failure, but also to increase its incidence >2.5-fold, independent of age or concomitant obesity, dyslipidaemia or coronary heart disease, and diabetic patients account for up to one third of patients in clinical trials for heart failure. Development and utilisation of appropriate pre-clinical models that closely resemble the human phenotype of these disorders is thus required. Considerations should include not just the degree of impairments in systolic and diastolic function and adverse cardiac remodelling, but whether concomitant hypertension, atherosclerosis, coronary disease, inflammation or obesity are common comorbidities. Integral considerations for ideal pre-clinical study design include the impact of sex, age, species (mice versus larger models), potential for off-target effects and timing of treatment intervention (e.g. effectiveness of pharmacotherapies commenced after the cardiac pathology is manifest). Lastly, the sensitivity and reproducibility of strategies available for measuring myocardial function will affect the clinical relevance of preclinical study design. In the light of each of these considerations, the usefulness of current preclinical models for each of diastolic heart failure and diabetic cardiomyopathy, and whether development of better cardiac failure models for translational pharmacology studies is warranted, will be discussed.

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***In silico* prediction of Ames mutagenicity based on molecular descriptors**

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Introduction. *In silico* prediction of Ames mutagenicity serves as a cheap, effective and fast way to test for potential mutagens in pharmaceutical compounds.

Aims. In this experiment, we gathered a dataset of 1000 non-confidential compounds (Hansen *et al.* 2009) which included their Ames mutagenicity test result and SMILES structure and modelled the relationship between physicochemical properties of the molecules and their genotoxic status.

Methods. The molecular descriptors of these compounds, calculated by PaDEL by analysis of the SMILES structures. The resulting physicochemical properties were mapped to the Ames results using several machine learning algorithms (k-Nearest Neighbours, C.45 Decision Tree, Multilayer Perceptron, Random Forest and Rotation Forest). Ten sets of 10-fold cross-validation were performed for each algorithm and performance was compared based on the area under receiver operating characteristic curve (ROC-AUC). The ROC curve provides an index of predictive performance based on a combination of sensitivity (positive predictive performance) and specificity (negative predictive performance).

Results. ROC-AUC values for k-Nearest Neighbours, C.45 Decision Tree, Multilayer Perceptron, Random Forest and Rotation Forest, were 0.76, 0.73, 0.75, 0.82 and 0.83, respectively. Using k-Nearest Neighbours as a baseline model, Random Forest and Rotation Forest were significantly better at predicting mutagenicity with this data.

Discussion. As Random Forest and Rotation Forest ensembles combine more than one algorithm to improve accuracy, these results point to the conclusion that combining algorithms enhances the *in silico* determination of Ames mutagenicity.

Hansen, K., *et al.* (2009). J Chem Inf Model 49(9): 2077-2081.

Augmented Caveolin 3 in cardiomyocytes limits age-related ischemic intolerance: Scaffolding hypothesis of aging

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The aged heart is more susceptible to stress and refractory to interventions that protect against ischaemic or hypoxic injury in young hearts. We specifically examined the contribution of caveolin-3 (Cav-3), an integral scaffolding protein, to cardiac ischaemic tolerance in young to aged mice. We first undertook detailed assessment of age-dependent ischaemic intolerance in male C57BL/6 mice aged 8, 16, 32 and 48 weeks of age. Animals were anaesthetised with Inactin (125 mg/g body weight, i.p.), and hearts perfused in a Langendorff mode with an intraventricular balloon for assessment of contractile function. The hearts were subjected to 20 minutes of ischaemia followed by 45 minutes of reperfusion. While recovery in hearts of males aged 16 weeks was not statistically different from hearts from 8-week old mice, hearts from mice aged 32 and 48 weeks displayed significant depression of intrinsic resistance to ischemia-reperfusion injury with greater diastolic dysfunction, contractile dysfunction and troponin release. RNA from ischaemic hearts revealed a decrease in Cav-3 at 48-weeks of age (expression relative to 8-week hearts 0.35), and proteomic analysis revealed ~50% lower Cav-3 protein at ~2 years of age and loss of caveolar morphology. Hearts from young (3 month) and aged (18 month) mice overexpressing Cav-3 (TG) were more tolerant of ischaemic insult: recovery of contractile and diastolic function was impaired in aged WT hearts while Cav -3 TG hearts were significantly protected from ischaemia-reperfusion injury. These data reveal a parallel decline in cardiac ischemic tolerance and Cav-3 expression, and indicate that augmenting Cav-3 expression can eliminate age-related ischemic intolerance. Therapies to drive Cav-3 expression in the heart may be a novel means to limit cardiac aging.

Endogenous Annexin-A1 (ANX-A1) is cardioprotective against myocardial infarction (MI) in mice *in vivo*

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Introduction: ANX-A1 is an endogenous anti-inflammatory protein that preserves left ventricular (LV) viability and function post ischaemic insult *in vitro*. Its cardioprotective actions over the longer-term *in vivo* are largely unknown.

Aim: In this study, we tested the hypothesis that ANX-A1^{-/-} mice have an exaggerated response (MI) *in vivo*.

Methods: In the first study, male ANX-A1^{-/-} and ANX-A1^{+/+} mice were anaesthetised with ketamine/xylazine/atropine (100:20:0.6mg kg⁻¹) and subjected to 1h ischaemia (left anterior descending coronary artery occlusion) followed by 24 or 48h reperfusion. In the second study, mice were subjected to permanent occlusion for 7 days. LV necrosis, inflammation and fibrosis were assessed.

Results: ANX-A1^{-/-} mice exhibit increased LV necrosis (infarct size, plasma troponin I, TnI), LV inflammation (macrophage infiltration, on CD68⁺ immunofluorescence) and LV collagen compared to ANX-A1^{+/+} mice after MI.

RESULTS mean ± SE (n)	ANX-A1 ^{+/+} sham	ANX-A1 ^{+/+} + MI	ANX-A1 ^{-/-} sham	ANX-A1 ^{-/-} +MI
24h reperfusion:				
Infarct size (%)	0.0±0.0(3)	34.8±1.7 (9)*	0.0±0.0 (3)	49.3±5.4 (8)*#
Tn I (fold WT sham)	1.0±0.3(3)	83.1±11.7 (9)*	5.5± 4.7(3)	103±9 (8)*
48h reperfusion:				
LV macrophage content (AU)	770±136(6)	2538±425 (6)*	947±87(4)	3525±505(8)*
7 days occlusion:				
Cardiac collagen content (%)	0.3,1.7(2)	19.2±1.5(7)	1.11(1)	40.5±6.9(5)*#
Heart:body weight	5.0±0.2(4)	5.9±0.2(14)	5.2±0.3(4)	7.3±0.4(11)*#
Lung:body weight	6.0±0.3(4)	7.0±0.5(14)	5.7±0.1(4)	9.3±1.5(11)

*P<0.05 vs sham; #P<0.05 ANX-A1^{-/-} +MI (two way ANOVA, Tukey's post-hoc for multiple comparisons).

Conclusion: Endogenous ANX-A1 limits LV damage *in vivo* and may represent novel therapy for MI in the clinic.

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AT₄ Receptor/ Insulin Regulated Aminopeptidase (IRAP): A target for protection in cardiovascular disease

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Introduction. The AT₄ receptor (AT₄R), now known as the enzyme insulin regulated aminopeptidase (IRAP) is upregulated in animal models of vascular injury (Moeller et al, 1999) and atherosclerosis (Vinh et al, 2008).

Aims. To investigate whether IRAP deficiency or pharmacological inhibition of IRAP would prevent Ang II-induced abdominal aortic aneurysms (AAA) and vascular injury.

Methods. Adult male IRAP deficient (IRAP^{-/-}) or WT (C57BI/6J) mice aged between 4-6 months were infused with either saline or Ang II (4 weeks; 800ng/kg/min). Other WT mice were co-treated with Ang II and either the IRAP inhibitor, HFI-419 (500ng/kg/min) or vehicle for 4 weeks. At end of the treatment, incidence of AAA, vascular reactivity, aortic hypertrophy, fibrosis and inflammation was quantified.

Results. Ang II infusion significantly increased systolic blood pressure in WT mice; IRAP deficiency or inhibition partially prevented Ang II-induced hypertension. IRAP^{-/-} or WT mice infused with HFI-419 exhibited reduced incidence of Ang II-evoked AAA, by ~65-80% (n=7-10, P<0.01). IRAP deletion or inhibition also attenuated Ang II-induced endothelial dysfunction, which was correlated with enhanced vascular phospho-eNOS and decreased superoxide production. Ang II infusion increased IRAP expression and inflammatory markers such as NFκB and MCP-1, all of which were reduced with IRAP inhibition. Furthermore, Ang II-treated IRAP^{-/-} and HFI-419-treated mice had significantly less adventitial thickening and aortic collagen deposition than their Ang II-treated controls (~50%, n=6, P<0.01).

Discussion. Pharmacological inhibition of IRAP exerts antihypertensive, vasoprotective and anti-inflammatory effects, consistent with effects in IRAP deficient mouse, thus highlighting the potential use of IRAP inhibitors as a future therapeutic treatment for cardiovascular disease.

Moeller I et al (1999) Regul Pept 83:25-30

Vinh A et al (2008) Cardiovasc. Res 77:178-187

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Biased agonism in the 4th dimension: the contribution of binding kinetics to the bias profile of antipsychotic drugs at the dopamine D₂ receptor

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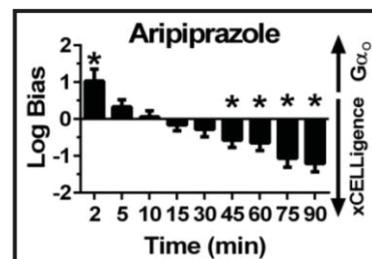
Introduction. Our initial experiments have revealed that clinically efficacious antipsychotics display a distinct profile of bias at the dopamine D₂ receptor (D₂R) compared to other dopaminergic ligands. The mechanism by which these drugs confer biased agonism is still poorly understood but often attributed to the ability of different ligands to stabilize distinct conformations of a G protein-coupled receptor (GPCR).

Aims. The aim of this study was to determine if ligand binding kinetics is of importance for biased agonism and if differential binding kinetics confer temporal changes in biased agonism.

Methods. Competition association binding experiments were performed to determine the binding kinetics of D₂R partial agonists with different profiles of biased agonism. To establish if differential binding kinetics confer temporal changes in biased agonism the bias profiles of D₂R partial agonists were established between three signalling pathways at various time points.

Results. Ligands that have a different pattern of bias from that of the endogenous agonist dopamine have substantially slower binding kinetics than ligands that have a similar bias profile to that of dopamine. Ligands with slower binding kinetics, such as aripiprazole, displayed higher potency with increasing time whereas those with faster binding kinetics became less potent. However, the relative change in potency was pathway dependent. This resulted in a significant change and even reversal of the bias profile over time.

Discussion. This study indicates that ligand binding kinetics should be considered in future studies of biased agonism and has major implications for the current interpretation of biased agonism at the D₂R and other GPCRs.



A switched on receptor – a role for adenosine A_{2B} receptor constitutive activity in cancer cell proliferation

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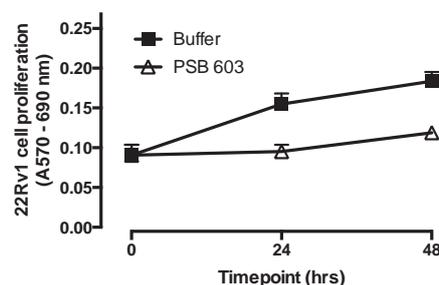
Introduction. Aberrant G protein-coupled receptor (GPCR) constitutive activity is implicated in disease pathology, including the initiation and/or progression of certain cancers. The adenosine A_{2B} receptor (A_{2B}AR) is highly upregulated in solid tumours and can promote cell proliferation. However, it is currently unknown whether A_{2B}AR constitutive activity has a significant role in cancer pathophysiology.

Aims. To characterise A_{2B}AR constitutive activity within a heterologous expression system and investigate the role of A_{2B}AR constitutive activity on prostate cancer cell proliferation.

Methods. A_{2B}AR signalling was profiled in FlpInCHO cells stably expressing the human A_{2B}AR (A_{2B}AR-FlpInCHO cells) and the 22Rv1 prostate cancer cell line using a fluorescence-based cAMP accumulation assay. Subsequent studies probed the role of A_{2B}AR constitutive activity on proliferation of 22Rv1 prostate cancer cells using a MTT cell growth assay. All assays were performed in the absence and presence of 1U/mL adenosine deaminase (ADA) to establish the influence of endogenous adenosine.

Results. In A_{2B}AR-FlpInCHO and 22Rv1 cells, the A_{2B}AR inverse agonists ZM241385 and PSB603 mediated a concentration-dependent decrease in baseline cAMP levels in the absence and presence of ADA (n=3-4). In 22Rv1 prostate cancer cells, when extracellular endogenous adenosine was removed from the system (+ 1U/mL ADA), PSB603 significantly inhibited proliferation (two-way ANOVA, P<0.05 at 24hr at 48hr timepoints, n=4).

Discussion. Evidence of A_{2B}AR constitutive activity was observed through inverse agonism of the G_s-coupled cAMP pathway in both A_{2B}AR-FlpInCHO and 22Rv1 prostate cancer cell lines. Importantly, inhibition of A_{2B}AR constitutive activity caused a significant decrease in 22Rv1 prostate cancer cell proliferation, suggesting that switching off A_{2B}AR constitutive activity with an inverse agonist may provide a valid therapeutic approach for slowing prostate tumour progression.

**The effect of ageing on isoniazid pharmacokinetics and hepatotoxicity in Fischer 344 rats**

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Background: Isoniazid is the first line treatment for tuberculosis however it has limitations of being hepatotoxic. The cause of age-related differences in isoniazid pharmacokinetics and hepatotoxicity is uncertain.

Aims: To investigate the effect of ageing on isoniazid pharmacokinetics and hepatotoxicity in male Fischer 344 rats.

Methods: Young (6±1 months) and old (24±1 months) rats were fasted for 16 hours followed by a toxic regimen of isoniazid (young n=8, old n=5) or saline vehicle (young n=7, old n=7) (4 doses/day over 2 days: 100, 75, 75, 75mg/kg ip every 3 hours). Fifteen hours later, animals were euthanased by ip injection of ketamine (75mg/kg)/xylazine (10mg/kg) and sera and livers were prepared for biochemical analysis, histology and enzyme activity.

Results: Isoniazid treatment significantly increased serum hepatotoxicity markers; aspartate transaminase, glutamate dehydrogenase and sorbitol dehydrogenase in young animals but not in old animals ($p<0.05$). A non-significant increase in necrosis was observed in young treated animals (young 5/7, old 1/5; $p<0.08$) while, steatosis was increased in old isoniazid treated animals only (old: saline 1/9, isoniazid 4/5; $p<0.05$; young: saline 0/7, isoniazid 1/7; $p>0.05$). Amongst isoniazid treated animals, concentrations of toxic intermediates acetylhydrazine and hydrazine were higher in old than young animals ($p<0.05$). N-acetyltransferase 2 activity decreased by ~40% with old age in both treatment groups ($p<0.05$). Amongst old isoniazid treated animals, the activity of amidase increased by 10% and CYP2e1 decreased by 50% compared to young. Glutathione-S-transferase did not change with age.

Conclusion: Age-related changes in isoniazid pharmacokinetics may contribute towards differential patterns of toxicity, with young rats showing a necroinflammatory pattern of injury and old rats developing steatosis.

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Bladder sensory nerve activity is enhanced by the cytotoxic drugs cyclophosphamide and ifosfamide

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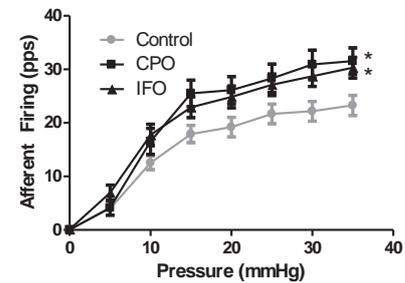
Introduction. The cytotoxic drugs cyclophosphamide (CPO) and ifosfamide (IFO) are uro-toxic and can cause ongoing bladder pain, urgency and dysuria. Both drugs have been shown to cause bladder hyperactivity in experimental models suggesting changes in sensory activity may be involved.

Aims. To determine the effect of CPO and IFO on the activity of high and low threshold bladder sensory nerves.

Methods. Male mice (12wks) were administered either CPO (100mg/kg) or IFO (200mg/kg) by i.p. injection and sacrificed for experimentation after 24hrs. Intravesical pressure and bladder afferent nerve activity were measured during bladder filling and emptying *in vitro*.

Results. As volume in the bladder increased both intravesical pressure and bladder sensory nerve activity increased. Nerve activity after treatment with CPO or IFO was enhanced throughout bladder filling. At maximum distension the total nerve activity was increased significantly from 182 ± 13 nerve pulses per second (pps) in control animals, to 230 ± 14 pps in CPO treated mice ($p < 0.05$) and 226 ± 17 pps in IFO treated mice ($p < 0.05$) ($n \geq 6$). Single nerve fibres were identified from each recording and characterised as either low threshold (activation at pressure < 15 mmHg) or high threshold fibres (activation at pressure > 15 mmHg). The activity of high threshold nerves was unchanged after treatment with CPO or IFO. Whereas CPO and IFO treatment caused enhanced activity of the low threshold nerves ($p < 0.05$) (Fig 1). Bladder compliance was not affected by CPO or IFO treatment.

Discussion. Both CPO and IFO enhanced low threshold bladder sensory nerve activity. Increased afferent sensitivity and firing may explain the pain, urgency and feelings of incomplete bladder emptying experienced by patients after treatment with CPO and IFO and provides a target for treating these adverse effects.



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Serelaxin signalling in human primary vascular cells: G-proteins and their location determines the shape of the concentration-response relationship

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Introduction. The phase III clinical trial, RELAX-AHF, demonstrated that 48 hour infusion of serelaxin, recombinant hormone H2 relaxin, improved short- and long-term clinical outcomes in patients with acute heart failure (Teerlink et al. 2013). However, the precise mechanism(s) associated with its cardio-beneficial effects in humans are poorly understood.

Aims. This study examined the effects of serelaxin in primary cell cultures of the human vasculature endogenously expressing the serelaxin receptor, RXFP1.

Methods. Radioligand binding studies were used to detect cell-surface RXFP1 expression. AlphaScreen cAMP and cGMP accumulation assays were conducted to examine serelaxin signalling.

Results. Radioligand binding showed cell surface RXFP1 expression in human umbilical vein endothelial (HUVECs) and smooth muscle cells (HUVSMCs), human umbilical artery smooth muscle cells (HUASMCs) but not in human umbilical artery endothelial cells (HUAECs). In venous cells (HUVECs, HUVSMCs), serelaxin increased cAMP and cGMP accumulation and the concentration-response curves (CRCs) were bell-shaped. However in HUASMCs, serelaxin increased cAMP and cGMP accumulation with sigmoidal CRCs. As expected, serelaxin had no effect on cAMP and cGMP signaling in HUAECs in agreement with a lack of RXFP1 expression in these cells. Pre-treatment of HUASMC (sigmoidal CRC) and HUVSMC (bell-shaped CRC) with NF449 (10 μ M, 30min, G α_s inhibitor) inhibited the initial phase of the serelaxin CRC, causing a rightward shift in the CRC and a drop in the E-max for cAMP and cGMP accumulation. Pre-treatment of HUASMC with NF023 (10 μ M, 30min, G $\alpha_{i/o}$ inhibitor) inhibited the latter part of the serelaxin CRC, causing a drop in the E-max of cAMP without changing potency. Pre-treatment of HUVSMC with NF023 or fillipin III (1 μ g/mL, 1hr) decreased E-max for cAMP and cGMP accumulation and converted a bell-shaped CRC to sigmoid.

Discussion. Serelaxin signaling is different in venous than in arterial cells and the bell-shaped CRCs that are a hallmark of serelaxin signaling *in vitro*, *in vivo* and clinically, are only observed in venous cells that involve G $\alpha_{i/o}$ associated with membrane lipid rafts.

Healthcare practitioners' perspectives on prescribing and deprescribing anticholinergic and sedative medications in older adults

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Introduction. Older adults commonly take anticholinergic and sedative medications, which have cumulative adverse effects. The Drug Burden Index (DBI) is a pharmacological risk assessment tool that measures an individual's total exposure to anticholinergic and sedative medications and is associated with functional impairments in older adults. There is a lack of understanding on the perspectives of healthcare practitioners (HCPs) about prescribing and deprescribing, anticholinergic and sedative medications for older adults and implementing the DBI in practice.

Aims. To investigate perspectives of HCPs surrounding prescribing and deprescribing of anticholinergic and sedative medications in older adults and the potential role of a DBI report in practice.

Methods. A semi-structured interview guide was developed to meet the study aims. A purposive sample of pharmacists, specialist physicians and general practitioners were recruited to participate in focus group discussions. Focus group sessions were audio recorded, transcribed verbatim and thematically analysed to derive conceptual domains using an iterative process. QSR NVivo Version 10 (QSR International Pty. Ltd. Australia) was used to assist with data management and analysis.

Results. Preliminary results indicate numerous issues surrounding the prescribing and deprescribing of anticholinergic and sedative medications during the medication review process, including perceived patient barriers, education and multi-disciplinary relationship dynamics. Feedback on the DBI report is largely positive with an overall consensus that the DBI should be considered as part of a whole patient's management plan. A sample quote follows: "So I think it (the report) should be used as a prompt to say, hey this patient is using a lot of agents can we reduce some of it" [Geriatrician].

Discussion. HCP perspectives on prescribing and deprescribing anticholinergic and sedatives in older adults are varied and may inform the implementation of the DBI into practice.

Quantification of the forgiveness of drugs to imperfect adherence

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Introduction. The sensitivity of therapeutic success to imperfect adherence is described by a property known as forgiveness [1]. When the duration of action greatly exceeds the dose interval then the drug is considered forgiving [2,3]. To date, no studies have considered variability in the pharmacokinetic-pharmacodynamic (PKPD) processes in conjunction with imperfect adherence patterns to develop a criterion to determine the forgiveness of a drug.

Aims. (1) To propose a criterion to quantify forgiveness, (2) to illustrate the criterion and (3) to apply the criterion to warfarin as a motivating example.

Methods. A criterion for relative forgiveness (RF) was developed that quantifies the probability of therapeutic success. RF is defined as the number of times more likely that a target is successfully attained under perfect adherence compared to imperfect adherence; or when comparing two drugs under a standard setting of imperfect adherence. Values of RF close to one indicate that a drug is forgiving to imperfect adherence and values close to zero indicate that the drug is particularly sensitive to imperfect adherence. Illustration and the application of the criterion (aims 2 and 3) were explored using simulation with MATLAB[®]. All simulations included 1000 individuals each with an individual adherence profile and individual set of theoretical, or warfarin PKPD parameters.

Results. For the illustrative example, the influence of only missed doses had a 2 fold greater impact on forgiveness than that of timing variability. In addition, theoretical modifications to the properties of a drug, e.g. twice half-life or potency, resulted in significant increases in therapeutic success even in the presence of imperfect adherence. The RF of warfarin was 0.78 suggesting that warfarin is a relatively forgiving drug.

Discussion. The relative forgiveness criterion has important implications for both drug development and clinical practice since the choice of drug can account for the likely influence of its forgiveness.

1 Urquhart J (1997) Clin Pharmacokinet 32:345-356

2 Urquhart J (1998) Adv Drug Deliv Rev 33:207-219

3 Osterberg L et al (2010) Clin Pharmacol Ther 88:457-459

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Targeting G protein-coupled estrogen receptor in stroke

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Introduction. Estrogen has been assumed to provide neuroprotection following stroke entirely via classical estrogen receptors. Interestingly, there is recent evidence that activation of a novel G protein-coupled estrogen receptor (GPER) with the selective ligand, G-1, can improve stroke outcome in ovariectomised mice. However, it remains to be determined if the neuroprotection provided by endogenous estrogen occurs via GPER signaling.

Aims. To test if the selective GPER antagonist, G-15, worsens stroke outcome and to examine whether tamoxifen, a clinically approved GPER agonist, provides neuroprotection post-stroke.

Methods. To address the first aim, intact female C57Bl6 mice were treated i.p. with G-15 (300µg/kg, n=7) or vehicle (dimethyl sulfoxide, n=8) 1h prior to 0.5h middle cerebral artery occlusion (MCAO). To address the second aim, ovariectomised mice were treated i.p. with tamoxifen (10µg/kg, n=8) or vehicle (dimethyl sulfoxide, n=7) 1h prior to MCAO.

Results. After 24h, intact female mice treated with G-15 showed worsened functional outcomes and increased infarct damage compared with vehicle. Furthermore, immunohistochemistry showed a significant increase in neutrophils, but not T lymphocytes in the ischemic hemisphere of G-15-treated mice. Tamoxifen-treated mice had significantly improved functional outcomes and a trend for ~60% smaller infarct volume ($P>0.05$). Immunohistochemistry revealed that tamoxifen limited the infiltration of T lymphocytes and neutrophils within the ischemic hemisphere.

Discussion. These results suggest that in females, GPER activation contributes to estrogen-mediated neuroprotection following stroke, and that tamoxifen can improve stroke outcome following surgical menopause.

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Role for endothelial cell mineralocorticoid receptors in aldosterone-induced oxidative stress and inflammation in the brain

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Introduction. Elevated aldosterone levels are an independent cardiovascular risk factor. Aldosterone is known to act on the mineralocorticoid receptor (MR) in renal tubular epithelial cells to regulate fluid and electrolyte homeostasis and blood pressure. Aldosterone can act on tissues such as the brain to cause oxidative stress and inflammation.

Aims. To test whether aldosterone causes endothelial cell MR-dependent oxidative stress in the cerebral circulation, and also whether aldosterone causes inflammation in the brain, and whether this is endothelial cell MR-dependent.

Methods. Male mice were treated with vehicle and aldosterone (0.72 mg/kg/d) by osmotic minipumps for 2 weeks. Endothelial cell MR-deficient ($MR^{flox/flox}/Tie2^{Cre/+}$) and wild-type ($MR^{flox/flox}$) mice, and C57Bl/6 mice pre-treated with spironolactone (25mg/kg per day, ip), an MR antagonist, were used to test for MR involvement.

Results. In C57Bl/6 mice, aldosterone modestly increased systolic blood pressure when compared to vehicle. Nox2-dependent increases in superoxide were ~60% greater in cerebral arteries from aldosterone- vs vehicle-treated mice (n=7-8, $P<0.05$). Pretreatment with spironolactone prevented aldosterone-induced increases in Nox2-dependent superoxide (n=5, $P>0.05$), suggesting this effect was MR-dependent. In wild-type ($MR^{flox/flox}$) mice, Nox2-dependent increases in superoxide were ~50% greater in cerebral arteries from aldosterone- vs vehicle-treated mice (n=7-8, $P<0.05$), and this effect was abolished in endothelial cell MR-deficient ($MR^{flox/flox}/Tie2^{Cre/+}$) mice (n=7-8, $P>0.05$). Aldosterone increased mRNA expression of CCL7 (by 2-fold, n=14-15, $P<0.05$), CCL8 (by 2.2-fold, n=10-11, $P=0.06$) and IL-1 β (by 1.9-fold, n=8, $P<0.05$) compared to vehicle. Both spironolactone pre-treatment and endothelial cell MR-deficiency prevented aldosterone-induced increases in CCL7 (both $P>0.05$, n=4-9) and CCL8 (both $P>0.05$, n=4-9), whereas only spironolactone prevented the increase in IL-1 β expression ($P>0.05$, n=4-5).

Discussion. Endothelial cell MR mediates aldosterone-induced increases in cerebrovascular superoxide levels and brain inflammation. Endothelial cell-specific MR antagonism may be a novel way to treat cerebrovascular disease.

CC Chemokine receptor 2 Inhibition Exacerbates Stroke Outcome in a Mouse Model of Transient Middle Cerebral Artery Occlusion

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Introduction: The recruitment of leukocytes to the injured brain via various chemokines and activation of their receptors is an important part of the inflammatory response after ischemic stroke. CC chemokine receptor 2 (CCR2) is expressed mainly on Ly6C^{hi} monocytes and plays an essential role in the extravasation and transmigration of those cells into sites of inflammation. Ly6C^{hi} monocytes enter the affected brain tissue early after cerebral ischemia and have been postulated to play a pro-inflammatory role in exacerbating the acute injury.

Aims: Our study aimed to investigate the importance of CCR2-dependent mechanisms on stroke outcome by testing the effect of a CCR2 antagonist, INCB3344, in a mouse model of transient cerebral ischemia.

Methods: Male C57Bl/6J mice (8-12 weeks) underwent middle cerebral artery occlusion for 1 h, followed by 23 h of reperfusion. Mice were treated with either vehicle or INCB3344 (10, 30 or 100 mg/kg i.p.) at 1 h before cerebral ischemia, and again at 2 h and 6 h after cerebral ischemia. At 24 h, we assessed neurological deficit, infarct volume and immune cell infiltration and expression of some common inflammatory markers in the brain.

Results: Cerebral ischemia resulted in a 4-fold increase in the total number of leukocytes, and a 6-fold increase in the CCR2⁺ monocyte subpopulation (i.e. ~4% of total leukocytes), present in the ischemic hemisphere compared with sham controls. In addition, there was no significant effect of stroke on the number of circulating leukocytes, although there was a 2.5-fold increase in circulating CCR2⁺ monocytes (~3% of total blood leukocytes). INCB3344 reduced the number of CCR2⁺ monocytes infiltrating into the brain and in the circulation after stroke in a dose-dependent manner while having no significant effect on total leukocyte numbers in either brain or blood. Importantly, mice treated with INCB3344 exhibited worsened functional outcomes and larger infarct volumes at 24 h after cerebral ischemia, compared to vehicle-treated mice. These effects of INCB3344 occurred in association with reduced expression of genes corresponding to M2 polarisation of macrophages, Ym-1 and Arg1.

Conclusion: These data suggest that circulating CCR2⁺ monocytes play a protective effect by entering the brain and limiting tissue injury and functional deficits early after cerebral ischemia-reperfusion.

Coenzyme Q₁₀ protects the heart against diabetes-induced diastolic dysfunction and structural remodelling in mice with diminished phosphoinositide 3-kinase p110 α (PI3K) signalling.

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Introduction: Diabetes induces cardiac complications including coronary heart disease, atherosclerosis and diabetic cardiomyopathy. Activation of PI3K is protective in various cardiac pathologies; its signaling promotes physiological cardiac hypertrophy and preserved contractile function. Conversely, dominant negative PI3K transgenic mice (dnPI3K) with reduced cardiac PI3K exhibit exaggerated diabetic cardiomyopathy, likely due to upregulated LV superoxide.

Aims: We hypothesised that diabetes-induced LV remodelling and dysfunction observed in dnPI3K mice is attenuated with the antioxidant coenzyme Q₁₀ (CoQ₁₀), in parallel with suppression of LV superoxide generation.

Methods: Diabetes was induced in 6-wk-old non-transgenic and dnPI3K male FVB/N mice (n=20/group) by i.p. injections of streptozotocin (STZ) for 5d. Four weeks after STZ, mice were treated for 8wks with CoQ₁₀ (10mg/kg/day, 3d/wk; LiQsorb). Cardiac function was determined via echocardiography and catheterisation in anaesthetised mice (ketamine/xylazine/atropine: 100/10/1.2 mg/kg, i.p.). At study end, cardiomyocyte hypertrophy, LV collagen deposition, superoxide (lucigenin-chemiluminescence) and lipid peroxidation (MDA) were determined.

Results: Diabetes was confirmed by hyperglycaemia (≥ 26 mM). Oxidative stress markers (LV superoxide, Nox2 expression, plasma lipid peroxidation) were increased with diabetes and further increased in dnPI3K mice; all were attenuated by CoQ₁₀ (all P<0.05). Diastolic dysfunction (prolonged deceleration time, LV-dP/dt) was also attenuated with CoQ₁₀ in diabetic and/or dnPI3K mice (all P<0.05). Cardiomyocyte size and hypertrophic gene expression were increased with diabetes and further increased in dnPI3K mice; these were similarly attenuated with CoQ₁₀ treatment (all P<0.05).

Discussion: Exogenous administration of CoQ₁₀ effectively reduced LV superoxide and lipid peroxidation, Nox2 expression and LV diastolic dysfunction. Strategies to enhance the PI3K/superoxide balance in the heart may represent a novel strategy to improve LV function and structure in diabetic patients.

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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.

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Introduction. Hyperglycaemia in diabetes leads to an increase in oxidant stress which is associated with impaired endothelial function, a likely cause of macro- and microvascular disease.

Aims. We hypothesized that DPP-4 inhibitors (linagliptin, sitagliptin, vildagliptin) and the GLP-1 receptor agonist exendin-4 would improve relaxation in mesenteric arteries from Wistar rats in the presence of high glucose.

Methods. Endothelium-dependent relaxation to acetylcholine (ACh) (0.1 nM–10 μ M) was determined in mesenteric arteries pre-contracted with phenylephrine (10–100 nM) and exposed to normal (11 mM) or high (40 mM) glucose.

Results. Incubation of mesenteric arteries in 40 mM glucose caused a significant impairment of endothelium-dependent relaxation (ACh pEC₅₀ 11 mM = 7.31 \pm 0.07 vs 40 mM = 6.32 \pm 0.2 p<0.05). In addition, 40 mM glucose caused a significant increase in superoxide levels in mesenteric arteries assayed by L-012 (11 mM, 2384 \pm 210 counts/mg vs 40 mM, 4458 \pm 420 counts/mg, n=6, p<0.0001). High superoxide levels were significantly reduced in the presence of linagliptin (1 μ M, 1101 \pm 172 counts/mg, n=7, p<0.001) and exendin-4 (1 μ M, 3064 \pm 250 counts/mg, n=7, p<0.0001), but not by sitagliptin and vildagliptin. Co-incubation with linagliptin (1 μ M) or exendin-4 (1 μ M) (ACh pEC₅₀ linagliptin = 7.25 \pm 0.06, exendin-4 = 6.80 \pm 0.05) prevented the impairment of endothelium-dependent relaxation caused by high glucose but sitagliptin and vildagliptin had no effect. The presence of the GLP-1 receptor antagonist exendin-fragment (9-39) prevented the beneficial effect of exendin-4 but did not alter the linagliptin-induced improvement of endothelium-dependent relaxation in the presence of high glucose.

Discussion. Our results indicate that the DPP-4 inhibitor linagliptin and the GLP-1 receptor agonist exendin-4 have antioxidant effects that are not shared by two other DPP-4 inhibitors sitagliptin and vildagliptin. Further, unlike exendin-4, the beneficial actions of linagliptin do not involve an action of the GLP-1 receptor. Linagliptin and exendin-4 may preserve endothelial function independently of any glucose lowering activity to improve vascular function in diabetes.

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PDE3 and PDE4 reduce the occurrence of β_2 - and β_1 -adrenoceptor mediated spontaneous contractions in isolated human right ventricular trabeculae from patients with terminal heart failure.

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Introduction. It is estimated that 30-50% of heart failure patients die of sudden cardiac death caused by a ventricular arrhythmia. Characteristic features of heart failure, namely increased sympathetic nerve- β -adrenoceptor (AR) activity and structural re-modelling of the heart, combine to initiate ventricular arrhythmias that can be fatal. There are two cardiostimulatory β AR subtypes, β_1 AR and β_2 AR. There are contrasting views concerning β_2 ARs in the context of heart failure with respect to whether they have a protective or a harmful role. Both β_2 AR and β_1 AR couple to the stimulatory Gs α -protein-cyclic AMP-protein kinase A (PKA) pathway in human heart. We therefore hypothesized that activation of both β_2 AR and β_1 ARs in human heart could cause spontaneous contractions.

Aim. To use a novel *in vitro* model of human ventricular arrhythmia to determine whether both β_2 AR and β_1 ARs cause spontaneous contractions.

Methods. Ventricular trabeculae from freshly explanted hearts of 12 patients with terminal heart failure were paced to contract at 1Hz. Spontaneous contractions induced by (-)-noradrenaline, mediated through β_1 ARs (β_2 ARs blocked with ICII 18551), and (-)-adrenaline, mediated through β_2 ARs (β_1 ARs blocked with CGP20712A), were assessed in the absence and presence of PDE3 (cilostamide 100 nM) or PDE4 (rolipram 10 μ M) inhibitors.

Results. (-)-Noradrenaline caused spontaneous contractions at concentrations of greater than 6 μ M (P < 0.05).

Inhibition of PDE3 or PDE4 lowered the threshold concentration of (-)-noradrenaline for the occurrence of spontaneous contractions to 200 nM and 2 μ M respectively (P < 0.05). Inhibition of PDE3 or PDE4 revealed the presence of spontaneous contractions to (-)-adrenaline at concentrations of greater than 6 μ M and 200 μ M respectively (P < 0.05).

Discussion. PDE3 and PDE4 reduce the occurrence of β_1 AR and β_2 AR mediated spontaneous contractions in an experimental model of human ventricular arrhythmia. Both PDE3 and PDE4 may reduce the likelihood of β_1 AR and β_2 AR mediated ventricular arrhythmias in patients with heart failure.

Phosphoinositide 3 kinase (p110 α) gene therapy attenuates diabetic cardiomyopathy in mice

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Introduction. Diabetic cardiomyopathy is a common complication of diabetes. It is characterised by left ventricular (LV) diastolic dysfunction with increased LV reactive oxygen species, fibrosis and cardiomyocyte hypertrophy.

Aims. In the present study the effect of cardiac-specific phosphoinositide 3-kinase (p110 α) adeno-associated viral gene therapy (rAAV6-caPI3K) for the treatment of diabetic cardiomyopathy was evaluated.

Methods. Diabetes was induced in male FVB/N mice by streptozotocin (55mg/kg/day i.p for 5 days) and confirmed by hyperglycaemia (>26 mM). After 8 weeks of diabetes, LV diastolic dysfunction was evident on E/A ratio (using echocardiography) compared to non-diabetic vehicle treated mice. A single tail vein injection of rAAV6-caPI3K was then administered (2×10^{11} vector genomes in 150 μ l saline) to both diabetic and non-diabetic mice. Results were compared to mice treated with null vector AAV (n=3-7)

Results. Six weeks after gene therapy, LV E/A ($\uparrow 23 \pm 15\%$) and deceleration time ($\downarrow 23 \pm 7\%$, $P < 0.05$) were improved by rAAV6-caPI3K, compared to null-vector. These improvements in diastolic function were accompanied by significant reduction in LV collagen deposition ($40 \pm 20\%$, $P < 0.05$) and connective tissue growth factor expression ($50 \pm 30\%$, $P < 0.05$). Hypertrophic gene expression (β myosin heavy chain) was also reduced by $50 \pm 25\%$, ($P < 0.05$) in rAAV6-caPI3K treated mice. Furthermore, LV Nox2 expression and LV superoxide production were reduced with gene therapy (by $50 \pm 30\%$, $P < 0.01$ and $50 \pm 35\%$, respectively)

Discussion. These results suggest that rAAV6-caPI3K gene therapy may be a promising approach for rescuing the heart from diabetic cardiomyopathy; evaluation of a higher dose or prolonged treatment period after gene therapy may further reduce diabetic cardiomyopathy.

Cardiac dysfunction and ischaemic intolerance in murine type II diabetes mellitus is reversed by sustained ligand-activated preconditioning

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Introduction. Classic preconditioning is impaired in aged and diabetic hearts; however, opioid-mediated sustained ligand-activated preconditioning (SLP) is effective in aged myocardium.

Aims. To test the efficacy of SLP in a mouse model of type II diabetes mellitus (T2DM).

Methods. T2DM was induced in young male C57/Bl6 mice by a single ip injection of streptozotocin (75 mg/kg) combined with a 60% high fat diet for 10 weeks. Placebo and SLP-treated (5 days subcutaneous 75 mg morphine pellet) mice were assessed for glucose handling and myocardial dysfunction using a glucose tolerance test (GTT) and echocardiography respectively. Hearts were then langendorff-perfused to assess contractile function, ischaemic tolerance and cell death. Post-ischaemic isolated mitochondria were subjected to Ca^{2+} -induced swelling and O_2 consumption analysis to examine mitochondrial dysfunction. Cardioprotective caveolin-3 and Akt levels were determined by western blot.

Results. T2DM induced significant weight gain (30%), impaired glucose homeostasis, *in vivo* cardiac dysfunction, *ex vivo* ischaemic intolerance, exaggerated cell death, mitochondrial dysfunction, and reduced caveolin-3 and P-Akt expression. SLP normalised blood glucose and insulin, significantly restored *in vivo* cardiac function, improved post-ischaemic functional recovery, reduced cell death and Ca^{2+} -induced mitochondrial swelling, and partially restored caveolin-3 expression in T2DM hearts. Post-ischaemic respiratory function was only sensitive to SLP in T2DM and not healthy mitochondria. SLP significantly reduced baseline P-Akt in healthy myocardium, while P-Akt in T2DM remained unchanged.

Discussion. T2DM-dependent cardiac dysfunction and ischaemic intolerance are reversed by SLP, in association with mitochondrial preservation, shifts in glucose homeostasis and protective signalling. This highlights novel SLP as a potential intervention in disease states refractory to conventional approaches.

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Protease-activated-receptor signaling in the airway: The good, the bad and the proinflammatory

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Protease-activated-receptor-2 (PAR2) is a G-protein-coupled receptor, highly expressed in airway epithelial cells, smooth muscle and infiltrating inflammatory cells. PAR₂ has a paradoxical role in inflammation, promoting both protective and pro-inflammatory events. The protective effects involve the release of prostaglandin E₂ from the epithelium, which acts as a broncho-dilator by promoting smooth muscle relaxation. The proinflammatory effects are more complex, involving the release of inflammatory cytokines, chemokines and additional PAR2 proteolytic agonists, which then recruit leukocytes to the airways, ultimately leading to robust cellular inflammation, epithelial thickening and mucus production. These PAR2-dependent events are activated in response to numerous environmental allergens, some of which contain proteases that directly act on epithelial PAR2 and others that indirectly lead to PAR2 activation via an IgE-mediated inflammatory cascade. Our recent studies have focused on allergic asthma induced by fungal spores from *Alternaria Alternata* and we have evidence that *Alternaria* filtrates activate a pro-inflammatory cascade leading to cytokine production, leukocyte infiltration, mucus production and epithelial thickening, all of which are dependent upon expression of both PAR2 and β -arrestin. Because the pro-inflammatory responses are entirely dependent upon β -arrestin signaling and not G-protein signaling, we propose that biased antagonism of PAR2 is a promising therapeutic target for treating allergic asthma.

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Opioid Bias Receptor Signaling and Regulation: β -Arrestin1/2 Selectivity.Christopher J. Evans¹, Wendy Walwyn¹, Arynah Pradhan^{1,2} Dept. Psychiatry and Biobehavioral Sciences, UCLA, CA, USA; Dept. of Psychiatry, UIC, Chicago, IL

We present evidence of distinct and separable roles of β -arrestin 1 and 2 in regulating opioid receptor signaling and ligand bias. Using mice lacking β -arrestin 1 or 2, prior studies have demonstrated that β -arrestin 2, but not 1, regulates several aspects of μ -opioid receptor signaling and ligand bias. β -arrestin 2 regulates constitutive activity of the μ -receptor, and enhances morphine but not fentanyl analgesia. The enhanced analgesic effect of morphine in β -arrestin 2 null mice can be reversed by inhibiting cJun-N-Terminal Kinase (JNK) suggesting that β -arrestin 2 normally prevents the activation of this cascade by morphine. The cellular phenotype in the β -arrestin 2 null includes dysregulation of the intracellular signaling via JNK, cJun and cSrc and functionally there is increased constitutive activity, disrupted heterologous desensitization and increased re-sensitization of receptor signaling after morphine exposure. Our recent studies have focused upon the δ -opioid receptor, which despite high sequence homology with the μ -opioid receptor in most intracellular domains, differs markedly at the C-terminus. Based on structural analysis, this C-terminal region is a major interaction domain with arrestins and other regulatory proteins. We have found that β -arrestin 1 but not 2, is able to regulate δ -agonist induced receptor export to the cell membrane via a Rho-ROCK LIMK pathway. If β -arrestin 1 is deleted, there is an enhanced and prolonged effect of SNC80 (a strong internalizing agonist at the delta receptor) on locomotion and analgesia in the naive and Complete-Fruend's Adjuvant (CFA) chronic inflammatory pain model. On the other hand, ARM390 (a weakly internalizing agonist) shows no behavioral enhancement in mice lacking β -arrestin1. In mice lacking β -arrestin 2, no behavioral phenotype for SNC80 is observed, however, ARM390 now acquires "short-term" tolerance in the CFA pain model, suggesting that β -arrestin 2 is protective of δ -receptor desensitization induced by ARM390. Further studies showed that that β -arrestin 2 promotes resensitization of δ -receptors thus showing an opposite phenotype to morphine-treated μ -receptors where β -arrestin 2 attenuates resensitization. Thus contrary to their role in promoting receptor desensitization and downregulation mechanisms for which arrestins are named, β -arrestin 2 appears protective of ARM390 mediated tolerance mechanisms of δ -receptors, indicating a new functional role for β -arrestin 2.

Developing opioid receptor signalling bias for improved pain relief

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Opioids interacting with the μ -opioid receptor (MOPr) are effective analgesics for acute and chronic pain but their utility is limited by on target adverse effects such as respiratory depression, constipation, tolerance and addiction. Recently there has been much interest in the possibility that biased signalling at the MOPr may be effective in skewing the analgesic versus side-effect profiles of MOPr agonists. Some studies suggest that MOPr agonists that show strong bias towards G-protein versus β -arrestin signalling appear to have an improved safety profile for analgesia versus respiratory depression and other adverse effects, perhaps including reduced tolerance. We have used a single cell type to examine the signalling efficacy of a series of opioids with varying efficacy for G-protein signalling versus endocytosis, including a novel peptidic agonist with extreme G-protein bias. We determined activation of G-protein signalling (GIRK activation), Ser³⁷⁵ phosphorylation (immunohistochemistry), β -arrestin-2 binding (BRET) and endocytosis (immunohistochemistry) under conditions, where possible, that association and dissociation kinetics could be determined. We and others have previously established that all regulatory steps of MOPr are reversible at the cell surface. Our major findings are that the dissociation kinetics of GIRK signalling, Ser³⁷⁵ phosphorylation, and β -arrestin-2 binding are highly correlated for agonists at each step of the regulatory process, with correlation coefficients of approximately 0.9. This demonstrates that reversal of each regulatory step is ligand-dissociation rate dependent rather than pathway (e.g. phosphatase kinetics) limited. Importantly, when we account for initial GIRK signalling efficacy, dissociation rate strongly predicts efficacy for endocytosis, with rapidly dissociating agonists displaying the greatest GIRK versus arresting bias. This suggests that development of high G-protein efficacy agonists with rapid dissociation rates can be systematically developed to limit adverse effects of opioids.

Hypothalamic neurons regulate blood glucose

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Every organism requires glucose to provide energy for cellular functions. Glucose levels fluctuate, but are generally controlled in a relatively narrow range. A number of mechanisms have been identified that regulate glucose. These include insulin, which is secreted from the pancreas, but up to 50% of glucose disposal may be mediated by non-insulin dependent mechanisms (Schwartz et al., 2013) potentially via changes in liver glucose production, or, muscle and the adipose glucose uptake. The work presented here seeks to determine the role of the brain and specifically the mechanism by which hypothalamic neurones regulate blood glucose. A range of mechanisms have been investigated to determine how a small population of neurons within the arcuate nucleus of the hypothalamus of the brain can regulate peripheral blood glucose. We have investigated the role of proopiomelanocortin (POMC) and agouti related peptide (AgRP) neurons by directly activating or inhibiting these neurons using engineered pharmacologically selective chimeric ion channels for activating and silencing neuron activity (Magnus et al., 2011). This enables us to specifically activate or inhibit particular neuronal populations, to investigate the discrete physiological response to that neuronal activity. Our findings have established that the POMC and AgRP neuronal populations that reside in the arcuate nucleus of the hypothalamus can control peripheral blood glucose levels by altering the uptake of glucose by numerous peripheral organs, which leads to changes in blood glucose levels. We are now investigating if these effects persist in obese states, where the animals become resistant to insulin in the periphery and the possible role of these neurons in diabetes.

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Auditing adulterants: toxicological assessment of herbal medicines sold in Australia during 2014

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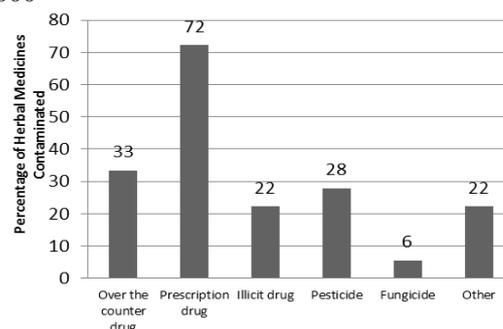
Introduction. Herbal medicine use has become increasingly popular in recent years, largely due to the belief that it is natural and therefore safe, legal and regulated. However, regulatory bodies warn that contamination and adulteration of herbal medicines poses a health risk to consumers (WHO, 2012). Despite regulations governing the contents of herbal medicines, the purity of herbal medicines in Australia is poorly understood.

Aims. The aim of this study was to qualitatively evaluate herbal medicines commercially available in Australia for the presence of undeclared and toxicologically relevant compounds.

Methods. Twenty-one psychotropic and analgesic 'over the counter' (OTC) herbal medicines were purchased at random in Australia during 2014. The contents underwent basic and ethanol extraction, and were analysed using Qualitative Time of Flight LCMS, GCMS with Nitrogen Phosphorous Detection (NPD) and LCUV. Data were compared to a library of over 9000 compounds and matches were recorded according to standard parameters.

Results. 85% (n=18) of samples contained one or more toxicologically relevant compound which was undeclared on the label. Compounds included synthetic OTC drugs, prescription drugs, illicit drugs, fungicides and pesticides.

Discussion. These findings highlight the risk that herbal medicines may pose to the Australian public, and the need for regulatory bodies to remain stringent and vigilant in pre- and post-market auditing. The levels of contamination found in this study suggests a larger and more comprehensive quantitative assessment of herbal medicines available to the Australian public is required in order to give an accurate reflection of the market.



World Health Organization (2012) Available: <http://apps.who.int/medicinedocs/en/m/abstract/Js14878e/> (Accessed 3 June, 2014)

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The Effect of Saxitoxin on the Differentiation of D3 Embryonic Stem Cells into a Neural Lineage

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Introduction. The neurotoxin saxitoxin (STX) is produced in both marine and freshwater environments. Its production by cyanobacteria in Australian freshwaters from which drinking water can be sourced makes it a potential public health concern. STX acts by blocking voltage-gated sodium channels (VGSC) preventing the inflow of sodium ions and consequently the generation of action potentials. While acute exposure to the toxin has been well researched and guidelines exist for both sources little is known about low dose extended exposure, despite this being a more likely scenario in drinking water. As VGSCs have been implicated in neurite outgrowth and development, STX could affect developing neurons.

Aims. To determine if STX at the Australian drinking water guideline level has an adverse effect on the differentiation of mouse embryonic stem (ES) cells into a neural lineage.

Methods. D3 ES cells were differentiated using retinoic acid in the presence or absence of STX (3µg/L) using a protocol previously shown to successfully induce stem cells to express neuronal morphology. Cells were assessed by scoring the development of morphological neuronal features and expression of 3 genetic markers (*oct4*, *mixL1* and *nestin*).

Results. Morphology results showed a statistically significant decrease in neuronal scores in STX treated cells (21% one way ANOVA p<0.05) and an increase in the relative expression of *Nestin* in STX treated cells.

Discussion. The increased expression of *Nestin*, an early neuronal marker expressed in neural progenitor cells, suggests that cells exposed to STX remained in an immature neuronal state which is supported by the lowered neuronal scores. Further investigation is required but these results could have implications for the safety of STX-affected drinking water.

Bain G et al (1995) Dev Biol 168(2):342-57

Okada Y et al (2004) Dev Biol 275(1):124-142.

Resveratrol and SIRT1 do not protect against paracetamol toxicity

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Introduction. Paracetamol is the most common cause of drug induced liver injury. Animal studies have shown that calorie restriction (CR) and CR-mimetic resveratrol protect against paracetamol toxicity and activate the SIRT1 pathway. It was hypothesized that protection against paracetamol toxicity may be through this pathway.

Aims. To assess the role of resveratrol and SIRT1 in protection against paracetamol induced liver toxicity.

Methods. Four experiments were carried out to address this aim. Briefly; (1) primary hepatocytes were isolated from C57BL/6 mice and treated with paracetamol (20mM) +/- NAC (20mM), resveratrol (1-100µM) or vehicle (0.5% ethanol) and hepatocyte survival was measured with the MTT assay after 24 hours; (2) C56BL/6 mice were given a dose of paracetamol (700mg/kg) or saline and concurrently treated with either resveratrol (30mg/kg), NAC (1200mg/kg), or vehicle (corn oil 2ml/kg). After 6 hours blood was collected for liver function tests (LFTs) and the liver perfused and collected; (3) Male C57BL/6 mice were fed a control diet or resveratrol diet (100mg/kg/day) for 6 months then given a dose of paracetamol (300mg/kg) or saline. After 6 hours blood and liver were collected as above; (4) SIRT1 knock-out (KO) and over-expressing (OE) C57BL/6 mice and wild-type (WT) controls, were given a dose of paracetamol (300mg/kg) or saline. After 6 hours blood and liver were collected as above, SIRT1 mRNA expression was measured with RT qPCR. (Anaesthetic: ip Ketamine (75mg/kg) & Xylazine (10mg/kg)).

Results. Paracetamol toxicity was successfully induced in all experiments, and hepatotoxicity was able to be prevented with concurrent treatment of NAC. However, neither acute or chronic resveratrol treatment protected against paracetamol toxicity in primary hepatocytes or mice. Furthermore, SIRT1 knockout and over-expressor mice did not have altered paracetamol toxicity compared to wild-type controls.

Discussion. These results imply that resveratrol does not protect against paracetamol toxicity and that SIRT1 does not play a role in paracetamol toxicity in mice.

Muscarinic acetylcholine M₄ receptor regulation of psychosis-like behaviours induced by a dopamine D₁ receptor-selective agonist in mice.

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Introduction. Muscarinic acetylcholine M₄ receptors (mAChRs) are found most abundantly in the striatum, where they are co-localised with dopamine D₁ receptors on GABAergic output neurons. M₄ mAChRs have been shown to functionally antagonise D₁ receptor-mediated activities in M₄ mAChR knockout studies. However, the modulation of specific dopamine D₁ receptor-mediated functions by selective activation of M₄ mAChRs has yet to be investigated.

Aims. To investigate the ability of M₄ mAChR activation to regulate dopamine D₁ receptor agonist-specific effects in vivo using the functionally selective muscarinic M₄ positive allosteric modulator, LY2033298, and dopamine D₁ receptor-selective agonist, R(+)-6-Br-APB.

Methods. Male C56BL/6 J mice (9 weeks old) were tested in two mouse models of aspects of psychosis-like behaviour that involve the striatum – prepulse inhibition (PPI) and locomotor activity (LMA).

Results. R(+)-6-Br-APB 0.3 and 1 mg/kg significantly disrupted PPI (n=11; *P*<0.01) and significantly increased LMA (n=8; *P*<0.001) compared to vehicle, respectively. LY2033298 10 mg/kg alone treatment failed to reverse the PPI deficits induced by R(+)-6-Br-APB. In contrast, the combined treatment of LY2033298 10 mg/kg and an acetylcholinesterase inhibitor, donepezil 1 mg/kg, reversed R(+)-6-Br-APB-induced disruption of PPI. Similarly, LY2033298 10 mg/kg alone treatment did not reverse the hyperactivity induced by R(+)-6-Br-APB, but the combined treatment of LY2033298 10 mg/kg and donepezil 0.6 mg/kg decreased R(+)-6-Br-APB-induced increase in LMA.

Discussion. These results demonstrate that selective M₄ mAChR activation can indeed regulate D₁ receptor-mediated behaviours, as well as add to the increasing evidence of M₄ receptors as a novel target for the treatment of psychosis.

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PITX3-eGFP dopaminergic neurons derived from human pluripotent stem cells: a versatile system for neurodegenerative modelling

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Introduction. The degeneration of dopaminergic neurons of the substantia nigra is a key pathological feature of Parkinson's disease. Although this degeneration is clearly evident *in vivo* it has been difficult to directly interrogate the underlying mechanisms due to the lack of an appropriate *in vitro* model of dopaminergic neurodegeneration. Stem cell technologies, in particular the recent development of robust and efficient methods of dopaminergic neuron differentiation, now offer scope to develop pathophysiologically relevant *in vitro* models of degeneration.

Aims. To develop a neurodegeneration modelling system using human embryonic stem cells expressing eGFP under the control of PITX3.

Methods. Pluripotent stem cells were cultivated and differentiated (Kriks *et al.*, 2011). Images of fluorescent neurons were captured with a Nikon A1R confocal microscope prior to, and 24 hr after incubation with TNF α (2 or 20ng/ml). In other experiments cultures were incubated with TNF α (20ng/ml, 3hr) and either loaded with FURA2-AM (10 μ M) prior to calcium imaging, or were fixed prior to immunolabeling.

Results. After differentiation, *PITX3^{eGFP/w}* neurons showed a transcript and immunocytochemical profile consistent with *bona fide* midbrain dopaminergic neurons. By 70 days in culture, neurons exhibited spontaneous Ca²⁺ transients and respond to multiple neurotransmitters. MPP⁺ (5 μ M) and tumor necrosis factor α (TNF α , 20 ng ml⁻¹), but not prostaglandin E₂ (300 nM) caused widespread degeneration of the *PITX3^{eGFP/w}* neuron population over a 24 hr period. The TNF α effect appeared independent of caspase activation but was inhibited by prior incubation with a negative allosteric modulator of the calcium sensing receptor, NPS2143 (10 μ M). Incubation with TNF α (3hr, 20 ng ml⁻¹) inhibited elevations of intracellular calcium induced by calcium, but elevated phosphoERK.

Discussion. This study shows that human pluripotent stem cell-derived *PITX3^{eGFP/w}* neurons represent an ideal population for the investigation of mechanisms underlying neurodegeneration, these cells may prove to be an important addition to the Parkinson's disease model toolbox.

Kriks S, *et al.* (2011). Dopamine neurons derived from human ES cells. *Nature* 480:547-551.

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Functional expression of TRPV4 by satellite glial cells of mouse dorsal root ganglia

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Introduction. Satellite glial cells (SGC) surround the dorsal root ganglia (DRG) sensory neurons, where they sense the extracellular environment as well as regulate neuronal activity. It is known that the Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel is expressed in nociceptor neurons and contributes to inflammatory pain, but expression and function of TRPV4 in SGCs has not been investigated.

Aims. To investigate the expression and function of TRPV4 in satellite glial cells.

Methods. Mouse DRGs were dispersed and co-cultures of neurons and SGCs were prepared. [Ca²⁺]_i was measured by ratiometric imaging of Fura2-AM after challenge with a TRPV4-selective agonist (GSK1016790A) in cells from wild type or *trpv4*^{-/-} mice and in the presence of the TRPV4 antagonist HC067047. Expression of TRPV4 was investigated by RT-PCR and immunofluorescence in tissue slices.

Results. We have optimized a tissue dissociation technique for enhancing yield of SGCs attached to neurons in acutely dissociated DRG cultures, confirmed by Kir4.1 immunoreactivity. This was also supported by TRPV4 and Kir4.1 immunoreactivity in DRG slices. As expected, TRPV4 expression was also localised to a subset of NeuN-positive sensory neurons. The TRPV4-selective agonist GSK1016790A induced concentration-dependent [Ca²⁺]_i responses in acutely dissociated cells (EC₅₀ = 200 nM) and enriched SGC cultures (EC₅₀ = 130 nM), which were abolished by the TRPV4 antagonist HC067047 (10 μ M) and in TRPV4 ^{-/-} mice. In acutely dissociated DRG culture, 22% of the SGCs responded to GSK (136 of 620 cells, n=6). RT-PCR of enriched SGC cultures confirmed TRPV4 expression (n=3).

Discussion. We have demonstrated for the first time the functional expression of TRPV4 by SGCs. TRPV4 is a mediator of inflammatory pain and its expression has been confirmed in nociceptive neurons and central glial cells such as astrocytes (Benfenati *et al.*, 2007). Future studies will determine if TRPV4 expression in SGCs play a role in pain transmission via the release of signalling molecules or "gliotransmitters" such as glutamate, ATP or inflammatory cytokines, which in turn can alter the activation threshold of nociceptor neurons.

Benfenati V. *et al.*, (2007) *Neuroscience* 148(4): 876-92.

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Exploring the synthetic cannabinome: actions of PB-22 and 5F-PB-22

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Introduction: Morbidity due to the ingestion of designer synthetic cannabinoids (SCs) continues to climb. Recent deaths have focused attention on PB-22 (8-quinolinyl 1-pentyl-1H-indole-3-carboxylate), its 5-fluoropentyl analog 5F-PB-22 as well as AB-PINACA (N-[(2S)-1-Amino-3-methyl-1-oxo-2-butanyl]-1-pentyl-1H-indazole-3-carboxamide) and its fluorobenzyl analog AB-FUBINACA, both constituents of the SC preparation *Marley*. The *in vitro* activity of these drugs is unknown.

Aims: Our aim was to determine the activity of PB-22 and 5F-PB-22 and *Marley* constituents at human CB1 and CB2 receptors and the cannabinoid-sensitive ion channel TRPA1.

Methods: Changes in membrane potential of AtT-20 cells stably expressing human CB1 and CB2 receptors were measured using a fluorescent dye in a Flexstation 3. Intracellular calcium elevations due to TRPA1 activation were measured in HEK293 cells expressing human TRPA1 using a calcium sensitive dye and a Flexstation.

Results: A maximal concentration of WIN-55212-2, a non-selective CB agonist (CB1: pEC_{50} 7.5±0.1 CB2: pEC_{50} 7.1±0.1), was used to normalize the data for comparison. PB-22 and 5F-PB-22 were both high efficacy agonists at CB1 (pEC_{50} 8.3 ± 0.06, 8.6±0.15 respectively) and CB2 (pEC_{50} 7.4±0.1, 8.0±0.1 respectively). Preliminary experiments indicated that the *Marley* constituents were also very potent and efficacious agonists at CB1 receptors. PB-22 and 5F-PB-22 (30µM) activated TRPA1 to 30±5 % of the maximal response of the prototypic TRPA1 agonist cinnamaldehyde.

Discussion: PB-22 and 5F-PB-22 are both effective agonists at CB1 and CB2, with greater efficacy than the major psychoactive ingredient of cannabis, Δ^9 -tetrahydrocannabinol. They have modest activity at TRPA1. This contrasts with the high TRPA1 activity of other SCs such as XLR-11, suggesting that SCs may not all produce toxicity in the same way. Continuing to build an *in vitro* pharmacological profile for SCs and their metabolites is an important step in elucidating their *in vivo* toxicity.

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Ghrelin receptor agonists to limit tissue loss after spinal cord injury.

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Introduction. Tissue loss after a spinal cord injury (SCI) occurs in two main phases; an initial loss at the time of injury, which is largely necrotic, followed by a prolonged period of secondary loss which is largely apoptotic. This project investigated whether inhibiting apoptosis might reduce the extent of tissue loss. Ghrelin, a peptide produced by the stomach, is known to have anti-apoptotic and anti-inflammatory properties that are mediated via the ghrelin receptor. It has also been shown to reduce tissue loss after SCI in animal models. Ghrelin's clinical application is limited by a short half life and inability to cross the blood-brain barrier. Small non-peptide ghrelin receptor agonists (such as Capromorelin) might be more suitable.

Aims. 1. To assess the neuroprotective effects of Capromorelin after SCI. 2. To determine the time course of blood-spinal cord barrier disruption after SCI to different size permeability tracers.

Methods. Anaesthetised Sprague Dawley rats (200-400g) were subjected to a moderate mid thoracic spinal contusion injury. One group of animals were then administered Capromorelin (50 ug/kg) via a mini osmotic pump over a 24 hour period and another group was administered sterile saline. At 24 hours post-injury, spinal cords were collected, serial sectioned (5µm) and stained with antibodies to neurons and caspase 3. A separate group of animals were injected with the permeability tracers HRP (44kDa) and rhodamine-dextran (10kDa) at 2h or 24h post-SCI.

Results. 1. At 2h post-SCI, both the 10kDa and 44kDa tracers showed visible extravasation in and around the injury site. At 24h post-injury, only the smaller (10kDa) tracer showed extravasation. 2. Neuronal counts revealed extensive loss at the centre of the injury site, but less loss with increasing distance from the injury centre. In the Capromorelin treated animals, significantly more neurons were present in the penumbra regions adjacent to the injury site (both rostral and caudal) compared to the saline treated animals.

Conclusions. 1. Blood-spinal cord barrier function for large compounds is restored between 2 and 24 hours post-SCI, but remains disrupted for smaller compounds. Thus circulating Capromorelin (505 Da) would have direct access to the injury site over the 24 hour period. 2. Capromorelin administration reduces the extent of neuronal loss after a SCI.

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Structural basis for activation and allosteric modulation of a muscarinic acetylcholine receptor

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Introduction. Muscarinic receptors are among the most extensively studied models of GPCR allosteric modulation. I will present recently solved crystal structures of the M₂ muscarinic receptor bound to an allosteric modulator.

Aims. The principle goals of this work were to study the molecular basis of muscarinic receptor activation and allosteric modulator binding using structural biology and pharmacology.

Methods. These structures were determined by lipidic cubic phase X-ray crystallography, and complemented by collaborative projects using mutagenesis, radioligand binding assays, chemical synthesis, and protein engineering.

Results. Active-state muscarinic receptor binding nanobodies were developed and used to enable structure determination.

Discussion. As one of the pre-eminent model systems for studying GPCR allostery, muscarinic receptors have long been the subject of extensive pharmacological investigation. Until recently, however, muscarinic receptors and other GPCRs have been poor candidates for crystallography and other structural biology techniques due to their biochemical intractability and conformational flexibility. Recent advances in GPCR structural biology have allowed the first structures of muscarinic receptors to be determined, revealing the molecular details of ligand recognition and receptor activation. These structures include the M₂ muscarinic receptor in both inactive and active states, as well as a complex with a positive allosteric modulator. Taken together, these structures offer insights into the molecular mechanisms of ligand recognition, receptor activation, and allosteric modulation, with implications for not only muscarinic receptor biology, but also for GPCR function in general.

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Exploring Peptide Agonist-Specific Activity at 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, and is a key therapeutic target for type II diabetes mellitus. The GLP-1R can be activated by both endogenous (GLP-1(1-36)NH₂, GLP-1(1-37), GLP-1(7-36)NH₂, GLP-1(7-37), oxyntomodulin) and exogenous (exendin-4) peptide agonists, each of which display a unique functional profile.

Aims. To elucidate GLP-1R residues that are important in directing peptide-specific activity and to determine whether any differences observed arise from peptides forming distinct contacts with receptor residues.

Methods. Alanine mutagenesis of the extracellular loops of the GLP-1R was performed and mutants characterized pharmacologically with all peptide agonists. Molecular models generated with this data in combination with current literature enabled prediction of putative agonist peptide/receptor interaction sites, which were then used as sites to introduce unnatural amino acid residues (*p*-benzoyl-Phe, BzF). Functional BzF-labeled GLP-1Rs have subsequently been used in targeted UV photocrosslinking reactions to begin mapping of direct sites of interaction between agonists and receptor.

Results. Mutagenesis data demonstrated that distinct residues of the extracellular loops of the GLP-1R are involved in peptide-mediated receptor activity, in a pathway and peptide agonist specific manner. BzF has been incorporated into several sites of the receptor and expression and functionality determined. 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 could be exploited in the design and application of superior therapeutics targeted to this receptor system.

Allosteric activation of the M₁ muscarinic receptor rescues the cognitive deficit in prion neurodegenerative disease in mice

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Neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's, and the less-common prion diseases, are characterized by progressive neuronal atrophy and cognitive dysfunction. Muscarinic acetylcholine receptors (mAChRs) regulate an array of CNS processes, including cognitive, behavioural and motor functions. The M₁ mAChR subtype is widely expressed post-synaptically in the cortex and the hippocampus (areas known to be important in learning and memory) and here we show that M₁ deficient mice have a deficit in hippocampal-dependent learning and memory. In this study, we have used a well-established mouse model of neurodegeneration to further explore the therapeutic potential of M₁ mAChR ligands in restoring cholinergic function and cognition in AD.

Prion-infected tg37 mice develop early pathological changes at 7 weeks post infection (wpi) with Rocky Mountain Laboratory (RML) scrapie prion. At 9 wpi, mice display a decline in burrowing behaviour and an abrupt reduction in synaptic proteins, rapidly followed by neurodegeneration, with 50% loss of hippocampal pyramidal neurons by 10 wpi (Moreno *et al.*, 2012).

Choline acetyltransferase levels in the hippocampus of prion-infected mice are reduced from 9 wpi, indicating degeneration of cholinergic neurons. However, M₁ mAChR expression and G-protein coupling at 9- and 10 wpi is maintained.

Prion-diseased mice display reduced fear conditioning responses at 9 wpi. This impairment in learning and memory is rescued by xanomeline, an M₁ and M₄ *orthosteric* agonist, and also by BQCA, an M₁-specific *allosteric* agonist. Furthermore, we show that xanomeline modulates postsynaptic activity and AMPA receptor phosphorylation in the hippocampus of prion-diseased mice.

In conclusion, prion-infected mice undergo cholinergic degeneration in the hippocampus, which is accompanied by a significant reduction in fear learning and memory. We show that targeting muscarinic receptor activity, with both orthosteric and allosteric ligands, can rescue the cognitive deficit in prion-diseased mice.

Development of novel tools to target drugs to internalised receptors

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Introduction. Endocytosis of ligand-stimulated G protein-coupled receptors (GPCRs) is an important regulatory process that enables sustained receptor signalling, or termination of signalling *via* lysosomal targeting for degradation. The Neurokinin 1 Receptor (NK₁R) is a mediator of pain and inflammation that internalises to endosomes following stimulation with ligands such as the painful, pro-inflammatory neuropeptide Substance P. Recent evidence suggests that endosomal pools of NK₁R form specific signalling complexes to initiate distinct signalling events and these are consistent with pain transmission. The rational targeting of antagonists to endosomes is a novel approach being investigated for the potential to manipulate intracellular NK₁R signalling.

Aims. To evaluate the potential for cholestanol-drug conjugation to promote endosomal drug targeting and provide new tools for probing endosome-specific receptor signalling.

Methods. NK₁R-selective peptide agonists and antagonists were conjugated to cholestanol (a sterol group for membrane anchoring) *via* a polyethylene glycol (PEG) chain to impart flexibility. Cyanin 5 fluorophore was used to track the distribution of membrane-anchored drugs by live cell confocal imaging. To determine how NK₁R responds to endosomal-targeted peptide agonists and antagonists, NK₁R trafficking was assessed using Bioluminescence Resonance Energy Transfer (BRET) relative to resident proteins of the endocytic pathway. NK₁R signalling was assessed using FRET-based compartmentalised ERK activity biosensors.

Results. Cholestanol-conjugated drugs are rapidly targeted to endosomes and remained in these regions for extended time periods (>8hrs). Membrane-anchored antagonists and agonists altered the magnitude and duration of endosomal signalling when compared to soluble or "free" drugs, but had minimal effect on trafficking and β -arrestin recruitment.

Discussion. Drug lipidation is a valuable modification for targeting drugs to specific intracellular locations and selectively (spatially and temporally) targeting NK₁R signalling events of pathophysiological importance.

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GPR37L1: an orphan G protein-coupled receptor with constitutive activity modulated by novel metalloprotease-mediated N-terminal cleavage

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Introduction. GPR37L1 is an orphan G protein-coupled receptor (GPCR) with a putative role in blood pressure regulation, and as such, offers hope as an unexplored therapeutic target in the treatment of cardiovascular disease. Despite this potential, GPR37L1's pharmacology remains poorly characterised.

Aims. To identify the cognate signalling pathways of GPR37L1 in the absence of a ligand and to investigate the consequences of observed metalloprotease-mediated truncation of the GPR37L1 N-terminus.

Methods. A mutant *S. cerevisiae* β -galactosidase assay was used to assess constitutive GPR37L1 G protein-coupling. Results were verified *in vitro* using a cAMP-activated luciferase reporter assay. Western blot analysis of lysates of HEK293 cells stably expressing GPR37L1-eYFP or an N-terminally truncated mutant, Δ 122-GPR37L1-eYFP, were used to profile receptor expression. *In vitro* inhibitor experiments were conducted as per our established luciferase reporter assay, with HEK293 cells cultured in the presence of a panel of metalloprotease inhibitors.

Results. We report the first evidence that GPR37L1 couples to $G\alpha_s$ and does so constitutively. Furthermore, we show that GPR37L1 is subject to N-terminal proteolysis in a manner that abrogates the receptor's constitutive signalling. Conversely, we demonstrate that inhibition of metalloproteases, specifically those of the A Disintegrin And Metalloprotease (ADAM) family, enhances GPR37L1 constitutive signalling *in vitro*, suggesting the receptor is a novel example of a GPCR in which signalling is regulated by metalloprotease activity.

Discussion. Despite the difficulties inherent in studying a receptor with no confirmed ligand, our laboratory's novel *in vitro* approach has generated considerable understanding of this orphan GPCR's biology.

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Identification of the mechanisms which govern allostery at the dopamine D₂ receptor

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Introduction: SB269652 has been identified as the first drug-like allosteric modulator of the dopamine D₂ receptor (D₂R), but has structural features associated with orthosteric D₂R antagonists. We employed ligand fragmentation studies and a novel functional complementation assay to demonstrate that SB269652 has a 'bitopic' (simultaneous allosteric/orthosteric) mode of interaction with D₂R, and binds to one protomer of a D₂R dimer to modulate the action of dopamine at the other (Lane, et al. 2014)

Aims: To validate the proposed bitopic mode of interaction of SB269652 at D₂R, and probe both the key residues of D₂R and structural motifs of SB269652 that confer its novel allosteric action.

Method: Structural features of SB269652 and key ligand receptor interactions were identified using molecular modeling, mutational impairment and structural derivatives. Residues of interest were mutated to alanine and mutant receptors were stably expressed in FlpIN CHO cells. Radioligand binding and ERK1/2 phosphorylation assays were used to investigate the influence of chemical modifications of the ligand and receptor mutations on ligand affinity and cooperativity.

Results: We identified several residues proposed to sit within a putative allosteric pocket at the extracellular end of transmembrane domains 2 & 7, with SB269652 extending into this region from the orthosteric pocket. Mutation of Glu95 to alanine caused a significant (nine-fold) ($pK_B = 5.14 \pm 0.28$; $n = 3$, $P < 0.05$) decrease in affinity and negative cooperativity (five-fold) of SB269652 ($\log \alpha\beta = -0.32 \pm 0.14$; $n = 3$, $P < 0.05$). Homology modeling predicted that Glu95 forms a hydrogen bond with the nitrogen from the indole heterocycle. Methylation of this nitrogen generated an orthosteric antagonist (MIPS1500) ($pK_B = 7.28 \pm 0.09$, Schild slope = 0.88 ± 0.12).

Discussion: Structural insights provided by this work provide validation of a bitopic mode of interaction for SB269652. As such they will inform rational drug design, leading to development of improved drugs at this therapeutically important receptor.

Lane JR et al (2014) Nat. Chem. Biol. 10(9): 745-752

Structural basis for G protein-mediated high-affinity agonist binding to GPCRs

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Introduction. G proteins allosterically enhance agonist affinity for many G protein-coupled receptors (GPCRs).

However, the mechanism by which G proteins allosterically regulate orthosteric ligand binding remains unclear.

Aims. We sought to provide a structural explanation for the influence of G proteins on the orthosteric binding site of GPCRs. Based on recent crystal structures, we hypothesized that transition to an active receptor conformation leads to closing of the orthosteric binding site around its ligand, thus slowing dissociation and enhancing affinity.

Methods. Using purified receptors reconstituted into recombinant high-density lipoprotein (rHDL) particles, we performed equilibrium and kinetic radioligand binding assays to characterize the effects of G protein and G protein-mimetic nanobodies on the ligand-binding properties of multiple GPCRs.

Results. When β_2 -adrenergic receptor (β_2 AR) was bound to nucleotide-free Gs heterotrimer, we observed a B_{max} decrease and a slowed association of the antagonist [³H]DHAP; both effects were reversible by GDP or GTP γ S. A Gs-mimetic nanobody (Nb80) slowed both [³H]DHAP association and dissociation. However, this effect was not antagonist-specific, as Nb80 also inhibited association of the agonists [³H]formoterol and [³H]4-methoxyfenoterol. Mutation of Tyr308 to alanine was sufficient to abolish the effect of Nb80 on [³H]DHAP association without influencing [³H]DHAP affinity. The M2 muscarinic acetylcholine receptor (M2R) and mu opioid receptor (MOPr) behaved similarly to β_2 AR when bound to nucleotide-free G protein.

Discussion. Together, these data suggest that transit into or out of the orthosteric site in the active β_2 AR conformation (bound either to Gs or Nb80) is restricted due to closure of the orthosteric site, and that Tyr308 is an important component of the "lid" over the orthosteric site in β_2 AR. The behavior of M2R and MOPr suggests that G protein-mediated closing of the orthosteric site may extend to GPCRs beyond β_2 AR and the amine receptor family.

Allosteric modulation of the mu opioid receptor

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Introduction. The mu opioid receptor (MOPr), a G protein-coupled receptor (GPCR), is one of the most highly targeted GPCRs in the clinic and is the site of action of analgesics such as morphine and oxycodone. Currently, all clinically used MOPr ligands target the orthosteric site, and thereby compete with the endogenous opioid peptides for binding. Our lab is currently pursuing the idea that allosteric modulation of MOPr may represent a novel mechanism for pain management. We have discovered the first positive allosteric modulators (PAMs) of MOPr, with BMS-986122 representing our lead compound (Ref 1). BMS-986122 enhances the affinity and efficacy of many MOPr agonists while having no effect on antagonist binding

Aims. To determine the probe dependence of BMS-986122; that is the variation in allosteric activity due to the nature of distinct orthosteric ligands

Methods. Utilizing membranes of C6 glioma cells expressing the rat MOPr, we performed competition binding at the orthosteric site and measured G protein activation using the GTP γ ³⁵S assay.

Results. BMS-986122 displayed marked probe dependence that were contingent on the efficacy of the orthosteric ligand. Thus, BMS-986122 enhanced the affinity and potency of full agonists including endogenous peptides and methadone while having no effect on maximal activity. For partial agonists like morphine and fentanyl, BMS-986122 did not alter ligand affinity, but caused an increase in the maximal activation of G protein. In addition, we studied the relationship between the positive allosteric modulatory effects of BMS-986122 and the effects of Na⁺ ions, an endogenous negative allosteric modulator of MOPr. We found a strong negative correlation between the effect of Na⁺ on ligand affinity to bind MOPr and the effect of BMS-986122. We also determined that the presence of BMS-986122 decreased the ability of Na⁺ to alter receptor states in an allosteric fashion.

Discussion. BMS-986122 acts as a PAM by allosterically disrupting the ability of Na⁺ to stabilize the inactive state of MOPr. This may represent a common mechanism for allostery at Class A GPCRs that are sensitive to Na⁺.

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Heterotrimeric G proteins and their allosteric interactions with GPCRs: Activation and regulation

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Recent advances in the structural biology of G protein-coupled receptors (GPCRs) have revealed many aspects of ligand-receptor specificity. The crystal structure of the β_2 -adrenergic receptor (β_2 AR) in a complex with the stimulatory G protein, Gs, has also provided insight into the mechanism of GPCR-stimulated GDP release on G α , the principle step that precedes GTP binding and G protein activation. These and more recent structural analyses of GPCRs in their activated states also reveal a possible mechanism through which G proteins in turn allosterically modulate hormone binding. The use of conformation-selective camelid antibodies (nanobodies) for high resolution structural studies, especially nanobodies raised against agonist-bound GPCRs, have been particularly interesting. The primary binding site for these antibodies target the same intracellular compartment that the G protein C-terminus occupies. In addition these antibodies display preferential affinity for agonist-bound GPCRs and likewise support high affinity agonist binding. These data suggest that occupation of the intracellular face of the receptor in turn stabilizes a conformation change on the extracellular face of the receptor. One of these changes is the formation of a 'cap' located just above the hormone binding site that effectively closes access to and from the binding site. G protein or antibody-mediated stabilization of this 'closed' conformation dramatically impairs otherosteric ligand dissociation, properties which give agonists their high affinity binding characteristics. Interestingly, basal receptor activity (ligand-free), capable of interacting and activating G proteins, can also adopt this 'closed' conformation upon GDP release. Formation of this 'closed' conformation in a ligand-free receptor also dramatically impairs radioligand association, whether the probe is an antagonist or agonist. Taken together these data suggest that agonist-mediated recruitment of G proteins and subsequent GDP release stabilizes a 'closed' conformation on the GPCRs extracellular face. This closed, activated conformation slows the observed hormone dissociation rates and thus accounts for the G protein-dependent high affinity binding properties of agonists.

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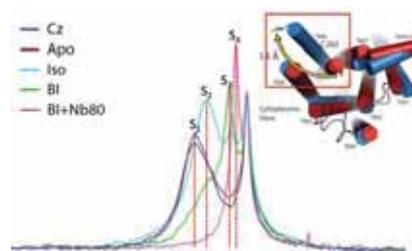
Conformational dynamics in the allosteric regulation of β_2 -adrenergic receptor signalingAashish Manglik^{1*}, Tae Hun Kim^{2*}, Christian Altenbach³, Zhongyu Yang³, Daniel Hilger¹, Tong Sun Kobilka¹, Foon Sun Thian¹, Wayne L. Hubbell³, Brian K. Kobilka¹, and R. Scott Prosser¹ ¹Department of Molecular and Cellular Physiology, Stanford University, CA, USA; ²Department of Chemistry, University of Toronto, ON, Canada; ³Department of Chemistry and Biochemistry, Jules Stein Eye Institute, University of California, CA, USA.

Introduction. G protein-coupled receptors (GPCRs) transduce signals from the extracellular environment to intracellular proteins. This signal transduction relies on allosteric regulation of the intracellular G protein-coupling domain by the extracellular ligand-binding domain. Despite recently acquired structures of active and inactive conformations of a prototypical GPCR, the β_2 adrenergic receptor (β_2 AR), it remains unclear how ligands regulate conformational changes in receptors.

Aims: to identify key states involved in activation and regulation of signaling and establish an energy landscape description of the adrenoreceptor. We wish to understand how ligands influence this landscape, resulting in a variety of pharmacological outcomes – inverse agonism, partial agonism, and full agonism.

Results. We assessed conformational changes and receptor dynamics by labeling the cytoplasmic domain of β_2 AR with a ¹⁹F-fluorine probe for NMR spectroscopy and nitroxide probes for double electron-electron resonance (DEER) spectroscopy. These studies show that in the unliganded and inverse agonist bound β_2 AR the cytoplasmic end of TM6 is represented primarily by two inactive conformations (S₁ and S₂), which exchange on a high-microsecond time scale. Generally agonists shift the conformational equilibrium towards a long-lived activation intermediate state (S₃) that is capable of engaging G proteins. Although agonists alter the conformation of the G protein-coupling domain, they do so incompletely resulting in a higher degree of conformational heterogeneity and coexistence of inactive states. Even an ultra-high affinity full agonist is incapable of fully stabilizing the active state, with complete transition to the active conformation (S₄) occurring only in the presence of an agonist and an intracellular G protein-mimetic nanobody, Nb80.

Discussion. These studies demonstrate a loose allosteric coupling of the agonist binding site and G protein-coupling interface of β_2 AR that stands in contrast to the tight regulation of rhodopsin conformation by the chromophore retinal. More generally, this loose allostery may be responsible for the complex signaling behavior observed for many GPCRs. Through NMR and DEER spectroscopy methods under development, it should become possible to extend the description of conformational dynamics associated with the functioning of ligand activated GPCRs.



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Exploring GPCR ligand interactions through structural and biophysical studies

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Recent years have witnessed some dramatic advances in the approaches available for GPCR drug discovery. High resolution X-ray structures are now available for more than 30 GPCRs, allowing the mechanisms of activation to be deduced, and enabling structure-based drug discovery approaches to be used for the first time.

To enable structural studies of GPCRs Heptares have developed the StaR technology, where the introduction of a small number of mutations locks the GPCR in an active or inactive state and stabilises the receptor sufficiently to allow purification and crystallization. X-ray structures are now available for class A, B and C receptors, in some cases with a range of ligands bound. This has enabled several interesting new features of GPCR structure and ligand recognition to be identified.

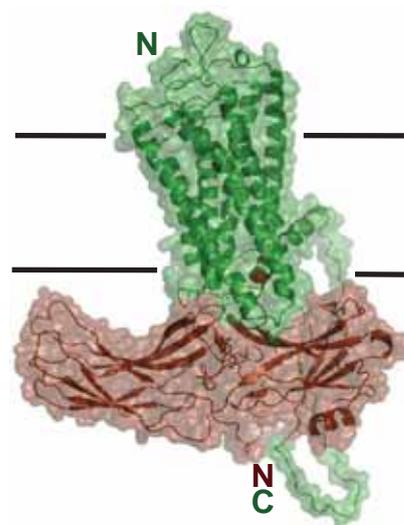
In addition to structural studies, the StaR approach enables other biophysical approaches such as surface plasmon resonance to investigate ligand binding. This has allowed the residues contributing to ligand binding to be identified, in the absence of detailed X-ray structures, as well as the kinetics of ligand binding to be determined.

The StaR-enabled developments in GPCR structural and biophysical studies have underpinned drug discovery efforts at Heptares, resulting in the discovery of multiple drug candidates, including a highly selective Muscarinic M₁ receptor agonist currently in clinical trials.

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Crystal structure of rhodopsin bound to arrestin determined by femtosecond X-ray laserH. Eric Xu^{1,2}, ¹Laboratory of Structural Sciences, Center for Structural Biology and Drug Discovery, Van Andel Research Institute, 333 Bostwick Ave., N.E., Grand Rapids, MI 49503, USA²VARI-SIMM Center, Center for Structure and Function of Drug Targets, CAS-Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China

G protein-coupled receptors (GPCRs) signal primarily through G proteins or arrestins. Arrestin binding to GPCRs blocks G protein interaction and redirects signaling to numerous G protein-independent pathways. Here we report the crystal structure of a constitutively active form of human rhodopsin bound to a pre-activated form of the mouse visual arrestin, determined by serial femtosecond X-ray laser crystallography. Together with extensive data from double electron-electron resonance, hydrogen-deuterium exchange mass spectrometry, cell-based rhodopsin-arrestin interaction assays, and site-specific disulfide bond crosslinking experiments, this structure reveals an overall architecture of rhodopsin-arrestin assembly, in which rhodopsin uses distinct structural elements, including TM7, Helix 8, and the C-terminal tail to recruit arrestin. Correspondingly, arrestin adopts the pre-activated conformation, with a ~20° rotation between the N- and C- domains, which opens up a cleft in arrestin to accommodate the second intracellular loop of rhodopsin. This structure provides a basis for understanding GPCR-mediated arrestin-biased signaling and demonstrates the power of X-ray lasers for advancing the frontiers of structural biology.



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GPCR-G protein signaling from endosomes

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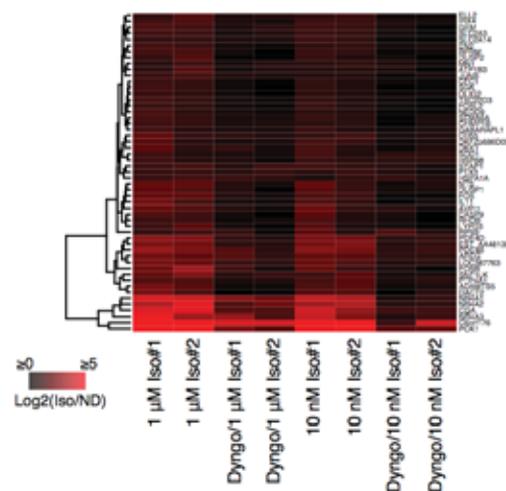
Introduction. Accumulating evidence suggests that some G protein-coupled receptors (GPCRs) can activate heterotrimeric G proteins in endosomes, after ligand-induced internalization, as well as from the plasma membrane. I will present data regarding our laboratory's efforts to assess functional significance of the putative endosome-initiated signal.

Aims. To assess the functional significance of GPCR-G protein activation in endosomes.

Methods. We profiled the genome-wide transcriptional response to endogenous β 2-AR activation in human embryonal kidney cells and then determined effects on this response of endocytic inhibition. We developed an orthogonal, optogenetic strategy to probe effects of generating cAMP selectively from the plasma membrane relative to endosomes irrespective of GPCR traffic.

Results. β 2-AR internalization is required for a full cAMP-dependent transcriptional response. This reflects a spatial bias with cAMP produced from endosomes preferentially coupled, relative to cAMP generated from the plasma membrane, to induction of a number of cAMP-responsive genes.

Discussion. These results suggest that cells discriminate the location of cAMP accumulation when initiating a response, with endosome-localized GPCR-G protein activation contributing to downstream transcriptional control.



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Receptor localisation shapes the spatiotemporal dynamics of GPCR signalling

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Introduction. Cells endogenously express many different receptors that can activate the same signalling pathway, but with remarkably diverse physiological outcomes. This suggests a high degree of organisation and regulation of intracellular signalling, which is achieved by the spatiotemporal compartmentalisation of signals –the restriction of second messengers in space and time. In this way, G protein-coupled receptors (GPCRs) can direct the assembly of focused “platforms” for unique signalling events.

Aims. To delineate the role of receptor localisation – within different sub-cellular compartments or higher-order protein complexes – in the control of compartmentalised GPCR signalling.

Methods. Receptor localisation, trafficking and signalling within sub-cellular compartments was measured using high-resolution bioluminescence or Förster resonance energy transfer (BRET or FRET), and visualised by super-resolution microscopy.

Results. A role for sub-cellular localisation: the neurokinin 1 receptor (NK₁R) activates distinct temporal signalling profiles when localised at plasma versus endosomal membranes. Within the early endosome, NK₁R couples to G $\alpha_{q/11}$ to induce sustained increases in cytosolic PKC, cytosolic cAMP and nuclear ERK. By conjugating a lipid anchor to a NK₁R antagonist, we can specifically block signalling from receptors localised to endosomes, but not those at the plasma membrane. Administration of the lipid conjugated antagonist blocks pain transmission, but not inflammation, which is dependent on signalling from the plasma membrane. A role for localisation within protein complexes: the β ₂-adrenoceptor (β ₂AR) constitutively forms a large protein complex (signalosome) that confers enhanced sensitivity of the receptor to very low concentrations of ligand. Activation occurs via the orthosteric binding site, is dependent on receptor internalisation to endosomes and causes an increase in receptors at the cell surface, suggesting a role in sensing the extracellular environment. Agonist activation of the β ₂AR-signalosome causes a sustained increase in nuclear ERK, increases global gene transcription and has unique effects at the proteomic level.

Discussion. Receptor localisation plays a major role in the control of spatiotemporal signalling, and facilitates tailored responses from the level of the cell to the whole organism.

From biochemistry to molecular imaging of GPCRs

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Introduction. Receptor signalling is usually analysed by biochemical means, which provides little spatial and temporal resolution. We have, therefore, developed a number of optical techniques to monitor and image signaling by G-protein-coupled receptors (GPCRs).

Aims. These studies aim to provide information, where and when a signal is transmitted in an intact cell or even an intact organism, and whether such signalling is localized or generalized.

Methods. Our studies use two main approaches: Fluorescence resonance energy transfer (FRET) and single molecule imaging. In FRET, we use fluorophores attached to GPCRs and downstream signaling proteins in order to monitor conformational changes in proteins and/or re-arrangements of protein complexes. In single molecule imaging we track individual receptor molecules by total internal reflection microscopy in order to follow their movements and their association and dissociation.

Results. Our studies indicate that GPCR signalling occurs in a sequence of events, where initially receptors rearrange and change their conformation within ≈ 50 msec, followed by similarly rapid interaction with G-proteins. Surprisingly, G-protein activation is ≈ 10 times slower, and from there signals propagate with similar speed to downstream signalling effectors. Receptors are highly mobile at the cell surface, facilitating their interactions with similarly mobile G-proteins. They may, in addition, form transient or stable dimers and oligomers in a receptor-specific fashion.

Discussion. Our studies illustrate that GPCR signalling is a highly dynamic and rapid process, which involves multiple opportunities for regulatory input and interference.

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A new approach to treat ischaemic heart disease: Adenosine receptor biased agonism

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Introduction. Adenosine A_1 receptor (A_1AR) activation represents a powerful cardioprotective mechanism. Unfortunately, the translation of A_1AR agonists into the clinic has been severely hindered due to on-target adverse effects, including bradycardia, atrioventricular block and hypotension (Jacobson & Gao, 2006). A novel approach to overcome the current limitations associated with prototypical A_1AR therapeutics is biased agonism. Biased agonism may provide a mechanism to develop A_1AR therapeutics that enhance cardioprotective signalling in the absence of unwanted hemodynamic effects, thereby enabling the separation of desired from adverse effects.

Aims. To quantify the bias profile of prototypical and atypical A_1AR agonists *in vitro* and investigate the ability of biased agonists to mediate cardioprotection in the absence of adverse hemodynamic effects *in vivo*.

Methods. Fluorescence approaches were used to determine phosphorylation of ERK1/2 and AKT, calcium mobilization, cAMP accumulation and cell survival stimulated by A_1AR agonists in CHO cells stably expressing the human A_1AR . An operational approach was used to quantify ligand bias (Kenakin et al., 2012). An acute myocardial ischaemia-reperfusion injury model in rats was used to determine the influence of A_1AR agonists on blood pressure, heart rate and infarct size.

Results. Prototypical A_1AR agonists did not display significant bias for any of the signaling pathways assessed. In contrast, the atypical agonist, VCP746, was significantly biased away from calcium mobilization. Both the prototypical agonist NECA and the atypical agonist VCP746 mediated a significant decrease in infarct size within an acute myocardial ischaemia-reperfusion injury model. However in contrast to NECA, which caused significant adverse cardiovascular effects, VCP746 had a negligible effect on mean arterial pressure and heart rate.

Discussion. Collectively, these studies demonstrate that “fingerprinting” of biased agonism within a model system has the potential to predict novel and physiologically relevant *in vivo* pharmacology.

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Dualsteric GPCR modulators with engineered efficacy to fine-tune muscarinic receptor signaling

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Crystal structures give fascinating insight into the three-dimensional shape of G-protein-coupled receptors (GPCRs). Published GPCR crystal structures often represent either agonist-bound active or inverse agonist-bound inactive states. As receptor conformation is sensitive to the shape and physicochemical properties of a bound ligand as well as to the type of intracellular adaptor protein, a multitude of conformations and functionalities may exist for a given GPCR. Furthermore, ligands may choose between binding topographies: orthosteric, allosteric, dualsteric (simultaneous ortho/allo-steric). Using the M2-subtype of the muscarinic acetylcholine receptor as a paradigm, superagonism [1], biased agonism [2], and dynamic ligand binding for designing partial agonism [3] will be discussed. As the general architecture of many class A GPCRs is similar to that of the M2 receptor, the discussed approaches may well have broader applicability for fine-tuning the signalling of G protein-coupled receptors.

Acknowledgments: We are grateful for the excellent collaboration with Prof. Dr. Marco De Amici (Università degli Studi di Milano, Italy), Prof. Dr. Carsten Hoffmann (University of Würzburg, Germany), Prof. Dr. Ulrike Holzgrabe (University of Würzburg, Germany), Prof. Dr. Evi Kostenis (University of Bonn, Germany), and their group members.

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Observed drug-receptor association rates are strongly influenced by membrane affinity: The importance of establishing “micro PK/PD relationships”

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Introduction. Current pharmacological models for determining affinity and kinetics of drugs for membrane receptors assume the interacting molecules are homogeneously distributed in the bulk aqueous phase. The phospholipid membrane can, however, provide a second compartment into which drugs can partition, especially lipophilic/basic compounds. This is particularly important for drugs that have been specifically designed to have tissue affinity in order to enhance duration of action after topical delivery, for example the β_2 -adrenoceptor agonists used to treat asthma and COPD.

Aim. To examine the effect that membrane affinity may have on observed pharmacology of several clinically relevant β_2 -adrenoceptor agonists and antagonists.

Methods. Phospholipid interactions were assessed using an HPLC method with immobilized phosphatidylcholine monolayers (Valko et al, 2000). Receptor binding kinetics were determined using a radiolabel competition association assay (Sykes & Charlton, 2012).

Results. We found that the degree of phospholipid interaction was directly related to the observed kinetic association rate (k_{on}) and affinity (K_D), but not the dissociation rate (k_{off}) from the target, presumably by concentrating drug in the local environment around the receptor. When the local drug concentration was accounted for, the k_{on} was comparable across the cohort and the corrected K_D was directly related to the k_{off} . We have also applied this approach to other receptors and shown that this relationship is not unique to the β_2 -adrenoceptor.

Discussion. We propose a new approach to determining the pharmacology of drugs for membrane targets that accounts for differences in local drug concentration brought about by direct affinity for phospholipids, establishing “micro PK/PD relationships” for drugs.

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