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Activation of AMPK prevents drug induced mitochondrial and hepatocellular damage
Ghada Haydar1, Geoff Farrell2 and Dong Fu1
Faculty of Pharmacy, The University of Sydney1, Sydney, NSW; School of Medicine, Australia National University2, Canberra, ACT.

Introduction. Drug induced liver injury (DILI) is responsible for many cases of acute liver failure; and it is the most common reason for withdrawing new agents during drug development or after approval. To date, few strategies have focused on mitochondrial damage for treatment of DILI. Understanding the cellular basis of mitochondrial damage during DILI would point to new approaches for treatment.

Aims. Using collagen sandwich culture of hepatocytes, our study investigates the mechanism of how various hepatotoxic drugs affect mitochondrial function, fusion/fission morphology and cell viability, and examines the preventative effect of AMP-activated kinase (AMPK) on mitochondrial and cellular damage in DILI.

Methods. Hepatocytes and mitochondrial morphology were examined by immunofluorescence and confocal microscopy. Mitochondrial potential and hepatocyte viability were measured using confocal microscopy. Protein expressions were detected by Western blot.

Results. Hepatotoxic drugs, paracetamol (10 mM) and diclofenac (250 microM), caused mitochondrial fragmentation; and significantly decreased mitochondrial membrane potential as well as the cellular ATP levels. These drugs either decreased expression of mitochondrial fusion proteins or increased mitochondrial fission protein activity, which were associated with mitochondrial fragmentation. Both drugs decreased hepatocyte viability and caused depolarization, confirming their hepatotoxicity. Pharmacological activation of AMPK restored mitochondrial function (mitochondrial potential and cellular ATP), morphology, hepatocyte polarization and cell viability; demonstrating that AMPK activation can prevent drug induced mitochondrial and hepatocellular damage.

Discussion. The current study reveals that hepatotoxic drugs paracetamol and diclofenac cause mitochondrial fragmentation and dysfunction, which result in hepatocellular injury. Through its effects on mitochondrial biogenesis/function and mitophagy, activation of AMPK prevents mitochondrial and hepatocellular damage, therefore, targeting AMPK can be a potential novel strategy for treatment of DILI.

NSAIDS: How clarifying a question of toxicity added to the vocabulary of pharmacology (A memoir).
Michael W Whitehouse, Schools of Medicine (Gold Coast) and Natural Science (Nathan), Griffith University, Qld.

Background: Sixty years ago, synthetic corticosteroids (SCS) were being (over)-prescribed for arthritis and other inflammatory disorders. They came at enormous cost, notably to a patient’s connective tissues (CT), fluid and fat regulation. But how were physicians and their patients to be weaned from their deceptive euphoric effects?

Procedures: a) Show how SCS were developed from toxicity assays in rats eg thymus involution, reducing experimental granuloma, etc.; b) identify chemical mechanisms of SCS side effects eg muscle wasting, impaired CT anabolism/regeneration; c) ‘flag’ alternative treatments providing analgesia and reducing inflammation without the metabolic havoc of SCS; d) use the descriptor ‘Non-steroid’ to clearly direct attention to other less noxious anti-inflammatories (1). What happened: Jonas Buer, Norway, recently reported the term NSAID had been used in over 4 million citations (2), tracing its first use to published abstracts in 1960 and subsequent publication in Nature (1961) in which Jay Lash (U.Pennsylvania) and I reported a chemical basis for the toxic effects of SCS on embryonic cartilage development in vitro (3). [This was my first serious venture into biochemical toxicology.]

Conclusion: The need to define a biochemical/toxicological distinction between SCS and all other anti-inflammatories has served its original purpose. This initial report showed how objective markers of toxic drug effects on tissue development and turnover could be quantified ex vivo. Extensive adoption of the NSAID acronym by most reputable sources of drug information was surprising but gratifying.

Disclaimer: I may not have been the first to colloquially use the term ‘non-steroid(al)’ as a prefix but merely note Buer’s findings that I probably authored its first appearance in the English medical literature.

**505**

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

John Mach¹,²,³, Aniko Huizer – Pajkos¹,², Catriona McKenzie⁴, Victoria Cogger³,⁵, David G Le Couteur³,⁵, Brett Jones⁶, Rafael de Cabo⁷ & Sarah N Hilmer¹,²,³, Laboratory of Ageing and Pharmacology, Kolling Institute of Medical Research, Sydney, NSW¹, Dept of Clin Pharmacol and Aged Care, Royal North Shore Hosp, Sydney, NSW², Sydney Medical School, Univ of Sydney, Sydney, NSW;³ Pathology Dept, Prince Alfred Hosp, Sydney, NSW, Australia;⁴Centre for Education and Research on Ageing and Anzac Research Institute, Concord Hospital and University of Sydney, Australia;⁵Gastroenterology Dept, Royal North Shore Hosp, Sydney, NSW⁶. Translational Gerontology Branch, NIA, NIH, Baltimore, Maryland, USA⁷.

**Background:** Isoniazid is the first-line medication for tuberculosis however it causes hepatotoxicity in 10-20% of patients. The mechanism of isoniazid induced hepatotoxicity has not been well defined.

**Aim:** To investigate the effect of a toxic isoniazid regimen on the expression of proteins involved in the intrinsic death pathway and apoptosis in young and old Fischer 344 rats.

**Methods:** Saline or a toxic regimen of isoniazid (4 doses/day: 100, 75, 75, 75mg/kg ip every 3 hours over 2 days) was administered to young (6 months) and old (24 months) male Fischer 344 rats (isoniazid: young n=7, old n=5; saline: young n=7, old n=8). Fifteen hours post last injection animals were euthanized by ip injection of ketamine (75mg/kg) xylazine (10mg/kg) and sera was collected for biochemical analysis and livers for histology and immunoblotting of target proteins.

**Results:** Isoniazid treatment significantly increased serum hepatotoxicity markers (aspartate transaminase, glutamate dehydrogenase and sorbitol dehydrogenase; p<0.05) and non-significantly increased the prevalence of necrosis (p<0.08) in young treated animals but not in old animals. Compared to saline treated animals, a toxic regimen of Isoniazid did not alter apoptosis or hepatic mitochondria expression of pro- (BAX, BAK, VDAC-1 and Bid) and anti-apoptotic (Bcl-2 and Bcl-Xl) proteins in either young or old animals.

**Conclusion:** Our preliminary results show that a toxic regimen of isoniazid does not activate the intrinsic death pathway or increase apoptosis in vivo and suggest no critical role of this pathway in the mechanism of toxicity in young or old Fischer 344 rats.

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

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**Introduction:** Atorvastatin (ASN) is widely used for the treatment of dyslipidemia and the prevention of cardiovascular disease. It has two active metabolites, ortho- and the para hydroxy atorvastatin as well as inactive lactone metabolites. Both active metabolites are known to be equipotent to the parent drug. Quantification of ASN and its metabolites in human plasma by LC/MS is well established but requires involved sample preparation.

**Aim:** The aim was to develop and validate a simple and rapid method for the simultaneous quantitation of ASN and three of its metabolites for monitoring ASN therapy and in critically ill patients with sepsis.

**Method:** Online sample clean-up was achieved on a Security Guard column (C18 4x3 mm, 5 µm). The chromatographic separation was performed on a Luna analytical column (C18(2) 50 × 2.0mm, 5 µm). An isocratic 5:95 mixture of 0.1% aqueous formic acid and acetonitrile elutes the analytes with good chromatographic separation. The LLOQ was 0.5 ng/mL on an ABSciex API2000 MS/MS system.

**Results.** The assay was validated based on FDA guidelines for repeatability, matrix effect and stability. The precision and accuracy for all analytes were acceptable (<15%).

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

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


Structure-function relationships of human dimethylarginine dimethylaminohydrolase (DDAH) isoforms, DDAH-1 & DDAH-2: an approach to rational drug design

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Introduction: The DDAH enzymes represents a potential target to mediate nitric oxide (NO) production, particularly in conditions characterized by excessive NO release, e.g. neurodegenerative diseases and septic shock. The isoform DDAH-1 is well characterised due to its role in cardiovascular homeostasis. However, little is known regarding DDAH-2, its tissue distribution in the body, or known substrates.

Aims: To identify the structure-function relationships of DDAH-1 and DDAH-2 and to elucidate the catalytic mechanism(s) that underpin substrate metabolism.

Methods: DNA cloning and site-directed mutagenesis was undertaken to generate a number of DDAH mutants. Recombinant protein was generated in HEK293T cells and their catalytic activity determined by mass spectrometry. The crystal template of DDAH-1 and an in silico model generated of DDAH-2, were utilized in automated docking experiments using the prototypic DDAH-1 substrate, asymmetric dimethylarginine (ADMA).

Results: Preliminary kinetic data with wild-type DDAH-1 and DDAH-1 mutants D73L, F76L, R145W and C274A revealed significant increases in Vmax relative to the WT enzyme. These results were not anticipated. DDAH-2 mutants L71D, L74F, W92G, T127G, W143R, and S274C were designed to investigate whether ADMA can be used as substrate in this enzyme. However no data is currently available. The catalytic mechanism of ADMA metabolism is currently unknown, however my structural investigations of DDAH-1 and DDAH-2 reveal a putative mechanism whereby the central core of each enzyme opens to accept the incoming substrate molecule.

Discussion: Computational docking experiments combined with enzyme kinetic data have provided insight into the physicochemical properties required for energetically favourable ADMA binding in DDAH-1. These data will be used to further design and develop DDAH inhibitors for use in neurodegenerative conditions, such as Alzheimer’s Disease and Parkinson’s Disease.
**POSTER ABSTRACTS**

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

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Introduction. Testosterone is converted into estrogen in breast cancer cells. Hence, removal of testosterone by glucuronidation has a potential impact on estrogen-driven breast carcinogenesis and cancer progression. As the testosterone-glucuronidating UDP glucuronosyltransferases 2B15 and 2B17 are expressed in breast cells, factors that control their expression may have an important role in modulating these pro-carcinogenic estrogen effects.

Aim. To study the potential regulation of UGT2B15 and 2B17 by tamoxifen and its active metabolite in breast cancer cells.

Methods & Results. UGT2B15 and 2B17 mRNA levels and testosterone-glucuronidating activity were significantly increased in breast cancer MCF-7 cells treated with tamoxifen and 4-hydroxytamoxifen (4-OH-tamoxifen). This increase was abrogated by either knockdown of estrogen receptor α (ERα) by siRNA or the ER antagonist, ICI 182,780. Furthermore, tamoxifen and 4-OH-tamoxifen stimulated the activity of the UGT2B15 promoter. This stimulation was enhanced by overexpression of ERα but significantly reduced by mutation of the previously reported functional estrogen response unit (ERU) at the UGT2B15 proximal promoter. Chromatin immunoprecipitation (ChIP) assay showed enrichment of ERα binding at the ERU of the UGT2B15 promoter upon 4-OH-tamoxifen exposure.

Discussion. Tamoxifen and its active metabolite up-regulate UGT2B15 and 2B17 expression via ERα in breast cancer cells. This tamoxifen-induced UGT2B15 and 2B17 enzymatic activity may facilitate the removal of testosterone from tumour cells. This is a novel mechanism that may contribute to the antiestrogenic effects of tamoxifen which is used in the treatment of estrogen receptor positive breast cancers.


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

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Introduction. Polypharmacy (use of ≥5 different medicines) is common in older Australians. In observational studies, it is associated with an increased risk of adverse geriatric outcomes such as falls and frailty.

Aims. (1) To establish a mouse model of polypharmacy; (2) To establish whether short term polypharmacy causes adverse geriatric outcomes in young and old age.

Methods. Male young (3 months) and old (23 months) C57BL/6 mice were administered control or polypharmacy diet for 4 weeks: simvastatin (20 mg/kg/day), metoprolol (350 mg/kg/day), omeprazole (10 mg/kg/day), paracetamol (100 mg/kg/day) and citalopram (10 mg/kg/day). Mouse food intake, weight, blood pressure, rotarod latency (balance and coordination), front paw hang (holding impulse, grip strength) and clinical frailty index were assessed.

Results. The polypharmacy regimen was well tolerated, with no change in or al intake or weight observed in young or old mice. Polypharmacy did not significantly affect outcomes in young mice. In old mice, polypharmacy lowered blood pressure and impaired physical function but did not significantly increase frailty.

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

<table>
<thead>
<tr>
<th>Mean (±SEM) change after 4 weeks of diet</th>
<th>Young Polypharmacy (n=5)</th>
<th>Old Polypharmacy (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>↑ 3± 6</td>
<td>↑ 0.2± 3</td>
</tr>
<tr>
<td>Rotarod latency (s)</td>
<td>↑ 43± 38</td>
<td>↑ 59± 11</td>
</tr>
<tr>
<td>Front paw hang (N sec)</td>
<td>↑ 1078± 664</td>
<td>↑ 250± 104</td>
</tr>
<tr>
<td>Frailty Index</td>
<td>N/A</td>
<td>↑ 0.03± 01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polypharmacy</td>
<td>↑ 9± 7</td>
<td>↑ 11± 4*</td>
</tr>
<tr>
<td></td>
<td>↑ 34± 11</td>
<td>↑ 2± 17*</td>
</tr>
<tr>
<td></td>
<td>↑ 529± 233</td>
<td>↑ 203± 122*</td>
</tr>
<tr>
<td></td>
<td>↑ 0.03± 01</td>
<td>↑ 0.05± 01*</td>
</tr>
</tbody>
</table>

*p < 0.05 for change seen in control diet mice vs polypharmacy diet mice within each age group; BP, blood pressure; N, newton
Detection of Testosterone dosing in racing male Greyhounds
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Introduction. Anabolic androgenic steroids that are derivatives of testosterone are used to increase performance in racing greyhounds. The analytical detection of these steroids in male dogs is complicated by the presence of endogenous hormone. As exogenous testosterone is administered as esters, which do not naturally occur, detection of the esters in biological samples would provide unequivocal evidence of illicit administration of drug.

Aims. The aim of this study was to develop and validate a confirmatory analytical method for detection in hair of testosterone-esters from greyhound dogs, and to identify the duration for which detection is possible.

Methods. A liquid chromatographic-tandem mass spectrometric method (LC-MS/MS) was developed and validated for the detection in hair of testosterone-esters; propionate, phenyl propionate, isocaproate, decanoate and enanthate. Drug administration studies were carried out by injection of testosterone-ester mix in five Greyhound dogs, and hair samples were collected on alternate days during the first week, followed by weekly for one month, and thereafter fortnightly until 3 months post dosing. These samples were analyzed using the above LC-MS/MS method.

Results. Preliminary results show that each testosterone ester was detectable from 2 hours and up to nine weeks post administration, with the mean maximum hair concentration occurring at 48 h for all esters; propionate 2.09±0.8 (ng/g), phenyl propionate 8.75±4.62 (ng/g), isocaproate 7.09±3.45 (ng/g), and decanoate 8.38±3.56 (ng/g). Propionate was readily detectable at nine weeks in all five dogs, compared to the other esters, which were detected in only some of the dogs. In addition, the concentration of the longer chain decanoate, was low or undetectable at nine weeks, which suggests that lipophilicity is not a major factor in the incorporation of anabolic steroids into hair.

Discussion. A sensitive LCMSMS method has been developed which provides an unequivocal confirmation of administration of testosterone-esters to greyhound dogs and bitches, and this new method should find useful application in racing regulation.
**Study of a novel regulatory mechanism that controls UGT1A8 synergistic activation by Cdx2 (Caudal-related Homeodomain protein-2) and HNF4a (Hepatocyte Nuclear Factor-4 alpha)**

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Introduction. UDP-glucuronosyltransferase (UGT) 1A8 is a UGT1A family enzyme exclusively expressed in extrahepatic tissues. It is a major mediator of first pass drug metabolism in the gastrointestinal system. We recently found that expression of this gene is controlled by the intestinal master transcription factor Cdx2 as well as HNF4a through a novel regulatory element that we have defined in the proximal UGT1A8 promoter. In the present study we further characterize the mechanism of Cdx2 and HNF4a synergistic action.

Aim. To determine the mechanism of UGT1A8 transcriptional activation by Cdx2 and HNF4a

Methods. All experiments were performed in Caco-2 cells. Functional analysis of regulatory elements used promoter-luciferase reporter assays, site directed mutagenesis and co-transfection assays. Endogenous UGT1A8 expression was assessed by qRTPCR. Electrophoretic mobility shift assays (EMSA), and chromatin immunoprecipitation (ChIP) were performed to assess promoter binding by Cdx2 and HNF4a.

Results and Discussion. We have defined a novel functional regulatory element in the proximal promoter of UGT1A8 (nt-44), which contains a consensus HNF4a binding motif. Mutagenesis assays show this HNF4a element to be essential for synergistic activation of the promoter by Cdx2 and HNF4a. Surprisingly however, a previously characterized Cdx2 element located upstream at nt -77 was not required for synergy. EMSA assays show that the -44 HNF4a element binds both HNF4a and Cdx2. Sequence analysis shows that the element is a composite of a consensus HNF4 motif and a cryptic Cdx2 recognition motif. Mutagenesis analysis shows that both motifs within this composite element are required for synergy of Cdx2+HNF4a, suggesting that the factors may interact at the composite element. This idea is supported by results from both EMSA and ChIP analysis. Moreover, mechanism analysis using dominant-negative Cdx2 mutants suggests that HNF4a may play the major role by recruiting Cdx2, which is in contrast to recent reports that in adult intestine Cdx2 induces chromatin remodeling to facilitate HNF4a binding. Ongoing analysis using clustered regularly interspaced short palindromic repeats (CRISPR) to remove this novel element from the genome will allow characterization of Cdx2+HNF4a functional synergy in vivo.

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

Suong N T Ngo, Chelsea A Smith, Pat H Cheung, Anna C Schumann, The University of Adelaide, School of Animal & Veterinary Sciences, Adelaide, SA.

Introduction. Unlike eutherian mammals, marsupial herbivores ingest large amounts of eucalyptus leaves containing dietary toxins. It is hypothesised that eucalyptus herbivores, such as the koala (Phascolarctos cinereus) utilise highly efficient enzyme systems in order to metabolise and excrete these toxins as non-toxic metabolites. Enzymes that have been identified to date as those that carry out this biotransformation are those of the Cytochrome P450 subfamilies (CYPs). Whilst CYP3A is considered the most abundant enzyme involved in mammalian xenobiotic metabolism, biochemical analysis has determined that koalas may express higher CYP2 activity. These studies indicate the possibility that CYP2 and CYP3 activity may play an important role in xenobiotic metabolism. Recent studies have highlighted that dietary terpenes act as peroxisome proliferators, activating an array of CYP genes upon binding to peroxisome proliferators activated receptor-alpha (PPAR-α). Higher expression of hepatic PPAR-α mRNA was detected in the koala in comparison with non-eucalyptus feeders, suggesting that dietary terpenes upon binding to PPAR-α possess the ability to up-regulate CYP subfamilies.

Aims. The aim of the current study was to provide an update on molecular and biochemical aspects of biotransformation enzymes in Australian marsupials and their possible roles in dietary toxin detoxification.

Methods. The study focuses on molecular and biochemical findings as well as approaches employed over the last 2 decades to investigate Phase I and II drug metabolising enzymes and processes involved in marsupial metabolism. Discussion. 1,8-cineole, the main terpenoid in Eucalyptus has been shown to have much lower LD50 values in mammalian species in comparison to that of marsupials. Possums and koalas have been found to tolerate a much higher daily intake of cineole. Administration of terpenes has been reported to induce certain CYPs, with studies of several mammalian species showing increased hepatic enzyme activity upon administration. Marsupials show elevated levels of CYP2C6 and CYP2C11 upon terpene administration, and also increased NAD activity. CYP4A increases hepatic NAD activity, thus elevating metabolic reactions. This elevation is an important finding in determining the efficiency of metabolic processes. The koala has been shown to have significantly higher NAD levels, suggesting that they have increased CYP activity and highly efficient hepatic metabolism. Moreover, koalas and possums have shown to rapidly produce and eliminate oxidised metabolites. The koala excretes 95% of administered cineole as carboxylic acid metabolites with complete absence of precursor metabolites in the urine. This indicates that oxidative reactions occur more rapidly in the koala than mammals. In summary, the unique
Xenobiotic metabolism in Australian marsupial koala, *Phascolarctos cinereus*: Comparative PKs/PDs and drug disposition.

Suong N T Ngo, Anna C Schumann, Pat H Cheung, Chelsea A Smith, The University of Adelaide, School of Animal & Veterinary Sciences, Adelaide, SA.

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

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

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

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

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

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


Introduction. The area under the free concentration time curve divided by the minimum inhibitory concentration ([AUC/MIC]) best predicts bacterial killing at 24 h and clinical success of quinolones and aminoglycosides. Aim. To determine whether delivering the same [AUC over 4 or 10 h vs. 24 h provides better killing and minimizes resistance.

Methods. *P. aeruginosa* ATCC 27853 was evaluated in 24 h *in vitro* static time-kills with ciprofloxacin (CIP; MIC=0.25 mg/L) at [AUC/MIC of 44 and 132 and tobramycin (TOB; MIC=0.5 mg/L) at [AUC/MIC of 36, 72 and 168, in duplicate. Log_{10} initial inocula (CFUo) were 4, 5 and 6 (95 profiles in total). Antibiotic was added at 0 h and removed at 1, 4, 10, 16 or 24 h by spinning and re-suspending bacteria 2- or 3-times in antibiotic-free broth. Drug concentrations were chosen to achieve the targeted [AUC/MIC for each exposure and serial viable counts were determined. Log_{10} mutation frequencies (MF, at 3x MIC, baseline: -6.6 ± 0.3 for CIP and -7.0 ± 0.3 for TOB) and MICs were assessed from the drug-free bacterial suspensions at 0 and 24 h.

Results. High CIP concentrations of the 4 h duration of exposure yielded extensive killing at 6 h and eradication at 24 h for CFUo=4, but were followed by regrowth for CFUo=6 (Table). For the 24 h duration of exposure, less regrowth occurred, but the CIP-resistant population almost completely replaced the susceptible population by 24 h and MICs increased 4 to 8-fold for CFUo=6 (Table). No resistance emerged for the 4 h (Table) and 10 h (not shown) duration of CIP exposure. For TOB [AUC/MIC of 36, the MF at 24 h was -6.4 ± 0.5 (mean±SD) for 4 h duration of exposure, but was higher (-0.53 ± 0.40; p<0.05) for 24 h exposure; TOB MICs at 24 h were 0.5 mg/L for 4 h duration of exposure and 4 mg/L for 24 h exposure.

### Table: Endpoint CIP Inoculum (CFU/mL) / duration of exposure (h) [AUC/MIC]

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>CIP 10^6 / 24 h</th>
<th>Inoculum (CFU/mL) / duration of exposure (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log_{10} CFU/mL at 6 h vs 0 h</td>
<td>132</td>
<td>-4.5 ± 0.02 / -6 * / -3.4 ± 1.1 / -4 *</td>
</tr>
<tr>
<td>Log_{10} CFU/mL at 24 h vs 0 h</td>
<td>132</td>
<td>-5.0 ± 0.1 / 1.9 ± 0.3 / -1.9 ± 0.4 / -4 *</td>
</tr>
<tr>
<td>MF at 24 h</td>
<td>44</td>
<td>-0.8 / -6.2 ± 0.4 / &lt; -3.1 * / -5.9 ± 0.1</td>
</tr>
<tr>
<td>MF at 24 h</td>
<td>132</td>
<td>-1.0 ± 0.8 / -6.4 ± 0.4 / &lt; -1.7 * / *</td>
</tr>
<tr>
<td>MIC (mg/L) at 24 h</td>
<td>44 &amp; 132</td>
<td>1 - 2 / 0.25 / 0.125-0.25 / 0.25</td>
</tr>
</tbody>
</table>

*: not detectable

Discussion. At the same [AUC/MIC, short 1, 4 [and 10] h durations of antibiotic exposure yielded extensive initial killing without resistance, whereas the 24 h duration of exposure caused less initial killing and substantial resistance emergence due to pre-existing resistant mutants at a CFUo of 6. High dose, short-course exposure of rapidly killing antibiotics seems promising for innovative combination therapies.
Comparing utilisation of mycophenolate, tacrolimus, cyclosporin, sirolimus and everolimus in Australia and Northern Europe
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1 School of Pharmacy, University of Queensland, Brisbane, QLD

Introduction. Mycophenolate, tacrolimus, cyclosporin, sirolimus and everolimus, used in management of transplant rejection, receive restricted public subsidy in Australia and Northern Europe.

Aims. To examine the utilisation and expenditure of each immunosuppressant in Australia between 2007-2013 and to identify specific patterns of immunosuppressant utilisation in Denmark, Sweden, Norway and the Netherlands.

Methods. Data were gathered from the Pharmaceutical Benefits Scheme (PBS) database, Medicare Australia; the Danish Health and Medicines Agency; the Norwegian Institute of Public Health; the Swedish Board of Health and Welfare; and the Netherlands Healthcare Insurance Board. Drug utilisation was expressed as WHO Anatomical Therapeutic Chemical Defined Daily Dose (2013)/1000 population/day (DDD/1000/day).

Results. In Australia in 2013, mycophenolate PBS usage was 0.28 DDD/1000/day, up 6.5-fold from 2007 with AUD$24.2million spent. Tacrolimus PBS utilisation was 0.23 DDD/1000/day, up 9.3-fold from 2007 with AUD$32.3million spent. Cyclosporin PBS utilisation was 0.13 DDD/1000/pop/day, up 1.9-fold from 2007 with AUD$15million spent. Sirolimus PBS utilisation was 0.02 DDD/1000/pop/day, up 4.6-fold from 2007 with AUD$3million spent and everolimus PBS usage was 0.04 DDD/1000/day, up 26-fold from 2007 with AUD$8.2million spent. Total Australian PBS expenditure across all immunosuppressants increased 4.2-fold between 2007 and 2013. In comparison to Northern Europe, 2012 Australian PBS immunosuppressant utilisation was approximately 50% lower but growing 2.8-fold faster, anticipating future convergence with the higher European usage. Transplantation rates have historically been higher in these Northern European countries.

Discussion. Immunosuppressant usage and subsequent expenditure are steadily rising with increased numbers of Australians living with a transplant. With transplant patients living longer, the potential for new subsidised indications and the observed growth potential predicted from Northern European data immunosuppressants can be predicted to continue consuming an increasing share of Australian PBS expenditures.
Analgesic use, pain and daytime sedation in people with and without dementia in aged care facilities
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Introduction. Managing pain in residents of aged care facilities is challenging, especially for people with dementia. Clinicians must weigh the benefits of analgesic use against the potential for adverse effects such as sedation.

Aims. To investigate the prevalence and correlates of analgesic use, pain and daytime sedation in people with and without dementia in Australian aged care facilities.

Methods. A cross-sectional study of 349 residents with and without dementia from five aged care facilities in South Australia was conducted. Trained study nurses administered validated and dementia-specific assessments of pain, sedation and other clinical outcomes. Medicine use data were extracted directly from each resident’s medication administration chart. Logistic regression was used to compute odds ratio (ORs) and confidence intervals (CIs) for factors associated with analgesic use.

Results. Analgesics were charted for 95.4% of residents. There was no difference in the administration of regular analgesics in the previous 24 hours between residents with and without dementia (72.2% vs 69.0%, p=0.21). Residents with dementia received fewer when-required analgesics in the previous 24-hours compared to residents without dementia (1.3% vs 5.5%, p=0.04). The prevalence of clinician-observed pain was similar in residents with and without dementia (25.2% vs 30.8%, p=0.25). Residents with dementia experienced more daytime sedation than residents without dementia (18.5% vs 7.0%, p<0.01). Analgesic use within the previous 24 hours was associated with both morning drowsiness (OR2.12, 95%CI 1.26 – 3.55) and napping frequency (OR2.01, 95%CI 1.20 – 3.40) when adjusting for age, sex, dementia diagnosis and pain.

Discussion. Encouragingly, there was a higher prevalence of regular analgesic use and lower prevalence of pain than reported in previous research, which did not differ in residents with and without dementia. These findings may indicate adequate pain control from analgesia. The presence of daytime sedation highlights the importance of ongoing assessment of the need for analgesia, especially in people with dementia.

Prevalence of statin-drug interactions in older people: a systematic review
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Introduction. Statins are amongst the most frequently prescribed medications internationally. Older people are commonly prescribed multiple medications, and are at an increased risk of drug-drug interactions, including statin-drug interactions.

Aim. To conduct a systematic review of the current evidence on the prevalence of statin-drug interactions in older people.

Methods. A systematic search of Embase, Medline and PubMed was conducted and augmented with a manual search of the reference list of identified articles. Articles were included if they were published in English in the past 14 years (July 2000–July 2014) and reported on the prevalence of any statin-drug interactions in people over 65 years of age. Two reviewers independently assessed the articles for eligibility and extracted the data.

Results. The search returned 1555 records. A total of 19 articles met the inclusion criteria. In studies that focused on statin-users only (n=7), the prevalence of potential statin-drug interactions defined using different international sources, ranged from 0.19% to 33.0%. Of these seven studies, only one reported specifically on clinically relevant statin-drug interactions and found a prevalence of 4%, with amiodarone and atorvastatin or simvastatin being the most common statin-drug interaction. In studies that examined drug interactions across a population of both statin-users and non-users (n=12), the prevalence of potential statin-drug interactions ranged from less than 0.1% to 7.1%, and the prevalence of clinically relevant statin-drug interactions ranged from 1.5% to 4% (n=4).

Discussion. Current published evidence suggests substantial variations in the prevalence of statin-drug interactions. This may be due to lack of consensus in defining statin-drug interactions across different study populations. Further studies are necessary to provide a better understanding of the prevalence and clinical impact of clinically relevant statin-drug interactions, and the medications most frequently contributing to statin-drug interactions in older people.
**Polypharmacy in older people with cancer: how many medicines are too many?**

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Introduction. Polypharmacy is often defined as use of five or more medicines. However, it is unclear if there is an optimal polypharmacy cut-off for predicting clinically important adverse events.

Aims. To determine the sensitivity and specificity of a range of common polypharmacy cut-offs for predicting frailty, physical function and falls in older people with cancer.

Methods. Between January 2009 and July 2010 385 patients aged ≥70 years presented to the medical oncology outpatient clinic at the Royal Adelaide Hospital and completed a structured data collection instrument including medicine use and falls. Frailty was determined using weight loss (patient self-reported over previous 6 months), exhaustion (CES-D), physical function (SF-36), instrumental activities of daily living and Karnofsky Performance Scale. Receiver operating characteristic (ROC) curves were derived to examine the sensitivities and specificities for varying definitions of polypharmacy in relation to frailty, physical function and falls (≥2 in previous 6 months).

Results. Patients had a mean age of 76.7 years and used a median of 5 medicines. Using Youden’s index, the optimal polypharmacy cut-point was 6.5 medicines for frailty (sensitivity 70.0%, specificity 67.0%), 6.5 for physical function (49.3%, 80.2%) and 5.5 for falls (73.0%, 59.2%). For polypharmacy defined as ‘five or more medicines,’ the sensitivity and specificity were acceptable for frailty (77.5%, 44.9%), physical function (68.6%, 58.0%) and falls (75.7%, 44.5%).

Discussion. Our results suggest no one polypharmacy cut-off is optimal for predicting all adverse events in older people with cancer. The value of a specific polypharmacy cut-off is likely to be dependent on the clinical and medicine use characteristics of the population to which it is applied. However, the common polypharmacy cut-off of ‘five or more medicines’ appears reasonable to use as a prompt for referral for a comprehensive medication review in the patient population.

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**Identifying the impact of medication complexity on chronic disease**

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Introduction. Medication complexity refers to the overall difficulty associated with following a medication regimen. Greater responsibility has been placed on patients to manage their chronic disease on a daily basis in the community. They face many challenges that result from polypharmacy due to multiple co-morbidities, medication alterations and food and drug interactions. This contributes to a complex medication regimen that without proper knowledge and management can impact on a patient’s outcome.

Aims. We aimed to review the literature to determine the different facets of medication complexity and its impact on patient management of their chronic disease.

Methods. A narrative review was conducted using the search engines of MEDLINE and EMBASE for articles related to medication complexity, and the Medication Regimen Complexity Index (MRCI). This was limited to the elderly in EMBASE to further narrow results. The PRISMA statement was used to provide a structure in which the articles were assessed.

Results. In total 23 studies met the inclusion criteria, comprising of 10 cross-sectional, 4 retrospective cohort, 4 validation, 2 prospective cohort, and 3 separate studies of quasi-randomised, before/after, and correlational design. All studies were based on improving patient adherence by altering medication complexity. Various factors were identified as affecting medication complexity including age, knowledge, comorbidities, hospitalisation, mental health, cognitive function and medication related factors such as dosage form, frequency and additional directions.

Discussion. Reducing medication complexity produced better adherence in the majority of studies. However, in order to address medication complexity, it needs to first be quantified through use of the MRCI. This enables objective assessment of potential challenges and allows a targeted approach towards the various factors that could negatively affect patient medication regimen adherence. The older population are particularly susceptible as they have increased comorbidity and are more vulnerable to adverse events. Medication complexity needs to be addressed in order to improve adherence and benefit patient outcomes in the chronic setting.
Medication Regimen Complexity Index: An indicator of bleeding risk
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Introduction. Medication complexity refers to the overall difficulty associated with adhering to a therapy and impacts negatively on patient outcomes. Anticoagulants are being increasingly used and are a large cause of medication related adverse events.

Aims. To determine if the Medication Regimen Complexity Index can be used as an indicator of patients at risk of bleeding.

Methods. A case control study of 487 participants on warfarin therapy was conducted. Factors impacting on warfarin management and patient outcomes were determined. The MRCI was used to quantify complexity and odds ratios (OR) with 95% confidence intervals calculated to determine factors impacting on bleeding risk.

Results. The calculated MRCI was right skewed with a mean of 19.12 (3-65.5) and median of 17. 33.33% of cases were found to have a high MRCI compared to 18.77% of controls. After adjusting for patient and psychosocial factors, participants having a high MRCI were found to be 2.24 time more likely to be at significant risk of bleeding (95% CI 1.32-3.00).

Discussion. Anticoagulants are associated with serious adverse events and given their rise in use, there needs to be an improvement in identifying patients at risk of bleeding. The MRCI is a validated tool. Having a high MRCI was associated with a greater likelihood of bleeding risk. The MRCI has been found to be a good indicator of bleeding risk and has the potential to be used in the clinical setting to direct warfarin management.

Medication-administration problems in older people in Sweden: a population-based case-control study
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Introduction. In older people, functional ability such as cognitive status often declines. Medication administration problems (MAPs) such as difficulties to remember, handle or administer medication can be the result.

Aims. To investigate factors associated with MAPs in older people.

Methods. Data were collected as part of the Swedish National Study of Aging and Care Kungsholmen (SNAC-K) study, a prospective population-based cohort study including people aged ≥60 years. MAPs were self-reported by participants during structured physician interviews. Using a case-control design, people with MAPs were individually matched 1:1 (age, sex and number of medications) with those without MAPs. The cognitive status was assessed using the Mini-Mental State Examination (MMSE). Medication regimen complexity was calculated using the 65-item validated Medication Regimen Complexity Index (MRCI). Mann-Whitney U tests were used to compare clinical, demographic and medication-related factors in people with and without MAPs.

Results. Of 3348 participants, 1891 participants had documentation regarding the presence or absence of MAPs. Overall, 10.7% (n=202) of participants self-reported one or more MAPs. The median age of people without MAPs was 73.0 years (range 60.0-105.0) versus 81.0 years (range 60-104.0) in people with MAPs. 1149/1689 (68%) of those who did not reporting MAPs and 161/202 (79.7%) of those who reported MAPs were women. The median number of medications in people without MAPs was four (range 1-23) versus seven (range 1-19) in people reporting MAPs. In the matched group, people with MAPs were more likely to have lower MMSE ($U=14938$, $p<0.001$, two-tailed). Mean MRCI for people with and without MAPs was 20.2 and 18.7, respectively. There was no significant difference in MRCI between people who did and did not self-report MAPs ($U=21637$, $p=0.29$, two-tailed).

Discussion. People with MAPs were more likely to have lower MMSE. In people with and without MAPs medication regimen complexity was not significantly different.
Inhibition of platelet aggregation by vanilloid-like agents is not mediated by transient receptor potential vanilloid-1 (TRPV1) channels or cannabinoid (CB1, CB2) receptors
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Introduction. Vanilloid-like agents, including capsaicin (CAP), N-arachidonoyl-dopamine (NADA) and N-oleoyldopamine (OLDA), inhibit platelet aggregation (Almaghrabi et al 2014), and hence may protect against CVD. Aim. To determine whether inhibition of platelet aggregation by vanilloid-like agents is mediated by TRPV1 channels and/or cannabinoid receptors.

Methods. Platelet-rich and -poor plasma were obtained from venous blood of healthy subjects. The effects of 50 μM CAP and NADA on aggregation induced by arachidonic acid (AA, 300 μg/mL) and of 50 μM OLDA and NADA induced by collagen (4 μg/ml) were determined in the absence and presence of TRPV1 (SB452553), CB1 (AM251) and CB2 (AM630) antagonists (10, 50 μM). Aggregation data (% maximum; mean±SEM; all n=4) were compared using ordinal logistic regression. Platelets were counted pre- and post-incubation.

Results. CAP and NADA significantly inhibited AA-induced aggregation (0 vs 50 μmol/L, 67.4±10.6% vs 29.6±14.5%, p<0.05, 66.8±9.9% vs 49.6±12.7%, p<0.05, CAP and NADA, respectively). SB452553, AM251 and AM630 did not affect CAP- or NADA-induced inhibition of aggregation. OLDA and NADA significantly inhibited aggregation induced by collagen, with the effect of OLDA enhanced significantly (p<0.05) by SB452553 (50 μM) (84.6±3.1% vs 32±17%) compared to OLDA alone (65.3±10%). The effect of NADA (86.9±2.8% vs 43.7±16.5%) was enhanced by 50 μM AM630 (13.4±2.8%, p<0.005). Finally, no vanilloid/endovanilloid or antagonist had an effect on platelet count.

Discussion. The inhibitory effects of capsaicin, OLDA and NADA on platelets are not mediated through TRPV1, CB1 or CB2 receptors. However, blocking TRPV1 and/or CB2 receptors may contribute to the action of vanilloids.


Evidence against a role for pannexin-1 channels in α1-adrenoceptor-mediated vasoconstriction in resistance arteries
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Introduction. Recent reports have provided evidence for a new concept that in small resistance arteries α1D-adrenoceptor-mediated contraction is intimately linked to pannexin-1 hemichannels that open to allow the release of ATP, from the smooth muscle effector cell, that acts back on P2Y purinoceptors to cause contraction (Billaud et al, 2011). This concept relied largely on the use of mefloquine 10-20 μM acting as a selective pannexin-1 blocking agent to completely inhibit the contraction to phenylephrine, but not K+ 40 mM. Lower concentrations of mefloquine had no effect.

Aims. The aim of the present study was to explore the specificity of mefloquine at 10 μM for pannexin-1 channels and the role of these channels in small artery contraction.

Methods. In mouse and rat isolated small resistance arteries, either pressurised or set up for wire myography, the effects of mefloquine on contractions to K+, phenylephrine and a range of vasoconstrictor agents were assessed and compared with the pannexin-1 inhibitor carbenoxolone.

Results. Mefloquine had a wide range of inhibitory actions at 10-20 μM, some 200-fold above the concentrations previously shown to inhibit expressed pannexin-1 channel activity. Mefloquine 3-10 μM inhibited phenylephrine, U46619, vasopressin, endothelin-1, sympathetic nerve stimulation and K+ 40 mM-mediated contractions in rat and mouse small mesenteric, and mouse thoracodorsal, arteries. Carbenoxolone 1-100 μM did not inhibit the contractile responses to these vasoconstrictor agents in small resistance arteries.

Discussion. The present study demonstrates that in small resistance arteries there is no evidence that pannexin-1 channels releasing ATP have any role in the constrictor actions of α1D-adrenoceptor activation and cautions the use of mefloquine at high concentrations that appear to elicit non-specific inhibition of various cellular mechanisms.

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

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

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

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

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


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

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

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

Results. Mean arterial pressure (MAP) was higher in low diet/angiotensin II-infused mice (93.7±4.3 mmHg) compared to saline-infused mice (86.9±6.8 mmHg, P<0.05). MAP was not different between high diet/angiotensin II-infused (85.4±6.4 mmHg) or saline-infused (85.4±2.9 mmHg) mice. Ach stimulated a relaxant response in low diet/saline-infused mice (28.9±6.0%; EC50, 0.22±0.05 µM). Relaxation was negligible in low diet/angiotensin II-infused mice (6.7±8.0%). A similar relaxant response was observed in high diet/saline-infused (13.2±2.1%, EC50, 0.49±0.24 µM), and angiotensin II-infused mice (19.3±3.1%, EC50, 0.92±0.23 µM).

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

VCP746: A cardioprotective adenosine receptor agonist with minimal haemodynamic effects
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Introduction. It is well accepted that adenosine receptor (AR) activation provides powerful cardioprotection. However, AR activation is also associated with undesirable side effects in the form of profound heart rate (HR) and blood pressure (BP) reduction. This has hindered the transition of AR agonists into the clinic.

Aims. The aim of this study was to determine the effect of VCP746, an A1/A2B AR agonist, on infarct size, HR and BP.

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

Results. Infarct size was found to be significantly reduced with VCP746 treatment compared to the vehicle-treated ischaemia/reperfusion control group (Figure inset; 14.7 ± 2.6% vs 32.1 ± 4.2% of area at risk, respectively; n = 5, P<0.05). Co-treatment with SLV320 and PSB603 (A1AR and A2BAR antagonist respectively) attenuated the infarct-sparing effect of VCP746. VCP746 also had minimal effects on HR and BP (maximum HR reduction: 6.3 ± 6.7 beats per min, BP reduction: 3.8 ± 1.6 mmHg; n = 5) as opposed to the profound bradycardia and hypotension mediated by N6-cyclopentyladenosine (CPA), a prototypical A1AR agonist (maximum HR reduction: 325.3 ± 64.4 beats per min, BP reduction: 103.0 ± 11.1 mmHg; n = 5).

Discussion. The results suggest that VCP746 is able to reduce infarct size and requires both the A1AR and A2BAR to mediate its cardioprotective effect. VCP746 also has minimal effects on HR/BP in vivo at doses that are known to result in cardioprotective plasma concentrations, unlike CPA.

Major role of endothelial nitric oxide release in the responses to noradrenaline in arteries isolated from different regions of the rat cerebral circulation
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Introduction. Cerebral arteries receive an abundant sympathetic innervation originating mainly from the superior cervical ganglia. However, the precise role of the sympathetic nervous system in regulation of cerebral blood flow is unclear. For example, studies in rat cerebral arteries have not been conclusive regarding the effects of adrenoceptor activation.

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

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

Results. Noradrenaline caused a weak contraction in ACA and MCA (~17% of maximum K+-induced contraction) that was significantly enhanced (~70%) by inhibiting the production of NO with L-NAME (Nω-nitro-L-arginine methyl ester) or in the presence of β-adrenoceptor antagonists, propranolol, atenolol (β1) or ICI 11855 (β2). Methoxamine (α1-agonist) also caused contraction in ACA and MCA, but not in PCA or BA. In all cerebral arteries pre-contracted with vasopressin, noradrenaline induced relaxation (60-80%) while isoprenaline (β-agonist) caused maximum relaxation. Endothelium removal or pre-treatment with L-NAME abolished relaxation to noradrenaline and isoprenaline. The relaxation to noradrenaline was also antagonised by all β-adrenoceptor antagonists.

Discussion. This study demonstrated that noradrenaline causes both contraction and relaxation of the arteries of the anterior cerebral circulation (ACA and MCA). The contraction is related to the α1-adrenoceptors which may be limited to the anterior parts of the rat cerebral circulation. The relaxation to noradrenaline is mediated by both β1- and β2-adrenoceptor activation and is dependent on the release of endothelial NO. There was no evidence of β-adrenoceptors located on rat cerebral artery smooth muscle mediating the relaxation induced by noradrenaline and isoprenaline. Therefore, the response to sympathetic stimulation in the cerebral circulation may be directly modified by endothelial dysfunction.
Genetic deletion of the complement receptor C5aR1 has no effect on angiotensin II-induced hypertension and vascular remodelling
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Introduction. Inflammation and immune system activation are now recognised as important contributors to the pathogenesis of hypertension and vascular damage. The complement system is a critical component of the innate immune system, and the complement peptide C5a is a potent pro-inflammatory mediator involved in both innate immune responses and adaptive immunity.

Aims. To determine whether genetic deletion of the primary receptor for C5a (C5aR1) affects the hypertension and microvascular remodelling caused by angiotensin (Ang) II.

Methods. Adult male C5aR1 knockout (KO) and wild type (WT) mice were anaesthetized with inhaled isoflurane and radiotelemetry probes implanted for the direct measurement of blood pressure. Osmotic pumps were implanted (sc) to administer either saline or Ang II (1000 ng/kg/min) continuously for 14 days. Pressurized myography was used to examine contractility, endothelial function, structure and mechanics in mesenteric resistance arteries.

Results. Basal 24 h mean arterial pressures (MAP) were similar in WT and C5aR1 KO mice (99.9±1.7 and 97.5±1.9 mmHg respectively, n=9). Infusion of Ang II for 14 days increased MAP by a similar amount in both WT and C5aR1 KO mice (to 147.2±11.2 and 136.2±4.4 mmHg respectively, n=3-4, *P<0.05 vs saline). Pressurized myography showed no differences between groups in microvascular contractility, endothelial function, or endothelium-independent relaxation (as measured by responses to phenylephrine, acetylcholine and sodium nitroprusside). There were no differences in microvascular structure (lumen diameter, media:lumen ratio, medial cross-sectional area) between saline-treated WT and C5aR1 KO mice (n=4-6). Ang II infusion caused inward vascular remodelling, and significantly reduced lumen diameters and increased media:lumen ratios in both WT and C5aR KO mice (n=4-6). Stress-strain curves were used to assess vascular stiffness, and were similar in all groups.

Discussion. These results demonstrate that a lack of complement C5a-C5aR1 signalling does not affect Ang II-induced hypertension and vascular remodelling in the mouse, suggesting complement-mediated inflammation does not contribute to Ang II-induced vascular pathology.

A flavonoid rich extract from Carpobrotus rossii improves glucose tolerance but not lipid profile
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Introduction. We have previously shown that a crude extract of the native plant Carpobrotus rossii (CR), lowers lipids in healthy rats (Pirie et al., 2014). Hyperlipidaemia, glucose intolerance and insulin resistance are features of type II diabetes and/or metabolic syndrome.

Aims. To determine whether consumption of CR extracts improves glucose tolerance and lipid profile in insulin resistant mice and hyperlipidaemic rats, respectively.

Methods. Glucose tolerance was assessed in 6 week old male C57/BL6 mice fed either a normal (Norm, 9% fat w/w) or high fat (HFD 22% fat w/w) diet, with or without crude (HFD+Crude) or flavonoid-rich extract (HFD+FLAV) for 28 days. Glucose responses to i.p. injection of glucose (2 g/kg bw) were measured at regular intervals over 2 h. Plasma lipids were measured in male Sprague-Dawley rats (~150g) on a high fat (10% fat, 2% cholesterol w/w) (HFR) or a standard rat chow diet (5% fat w/w) (NDR) for 28 days. Additional HFR animals were supplemented with crude CR (HFR+Crude), CR flavonoids (HFR+FLAV) or simvastatin at 2mg/kg body weight (HFR+S). Supplementation (70 mg/kg/day crude equivalent) was via drinking water. Responses were compared using two way ANOVA followed by Fisher’s post-hoc test.

Results. HFD+FLAV supplemented mice had significantly lower blood glucose 45 to 90 min post glucose challenge (all P<0.05), compared with HFD controls. None of the treatments improved the lipid profile in HFR rats.

Discussion. CR flavonoids, but not the crude extract, improved glucose tolerance in insulin-resistant mice. Investigations to determine the effects of the CR flavonoids on other aspects of metabolic syndrome are recommended.

Endothelium-dependent cAMP and cGMP responses to serelaxin in human smooth muscle cells: role of nitric oxide and prostanoids
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Introduction. The phase III clinical trial, RELAX-AHF, showed that 48 hour infusion of serelaxin (recombinant H2 relaxin), caused marked vasodilation in patients with acute heart failure (Ponikowski et al. 2013). However, the precise cellular mechanism(s) associated with its vascular effects in humans are poorly understood.

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

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

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

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

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

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Introduction. Hydrogen sulfide (H\textsubscript{2}S) is an antioxidant (Al-Magableh 2014) and may be a useful therapeutic agent under conditions of increased oxidative stress.

Aim. To determine if the reported enzymatic pathways for H\textsubscript{2}S production, CSE, cysteine aminotransferase (CAT) or D-amino acid oxidase (DAO) in concert with 3-mercaptosulfurtransferase (MST) or cystathionine-\gamma-synthase (CBS) are present in the resistance vasculature and if so, what their functional role is in H\textsubscript{2}S mediated vasoregulation.

Methods. Small mesenteric arteries were collected from male Sprague-Dawley rats (10-14 weeks old). CSE, CAT, DAO, MST and CBS protein levels were assessed via western blotting and cellular location via immunohistochemistry. Vasorelaxation responses elicited by the H\textsubscript{2}S precursors, L-cysteine or D-cysteine, in the presence and absence of inhibitors of CSE, CAT, DAO, MST and CBS were examined using myography.

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

Discussion. These data show that CSE-derived but not CBS nor DAO-MST derived H\textsubscript{2}S contribute to vasoregulation in resistance arteries. Further, this is the first demonstration that CAT-MST-derived H\textsubscript{2}S has vasoactive effects.

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

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

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

Results. Brain infarction was reduced by ~35% by IL-33 or IL-4 treatment, as compared to vehicle (26.09 ± 3.136 mm³, 29.10 ± 3.657 mm³ and 43.77 ± 4.589 mm³, respectively, n = 13-16, both P < 0.05). However, mortality and neurological deficit were exacerbated by IL-33 and IL-4 (n ≥ 18; both P < 0.01). Flow cytometric analysis indicated that IL-33 reduced pro-inflammatory Ly6Chi monocytes in ischemic brains, as compared to vehicle (166.6 ± 94.68 versus 782.2 ± 279.1 cells, respectively, n = 5-9; P < 0.05). Ongoing studies suggest that combined IL-33 and antibiotic therapy improves functional recovery compared with IL-33 alone.

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

Improving warfarin education in community patients
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Introduction. Patient education has been identified as an important factor associated with the safe use of warfarin. Aims. To assess the warfarin education received by a Melbourne population of older, community-based warfarin patients and to examine the relationship between warfarin education and bleeding risk.

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

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

Discussion. Adequate patient education was not associated with a decreased risk of bleeding in older warfarin patients. Conversely, doing poorly on specific WET questions was associated with increased risk. Patients that display poor health literacy, possible depression or are visually impaired may require more personalised education. Thus, future research should aim to improve the warfarin education delivered to patients in order to reduce warfarin related adverse events.
Improving student outcomes and perceptions by enhancing engagement
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Introduction. This paper reports the results of a case study on the use of reflective practice to enhance student engagement and student success in a first year biosciences course at Griffith University.

Aims. The study was undertaken to evaluate different teaching methodologies, learning activities and assessment strategies and determine those that gave positive outcomes, with the aim of incorporating them into the course to enhance student engagement and success. Specifically, the study examined the development and modification of a course aimed at engaging student interest in contemporary issues and current research in the biosciences.

Methods. Through the course of the study, the curriculum was developed and adapted to maximise student engagement and a suite of teaching modalities and philosophies were implemented and trialled. All teaching methods and activities trialled have received recent interest and all are purported to enhance student engagement. Each modification was critically examined in terms of its effect on student outcomes and on student perceptions (as determined via anonymous student questionnaires).

Results. The course Topics in Biosciences (1003 BPS) at Griffith University, Australia was developed with the aim of engaging first year university students in the biosciences and thereby aid in increasing student retention rates and the transition of students to the second year of their studies. The course incorporated learning activities that have previously been shown to have positive effects on student engagement including collaborative group work, writing to learn activities as well as oral and written presentations. Incorporation of other teaching practices which have been established to positively influence student engagement and success such as clear and rapid feedback on assessment, directed approaches to group assignment and in lecture activities to engage student participation were all included. The result was a well-rounded course that achieved good student engagement and success rates and that was positively received by the student cohort.

Discussion. Results from this study strongly indicate a positive influence for incorporating teaching activities that encourage active learning and engagement (such as in-lecture quizzes, collaborative group presentations, writing-to-learn activities) into the course structure. A clear correlation between incorporating these teaching practices with both student outcomes and student perceptions with the course was noted.

Development of a smartphone ‘app’ for converting brand names of drugs to approved drug names to assist trainee doctors in taking drug histories and in prescribing
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Introduction. The World Health Organisation’s Approved names of drugs are used exclusively in the teaching of pharmacology to medical students. Likewise, refereed medical literature use Approved names and not brand names of drugs primarily because brand names can vary between countries depending on copyright. Unfortunately, most patients only know the drugs that they are taking by the brand name, shown on the label of drugs from their community pharmacy. With the increase in widely used drugs coming off patent, there has been a proliferation in brand names with some widely used drugs having 6 or more brand names in Australia. This makes it difficult for medical interns to take drug histories, to assess both clinical needs for these drugs and potential drug interactions.

Aim. The latest generation of smart phones with touch screen input, large storage and computing capacity makes them ideal as portable computers to assist trainees in prescribing (Haffey et al 2013). We have written an application (‘app’), specific for use in Australia, which will assist trainees deal with brand names that they do not know.

Methods. Approved drug names (n = 840) and corresponding brand names (n = 1762) were taken primarily from the Pharmaceutical Benefits Scheme website (www.pbs.gov.au). This data was compiled into an ‘app’ that runs on any modern browser and is agnostic to the operating system (e.g. iOS, Android, Windows). Users must access the ‘app’ once with an internet connection and the dataset is then stored locally on their device and can be accessed offline until the device is turned off.

Results and Discussion. When a brand name is entered into the phone screen, it quickly goes to a new screen showing the Approved name in uppercase and below are listed the appropriate brand names in alphabetical order in lower case. Additional brief information to prompt the memory of interns is the clinical classification and uses of the Approved drug (e.g. antidepressant, broad spectrum antibacterial, proton pump inhibitor etc) followed by the accepted pharmacological Mode of Action of that drug.

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

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Introduction. Pannexin-2 (Panx2) is a member of the novel group of membrane spanning protein channels, pannexins, and is primarily found in the CNS (Bond et al, 2014). Definitive Panx2 function has yet to be elucidated, and since no studies have examined Panx2 in the intestine, it may have important physiological roles in the enteric nervous system (ENS) which contains chemically coded neuron populations similar to those found in the CNS.

Aims. The present study characterised Panx2 expression and localisation in the human colon in health and disease.

Methods. Immunohistochemistry was conducted on cross-sections of intact human colon tissues to determine Panx2 localisation and co-localisation with neuronal markers for intrinsic and extrinsic neurons. Colon specimens were separated into mucosa and muscularis layers for determining Panx2 gene and protein expression using quantitative real-time PCR and Western blot, respectively, in control, ulcerative colitis (UC) and Crohn’s disease (CD) colon.

Results. Intense Panx2 immunoreactive staining was found in myenteric and submucosal plexuses, where Panx2 was localised to neuronal cell bodies, as well as nerve fibres. Double labelling immunohistochemistry demonstrated co-localisation of Panx2 with Class III β-tubulin, neuronal nitric oxide synthase, substance P, vesicular acetylcholine transporter, and calcitonin gene-related peptide, indicating widespread Panx2 expression in neurons such as motorneurons, and neurons involved in pain sensation. Molecular studies revealed a 3.4-fold higher level of Panx2 mRNA in ascending compared to sigmoid muscularis (**p < 0.05), despite similar protein levels. Similarly, UC muscularis showed a ~35-fold up-regulation in Panx2 mRNA (**p < 0.05), but not in protein.

Discussion. Dense and broad Panx2 expression in enteric neurons suggests important roles in intercellular signalling, especially in the modulation of ENS mediated sensation, motility and secretion. The dense expression of Panx2 in the ENS implies that Panx2 may be important in regulating neuronal function, and may contribute to the dysmotility symptoms seen in UC.


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

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Introduction. ATP released from intestinal epithelial cells behaves as a sensory mediator that excites subepithelial sensory nerve terminals to regulate enteric motor reflexes. The mechanisms of ATP release in the human colon remain totally unexplored, but may involve calcium homeostasis modulator 1 (CALHM1), an ion channel recently shown to behave as an ATP-release conduit in primate taste bud cells (Taruno et al., 2013).

Aims. This study was aimed to localise CALHM1 expression in the human colon, and to determine whether this ion channel mediates colonic ATP release during Ca2+ depletion and mechanical stimulation.

Methods. Immunohistochemistry was conducted on cross-sections of intact human colon tissues to determine CALHM1 expression in the human colon, and to determine whether this ion channel mediates colonic ATP release during Ca2+ depletion and mechanical stimulation.

Results. Immunofluorescence staining showed CALHM1 expression on many cell types of the human colon, including smooth muscle cells, blood vessels, and enteric ganglia, as well as very dense staining on the membranes of epithelial cells. Both Ca2+ depletion and stretch significantly stimulated ATP release from CRL1790 cells, with levels peaking at 2 min and 10 min, respectively. Ca2+-free solution produced a 1.5-fold increase in ATP release (P<0.001, one-way ANOVA) which was reduced to baseline levels by incubation with RuR. Stretch produced a 3.5-fold increase in ATP release (P<0.01), which was significantly enhanced by RuR.

Discussion. CALHM1 is widely expressed in the human colon. The different time courses suggest that ATP release in response to Ca2+ depletion and stretch may occur via different mechanisms/pathways. CALHM1 appeared to be a primary mechanism of ATP release in the Ca2+-free condition, whereas enhancement of ATP release by RuR implies that other pathways participate in stretch-mediate ATP release. These pathways may involve a compensatory mechanism that overrides the pharmacological blockage of CALHM1. Alternatively, there may be a negative autoregulatory mechanism associated with CALHM1 channels, which was impaired by blockage of CALHM1.

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

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Introduction. Intravesical chemotherapy for bladder cancer limits systemic absorption of cytotoxic drugs, but significant local urological side effects including dysuria and urgency still occur. No studies have investigated the potency of cytotoxic therapies on normal urothelial cells, which changes to may be responsible for the reported side effects. During bladder filling the normal urothelium releases mediators (ATP, acetylcholine, PGE₂) which activate sensory nerves, but intravesical treatment with cytotoxic drugs may possibly disrupt this process.

Aims. This study compared the toxicity of gemcitabine in cultured bladder urothelial cell lines (cancer versus non-cancer cells), and investigated drug induced changes in basal and stretch-induced release of urothelial mediators.

Methods. The human urothelial bladder cancer cell lines RT4 and T24, and non-cancer UROtsa urothelial cells were treated with gemcitabine. Cell viability was measured 72 hours post-treatment using resazurin reduction assay. Basal and stretch-induced release (using hypotonic solution) of mediators was measured from urothelial cells post-gemcitabine treatment.

Results. A concentration-dependent reduction in cell survival was seen 72 hours post-gemcitabine treatment. The potency of gemcitabine on cancer cells was approximately 100,000 fold greater than its potency on non-cancer urothelial cells (p<0.001). In urothelial cells treated with 4mg/mL gemcitabine, basal ATP release was significantly increased compared to control (16.4±2.5nM to 6.0±0.7nM, n=8-13, p<0.05). Levels of basal and stimulated acetylcholine and prostaglandin E₂ release from urothelial cells were unchanged after incubation with gemcitabine.

Discussion. Gemcitabine was selectively toxic to malignant urothelial cells with comparison to the normal urothelial cells, although the mechanism of this currently unknown. The increased release of urothelial ATP after incubation with gemcitabine may contribute to the urinary urgency and pain experienced by patients after intravesical treatment with this cytotoxic drug.

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**The α₁-adrenoceptor antagonist prazosin enhances sensitivity of hypoxic prostate cancer cells to irradiation**

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Introduction. Tumour hypoxia is an important determinant in the outcome of many malignancies and is associated with increased resistance to radiotherapy in solid human prostate tumours and biochemical relapse. In vitro, α₁-adrenoceptor antagonists are suggested to have cytotoxic effects on prostate cancer cells but little is known about their effects in hypoxic conditions and in the presence of irradiation.

Aims. The aim of this study was to investigate the potential radiosensitising actions of prazosin in prostate cancer cells irradiation under normoxic and hypoxic conditions.

Methods. Human androgen receptor negative prostate cancer PC-3 cells were treated acutely (2 h) with prazosin (0-100 μM) and irradiated (6MV, 5Gy) under normoxic or hypoxic conditions. Hypoxia (<0.2% oxygen concentration in culture medium) was generated by purging controlled-atmosphere chambers containing cells with oxygen free nitrogen for 38 min. Following acute prazosin treatment, cells were washed and incubated in drug-free medium for 5 days. Cell survival was determined by resazurin reduction.

Results. Following acute treatment and 5 day recovery, prazosin toxicity appeared to be selective for hypoxic PC-3 cells (n=5, P<0.05). Under normoxic conditions, irradiation resulted in approximately 75% reduction in PC-3 survival and the presence of prazosin (10-100 μM) during irradiation did not affect cell survival. Hypoxia protected PC-3 cells from irradiation-induced cytotoxicity, with a 3-fold increase in cell survival compared to normoxic cells (n=5, P<0.001). Prazosin increased sensitivity of hypoxic PC-3 cells to irradiation in a concentration-dependent manner, with 30 and 100 μM prazosin reducing survival to that of normoxic cells (n=5, P>0.05).

Discussion. Prazosin has cytotoxic actions, which are enhanced in the presence of acute hypoxia. Furthermore, prazosin abolished hypoxia-mediated resistance to irradiation in PC-3 cells. These findings suggest prazosin, or other α₁-adrenoceptor antagonists, may be useful in combination with radiation therapy to overcome radioresistance of hypoxic solid prostate tumours.
The differing effects of doxorubicin treatment on neurogenic detrusor responses of young and old porcine bladders
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Introduction. Intravesical chemotherapeutic agent doxorubicin is commonly used for the treatment of superficial bladder cancer. It causes significant urological adverse effects including dysuria, increased urinary frequency, and urgency. Despite the large number of patients receiving this agent intravesically and the high percentage suffering urological adverse effects, there have been no investigations of the actions of this agent on the non-cancerous tissues of the bladder.

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

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

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

Discussion. The results suggest that doxorubicin, when administered intravesically for bladder cancer, may alter the release of neurotransmitters within the detrusor smooth muscle depending on the age of the bladder. Hence, the effects of the drug on older porcine bladders may be more relevant to real life patients as the majority of those affected by bladder cancer are within the senior age range.
Alterations in bladder urothelial acetylcholine, ATP, prostaglandin E2 and inflammatory cytokines by the chemotherapeutic agent epirubicin
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Introduction. Epirubicin is a cytotoxic agent administered intravesically for the treatment of superficial bladder cancer. During treatment, the drug comes into close contact with the urothelium, and after the treatment, patients may suffer urinary adverse effects.

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

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

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

Discussion. These findings indicate that inflammatory cytokines interleukin-6 and interleukin-8 are induced and urothelial mediator release is affected by treatment with epirubicin at clinically relevant concentrations and durations of treatment. These changes may play a role in the adverse effects observed in patients following intravesical epirubicin treatment.
KCl potentiates subsequent responses to noradrenaline of the porcine urethra by enhancing ROCK and Ca\textsuperscript{2+} release.
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Introduction. Potassium chloride (KCl) has long been used in pharmacology to induce depolarisation and produce a non-receptor mediated contraction of smooth muscle to test tissue viability and also allow normalization of responses. However, recently it has been shown that KCl may also cause calcium sensitisation by activating the Rho kinase (ROCK) pathway in some smooth muscle preparations (Borysova \textit{et al.}, 2011). The effect of KCl on smooth muscle sensitization and contraction in the urethra has not been investigated.

Aim. To investigate the effects of KCl-pretreatment on subsequent noradrenaline-induced contractions of the porcine urethra.

Methods. Strips of urethra were mounted in organ baths at 37\textdegree C in Krebs-bicarbonate solution. Test tissues were pre-contracted with KCl (60mM) for 5mins (control tissues were not pre-contracted). After 5mins, the tissues were washed for 30mins and then contracted to noradrenaline (100μM) in the presence or absence of the rho-kinase inhibitor fasudil (10μM), the PKC inhibitor calphostin C (1μM), and the inhibitor of intracellular calcium release cyclopiazonic acid (10μM).

Result. Pre-incubation of tissues with KCl resulted in significant potentiation of subsequent noradrenaline-induced contractions by (83.6±43.48%, Mean±SEM, n=5-10; P=0.05). This potentiation was still observed when PKC was inhibited. However, inhibition of ROCK or intracellular Ca\textsuperscript{2+} release abolished the potentiation of responses by KCl and reduced noradrenaline contractions by 54.6±8.4% and 48.4±11.0% respectively. The potentiating effects of KCl were long lasting and were even greater 3 hours after pre-incubation with KCl (90.34±14.292%, P<0.05).

Discussion/Conclusion. Prior exposure of urethral tissues to KCl causes potentiation of subsequent noradrenaline induced contractions of the urethra, which may be associated with both Ca\textsuperscript{2+} sensitization and enhanced Ca\textsuperscript{2+} release from the sarcoplasmic reticulum.


The role of Rho-kinase in phenylephrine and 5-HT-induced contractile activity of the porcine ureter
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Introduction. Ureteral calculus is frequently accompanied by ureteral colic, which is understood to be caused by constriction of the ureteral smooth muscle (Canda \textit{et al.}, 2007). In a previous study, we showed that porcine ureters from older animals generated greater contractions to α-adrenoceptor stimulation, and smaller contractions to 5-HT (Lim \textit{et al.}, 2013). Recently, calcium sensitisation via the Rho-kinase pathway has been proposed to play a significant role in smooth muscle contraction (de Godoy & Rattan, 2011).

Aims. To investigate the role of Rho-kinase in phenylephrine and 5-HT induced contractile activity in isolated ureters from old and young pigs.

Methods. Contractile responses of isolated smooth muscle to EC\textsubscript{50} (low) and maximal (high) doses of phenylephrine and 5-HT were examined in distal ureteral tissues from young (20 weeks) and older (56 weeks) pigs, in the absence and presence of Rho-kinase inhibitors, Y-27632 (10μM) or fasudil (30μM). Tissues developed spontaneous contractile activity and responses were expressed as AUC as a percentage of the maximal contraction.

Results. Fasudil significantly attenuated (P<0.0001 vs control, n=6) ureteral contractions to low and high doses of phenylephrine by 86.81±4.18% and 81.96±0.97% in younger animals. Fasudil also reduced contractions to low and high doses of 5-HT by 83.69±2.97% and 77.67±3.16% (P<0.0001, n=6). In older animals, similar effects were observed, with contractions to phenylephrine being reduced by 82.01±1.71% and 78.69±3.01%, and to 5-HT, by 80.16±3.43% and 79.31±3.24% at low and high doses respectively (P<0.0001, n=6). The Rho-kinase inhibitor Y-27632 produced similar effects with both agonists in tissues from young and old animals.

Discussion. These results show that Rho-kinase mediates contractile responses to phenylephrine and 5-HT in isolated ureteral tissues and contribution to contraction is similar in ureters from both young and old animals.

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

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

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

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

Conclusions: The present study indicates that EGCG is most efficacious polyphenol among different polyphenols of green tea. Nano Epigallocatechin gallate is effective as it has additive effect in experimental induced colitis, however these results require further confirmation in human studies.
Loss of fenestrations in liver sinusoidal endothelial cells impairs hepatic insulin signaling and glucose homeostasis
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Introduction. The liver plays a major role in the regulation of glucose homeostasis, which was tightly regulated by insulin. Prior to hepatic metabolism, substrates travel through fenestrations in the liver sinusoidal endothelial cells to gain access to hepatocytes. We propose loss of fenestrations (defenestration), such as seen in ageing, will impair the transfer of insulin and glucose across the hepatic sinusoidal endothelium, thus contributing to hepatic insulin resistance.

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

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

Results. Animals treated with p407 showed a significant increase in triglyceride and cholesterol levels compared to control (p<0.001), together with a marked defenestration in the liver sinusoidal endothelial cells. Hepatic volume of distribution as a fraction of the extracellular space for both insulin and glucose were significantly decreased in the hyperlipidemic rats, indicating impeded substrate transfer as a result of defenestration (glucose: 1.54±0.06 control vs 1.10±0.10 P407; insulin: 1.08±0.08 control vs 0.81±0.05 P407, p<0.001). There was a significant up-regulation of Insulin Receptor Substrate-2 mRNA (Control 1.30±0.03 vs P407 2.20±0.30, 1.76 fold change, p=0.02), with a trend for down-regulation of the rate limiting gluconeogenic enzyme Glucose-6-phosphatase mRNA. Limited access of insulin to hepatocellular membrane is shown by a decreased phosphorylation of Insulin Receptor Substrate-1 protein (p=0.045) involved in early insulin signalling pathway. Defenestration also induced hyperinsulinemia (T0: Control 0.61±0.07 vs P407 1.35±0.22, p=0.005; T90: Control 0.94±0.15 vs P407 2.85±0.73, p=0.036) and reduced insulin sensitivity (HOMA index; Control 5.43±0.68 vs P407 11.7±2.06, p=0.014). In addition, there was decreased incorporation of glucose into glycogen in the liver (Control 4.2±0.38 vs P407 2.8±0.48, p=0.042), but not in muscle and WAT in the P407 treated group.

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

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Introduction. The maximum penetration flux of topically applied solutes should be independent of the vehicle, unless the vehicle affects the skin (Zhang, 2009). In this study, we examined how skin penetration fluxes are affected by vehicles reported to interact with the skin barrier to different degrees. We compared in vitro maximum penetration fluxes ($J_{max}$) and mole fraction solubilities in the vehicles ($X_v$) and stratum corneum ($X_{sc}$) for caffeine from various solvent vehicles to evaluate vehicle effects on permeability parameters.

Methods. Solutions of caffeine were dissolved in a range of vehicles and applied to heat separated human epidermal membranes in Franz diffusion cells. Permeated solute concentrations were measured by HPLC and used to calculate permeation parameters. Caffeine solubilities in the vehicles and in stratum corneum were estimated after equilibration with saturated caffeine solutions for 24 hrs.

Results. $J_{max}$ was independent of $X_v$ for inert vehicles with no reported effects on the skin barrier, whereas enhanced caffeine flux was seen with vehicles containing the penetration enhancers oleic acid (OA) or eucalyptol (Eu). There was a linear relationship between $J_{max}$ and $X_{sc}$ for all vehicles, including those containing OA and Eu, while the 60% ethanol/water vehicle deviated from linearity with a reduced flux (see Figure).

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


Redefining normal variability of drug disposition

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Introduction. The pharmacokinetics of many drugs are said to be predictable. This is often used to imply the ease of dosing or dose-adjustments. However, predictability requires both accuracy (lack of bias) and precision (reproducibility). In the context of pharmacokinetics, precision refers to the ability to achieve a specified target concentration in different individuals. Precision is the inverse of the variance between-subjects, i.e. the greater the between subject variability (BSV) the less precise/predictable a parameter is across a patient population. BSV in PK parameters is quantified by the coefficient of variation (CV%). The current convention is that BSV in PK parameters is considered “low” (CV% ≤ 10%), “moderate” (CV% ~ 25%), or “high” (CV% > 40%). [1]

Aims. To explore the range of BSV values in PK parameters in patient populations of preselected drug classes.

Methods. A literature review of population PK studies from various data sources was conducted. Drug classes studied included psychotropics, immunosuppressants, cardiovascular drugs, and antibiotics. Estimates of clearance (CL) and volume of distribution (V) and their corresponding CV% were recorded.

Results. A total of 181 studies involving 95 drugs were found. The mean CV% in CL/F was 40.3% and in V/F was 51.3%. The mean CV% in CL/F in predominately renally cleared drugs was 31% (after accounting for renal function) and those predominately hepatically cleared drugs was 47.4%. Age, sex, weight, and renal function were among the most significant covariates reported across the drug classes.

Discussion. According to the current convention most drugs show “moderate” to “high” BSV. The current convention needs to be recalibrated to consider that a low BSV in CL is < 25%, 25 – 50% is normal, and > 50% is high. Clinically, this means that a normal level of variability in CL would result in a 4- to 5-fold variability in steady state average plasma concentrations and therefore for all drugs with a low therapeutic index, monitoring plasma concentration or response and dose-individualisation will be essential.

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

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Introduction: Inflammation has been implicated in tumour initiation, angiogenesis and metastasis and linked to the development of more aggressive, therapy-resistant estrogen receptor positive breast cancer (Baumgarten and Frasor, 2012). Resolvin D2 (RvD2) is a potent anti-inflammatory lipid mediator. RvD2 is present in plasma at bioactive concentrations and the enzymes involved in its synthesis are expressed by both tumour and stromal cells.

Aim: To investigate the impact of RvD2 on cell processes underlying breast tumour growth and spread.

Methods: Viable cells were enumerated by Trypan-blue exclusion. Transactivation of estrogen response element (ERE) was assessed by transient transfection with an ERE reporter. RT-qPCR was used to examine gene expression. Binding to the estrogen receptor (ER) was investigated by radioligand binding assays. Western blotting and immunofluorescence techniques were used to ascertain ER α nuclear localization.

Results: RvD2 (10-1000 nM) supported the proliferation of the ER-positive breast tumour, MCF-7, but not the ER-negative, MDA-MB-231 cells. RvD2 mitogenesis in MCF-7 cells was prevented by the estrogen receptor antagonist ICI 182,780 (100nM). Furthermore, RvD2 increased ERE transcriptional activity in MCF-7, T47D, BT474 and SKOV3 cells. RvD2 altered the expression of a subset of estrogen-responsive genes. Prior exposure of MCF-7 cells to RvD2 reduced the apparent cytosolic ER density. RvD2 did not directly compete with 3[H]-17 β-estradiol (E2) for ER binding. Confocal immunofluorescence localisation and western blotting studies showed that RvD2 promoted nuclear localization of ERs with a corresponding decrease in cytosol ER density.

Discussion: RvD2 displays significant but indirect estrogenic activities and that it has the potential to play a role in estrogen-dependent breast cancer progression.


Safety and Pharmacokinetics of Metformin in Liver Disease

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Introduction. Metformin is the first-line treatment of type II diabetes mellitus. In the product information, metformin use is contraindicated in patients with hepatic impairment due to concerns of heightening the risk of precipitating lactic acidosis. However, this causal relation is contentious, and there is little research conducted in this area.

Aims. The primary objectives of this study was to evaluate the safety and PK parameters of metformin in subjects with liver fibrosis.

Methods. Liver disease patients currently taking metformin were recruited for this observational cohort study. Subjects were stratified according to degree of liver fibrosis; minimal fibrosis (n = 3) and cirrhosis (n=5). At least one blood sample was collected from each patient. Metformin concentrations in plasma were determined using HPLC. Biochemical parameters such as lactate, bicarbonate and anion gap were also assessed. PK parameters were determined by using TCIWorks (Version 1.0) and a published population PK model1.

Results. The mean (± SD) metformin CL/F was 55 (±16) L/h and the apparent volume of distribution was 185 (±71) L. The mean ratio of metformin CL/F to creatinine CL was 13.5 (±3.4) which was very similar to values found in without significant liver disease, 12.6 (±4.3) 1. The plasma concentrations of lactate and bicarbonate were 2.2 (± 0.7) mmol/L and 25.8 (± 3.9) mmol/L, respectively. The anion gap was 17.1 (±2.3) mmol/L. There was no correlation between metformin concentrations and lactate, bicarbonate or anion gap concentrations.

Discussion. Metformin clearance was similar to that seen in previous literature, with concentrations remaining within the therapeutic range. This study shows that liver disease has no significant impact on metformin PK and safety although further studies are required. Renal function and body weight are still the most important determinants of metformin PK.

Domperidone, QT prolongation and sudden cardiac death – is there really a risk?
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Introduction. Domperidone (DOM) fills a prescribing niche for gastrointestinal motility and chemotherapy nausea and vomiting. The European Medicines Agency (EMA) recently concluded that it poses a significant risk of QT prolongation and sudden cardiac death (SCD) and subsequently restricted the use in Europe.

Aims. To review the risk of QT prolongation and cardiac adverse effects with DOM and provide information to allow prescribers to make informed decisions on usage.

Methods. A search of two bibliographic databases, the EMA website, Micromedex, Lexicomp and reference texts was undertaken for DOM related reports of QT prolongation, cardiac arrhythmias and/or SCD. The New Zealand Centre for Adverse Drugs Reaction Monitoring was also contacted for cardiac adverse event reports with DOM.

Results. Over 30 published papers, EMA documents and other information sources were collated including two studies that met thorough QT study (TQT) criteria (ICH-E14). The first TQT1 was negative while the second2 was marginally positive. Reports of QT prolongation, ventricular arrhythmias and SCD were located (predominantly high/very high-dose IV DOM). With oral DOM, a Dutch case controlled study3 reported an adjusted odds ratio of SCD of 11.4 (95% CI 1.99-65.2), based on only 3 patients out of 1366 cases of SCD. A second nested case-controlled study4 calculated an odds ratio of ventricular arrhythmia or SCD of 1.59 (1.28-1.98) vs. placebo.

Discussion. Based on the results of the two TQT (regulatory agency gold standard for assessment of QT prolongation) DOM does not appear to be strongly associated with QT prolongation at oral doses of 20 mg QID in healthy volunteers. Further, there are limited case reports supporting an association, and the frequently cited case-control studies have significant flaws. While there remains an ill-defined risk at higher systemic concentrations, especially in patients with a higher baseline risk of QT prolongation, our review does not support the view that DOM presents intolerable risk.

1. EMA Assessment Report 06/03/2014 (EMEA/H/A-31/1365)
2. BJCP 2012; 73 (3): 411-21

Microglia-Derived BDNF Modulates Dopamine Circuitry in Opioid Dependent States
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Opioid dependent states are associated with adaptations within the mesocorticolimbic dopaminergic system that contribute to the negative affective state when the drug is absent (withdrawal). Here, we analyze molecular adaptations in the ventral tegmental area (VTA) and their effect on drug reward.

C57Bl/6 mice were made opioid dependent with increasing injections of morphine (10-40mg/kg, i.p) twice daily for 4 days. Opioid dependent animals displayed significant microglial activation in the VTA and blocking microglial activation decreased BDNF expression. Chronic morphine exposure decreased expression of the potassium/chloride (Cl-) co-transporter, KCC2, within VTA GABAergic neurons, which resulted in a loss of Cl- extrusion as measured by fluorescent lifetime imaging. Interfering with BDNF signaling recovered Cl- extrusion in morphine-dependent GABAergic neurons.

Loss of Cl- extrusion is known to undermine GABAergic inhibitory potential, which translates into an increased inhibition in dopaminergic VTA neurons. In support of this, cocaine reinforcement (as measured using the conditioned place preference paradigm) was diminished in opioid dependent animals. Cocaine place preference was restored in opioid dependent animals by cotreatment with microglial inhibitors.

This study provides evidence for disrupted reward circuitry in opioid dependent animals that is driven by microglial activation within the VTA. Reactive microglia release BDNF that precipitates a shift in E_{GABA} within VTA GABAergic inhibitory interneurons leading to dysregulation of dopaminergic circuitry.
Audit of clinical adherence to National Health Medical Research Council (NHMRC) venous thromboprophylaxis (VTE) guidelines in acutely ill older medical inpatients.

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Introduction: Clinical adherence to national and local VTE thromboprophylaxis guidelines is reported to be 40-60% for medical inpatients. Adherence to and applicability of these guidelines to older frail and robust medical inpatients is unknown.

Aims: Amongst frail and robust older medical inpatients, to evaluate the clinical adherence to NHMRC thromboprophylaxis guidelines, assess clinical prescribing behaviour and identify patient groups not described in the guidelines.

Methods: In this prospective cohort study, medical patients aged ≥65 years were recruited from the Emergency Department of Royal North Shore Hospital. The Reported Edmonton Frailty Scale was used to assess frailty. A predetermined protocol was used to assess adherence to the NHMRC guidelines for thromboprophylaxis and cases that did not fit this protocol were classified using expert consensus.

Results: To date, 90 patients with complete data were analysed: 46.7% female, median (range) age 79.5 (65-99). Concordance with the NHMRC guidelines was observed for all 59 participants who received VTE prophylaxis but for only three of the 31 participants who did not receive prophylaxis. This equates to 68.9% overall adherence. There was no correlation between non-adherence and frailty. Frail patients were not significantly more likely to receive heparin or enoxaparin dose reductions than robust patients (frail 34.5%, robust 25.9%, p>0.18). Eleven patients had pharmacologic dose reduction but only one was clearly appropriate due to renal impairment. Of those who received thromboprophylaxis, 55/59 patients received prophylaxis though their whole inpatient stay. There was variable prescribing for drug and dosage observed for groups not explicitly covered by the guidelines (n=4), such as palliative patients and those undergoing minor surgical procedures such as angiograms or pacemaker implantation.

Discussion: There is a need for more evidence to inform clearer thromboprophylaxis guidelines to guide clinical prescribing for older medical inpatients, particularly for those receiving palliative care and minor procedures.

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

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Introduction. Previously, we have suggested that the older-aged living in a low socioeconomic, rental retirement village have a low adherence to medicines and a poor understanding of their illnesses (Doggrell & Kairuz, 2012). However, as the sample size was relatively small for the rental retirement villages it was not clear whether this is a consistent finding.

Aims. The aim was to determine the ongoing management of medicines by the older-aged living independently in a rental retirement village.

Methods. We returned to the rental retirement villages after one and two years, and reassessed the management of medicines by the older-aged living in the village, using semi-structured interviews.

Results. Although similar numbers participated in the study in 2011 (25 participants from 60 units), 2012 (n = 25) and 2013 (n = 23), the cohort changed. Thus, only 9 of the older-aged interviewed in 2011 were re-interviewed in 2012, and similarly, only 9 interviewed in 2012 were re-interviewed in 2013. Nevertheless, the findings over the 3 years were very similar. The participants at the rental retirement village had a mean age ~75 years, each time we interviewed, and less than 50% were adherent at the time of the study and unlikely to have problems in the next 6-12 months. Only about 50% of the participants had a good knowledge of the illnesses, which they were being prescribed medicines for. Participants were taking about 7 medicines each, and cardiovascular/antithrombotic drugs were the most common medicines prescribed in the village, followed by psychotropic and gastrointestinal medicines.

Discussion. The management of medicines by the older-aged living in a low socioeconomic rental retirement villages is poor, and this finding is ongoing and consistent. This supports the need for extra assistance and resources for the older-aged living to manage their medicines in rental retirement villages.

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**Metformin pharmacokinetics in haemodialysis**

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Introduction. Metformin is eliminated largely if not entirely by renal excretion. Currently, it is contraindicated in dialysed patients because there is no data on PK during hemodialysis (HD) at therapeutic doses. Furthermore, excessive retention of metformin could lead to lactic acidosis, a major, although rare, adverse effect of the drug.

Aim. To investigate the PK and safety of metformin in patients on HD.

Methods. Two patients were dosed with 500 mg of metformin after each dialysis session (3 x 500 mg per week) for 4 weeks. Biochemical parameters were measured weekly. Blood was collected 4, 24 and 48 hours post dose after Week 2. HD CL was calculated on 3 occasions by collecting blood entering and exiting the dialyser at various time points for the entire dialysis session (Weeks 2–4). Metformin concentrations were measured by HPLC.

Results. Metformin concentrations did not exceed 5 mg/L and were generally constant between 4 and 48 hours. The dialyser CL of metformin from plasma was very high (191 ± 20 and 183 ± 11 mL/min for patient 1 and 2, respectively) and approached the plasma flow rate (approximately 200 mL/min). The CL of metformin from red blood cells was approximately 80% lower than CL from plasma. The patients tolerated metformin well with no adverse effects. Plasma lactate concentrations remained within the normal range (up to 2.2 mmol/L) and did not increase during dosage with metformin. The anion gap also did not increase but was slightly higher than the normal range (10 to 18 mmol/L) throughout.

Discussion. Metformin is readily dialyzable from plasma. The lower dialyser clearance from red blood cells is due to slow equilibration of metformin between red blood cells and plasma. Further studies are in progress.

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

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Introduction. Older adults, particularly the frail have multiple co-morbidities and are under-represented in pharmacological clinical trials. The safety and efficacy of VTE prophylaxis is unknown in this population.

Aims. To evaluate the utilisation of pharmacological VTE prophylaxis in frail and robust older medical inpatients and investigate the proportion that would have been excluded from previous clinical trials. To describe the prevalence of haemorrhage and VTE in this population.

Methods. In this prospective, cohort study, medical patients (≥65 years) were recruited from the Emergency Department of Royal North Shore Hospital. The Reported Edmonton Frailty Scale, IMPROVE bleeding risk assessment and Padua prediction score were utilised to assess frailty, bleeding risk and VTE risk respectively. Use of VTE prophylaxis and adverse events in hospital were obtained from medical notes and participant questionnaires.

Results. To date, 94 patients have been recruited, 46.8% female, median (range) age 79.5 (65-99) and 44.7% frail. Frail patients had more co-morbidities than robust (3.2±1.6 frail, 2.4±1.6 robust, p<0.05); higher risk of bleeding (31.0% frail, 11.5% robust, p<0.05) and higher risk of VTE (65.9% frail, 24.0% robust, p<0.05). Frail patients were not significantly more likely to receive VTE prophylaxis (73.8% frail, 55.8% robust, p=0.07). Of the patients on prophylaxis, 68.3% to 88.3% would have been excluded from past clinical trials. Minor bleeding occurred in two patients who both received VTE prophylaxis, one frail and one robust. One VTE event occurred in this study in a frail participant who did not receive VTE prophylaxis.

Discussion. Frail older medical inpatients have higher risk scores for VTE and bleeding than the robust. The majority of older medical inpatients would not have been represented in previous clinical trials for VTE prophylaxis, as such further research is warranted to explore the safety and efficacy of VTE prophylaxis in older adults.
Factors associated with a high anticholinergic burden in elderly Australians with and without dementia
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Introduction. Elderly patients taking anticholinergic drugs have been shown to have an increased risk for cognitive decline and dementia and it is recommended that these drugs be prescribed cautiously (Carriere et al, 2009). Aims. To determine the predictors of anticholinergic medication use in community-dwelling older primary care Australian patients with and without dementia. This will help identify patients most at risk.

Methods. The cognitive status of patients aged 75 years or older (n=1044) was determined using a subsection of the revised Cambridge Examination for Mental Disorders of the Elderly (CAMCOG-R; Roth et al, 1986). A CAMCOG-R score of 79 or less was used as a relatively sensitive indicator of dementia. Patient demographics and lists of their medications were collected. Anticholinergic load was determined using the Anticholinergic Drug Scale (Carnahan et al, 2006). Quality of life and depression were determined using an Australian version of the WHOQOL-BREF (Hawthorne, et al 2006) and a 15 item scale (Sheikh et al, 1986), respectively.

Results. The dementia group had a significantly higher anticholinergic load (1.5 vs 0.8; P = 0.002). Multivariate analysis identified several patient factors that were associated with a higher anticholinergic burden including polypharmacy (i.e. taking five or more medications) (P < 0.001), increasing age (P = 0.018), CAMCOG-R dementia (P = 0.003), depression (P = 0.003) and lower physical quality of life (P < 0.001).

Discussion. There is considerable scope for the improvement of prescribing practices in the elderly.

Declining intra-lymphocyte concentrations of mycophenolic acid correlate with the incidence of graft rejection in renal transplant recipients: preliminary results of a prospective study
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Introduction: Mycophenolic acid (MPA), a key immunosuppressant used to prevent rejection following solid organ transplantation, exerts antiproliferative effects on lymphocytes. Routine therapeutic drug monitoring (TDM) of MPA concentrations in plasma is currently recommended to individualise MPA doses, but little is known regarding MPA concentrations at the site of action, within lymphocytes. Intra-lymphocyte MPA concentrations might be better predictors of graft rejection compared to plasma concentrations.

Aims: This study investigated the relationships between intra-lymphocyte and plasma MPA concentrations, and their association to graft rejection in renal transplant recipients.

Methods: Thirty-two renal transplant recipients receiving MPA were included and rejection was determined histologically from routine biopsies performed within 2 weeks post-transplantation. Lymphocytes were isolated from blood (9 mL) and intra-lymphocyte MPA concentrations were quantified using a validated LC-MS/MS method. Corresponding trough MPA plasma concentrations were collected from TDM records.

Results: The association between intra-lymphocyte MPA concentrations and trough MPA plasma concentrations was intra-lymphocyte MPA = 0.33 x plasma MPA + 0.27, \( r^2 = 0.34, P = 0.06 \). In 7 patients experiencing rejection (22%), intra-lymphocyte MPA concentrations were lower compared to those who did not reject, with median ± SD of 0.38 ± 0.48 and 1.1 ± 1.0 ng/10⁷ cells (\( P = 0.05 \)), respectively. There was no significant relationship between trough MPA plasma concentrations and graft rejection (\( P = 0.37 \)).

Discussion: These observations suggest that, despite TDM to minimise inter-individual pharmacokinetic variability in systemic MPA concentrations, intra-lymphocyte MPA concentrations may represent an additional tool to further improve individualisation of MPA dose after transplantation with the purpose of either preventing graft rejection or minimising drug toxicities.

Response to antplatelet drugs in frail and non-frail older inpatients with atrial fibrillation
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Introduction. The utilisation of antplatelet therapy in treatment for cardiovascular diseases is increasing, especially in older people. In frail older people it is unclear whether response to antplatelet therapies is altered.

Aims. To study the platelet function of older inpatients with atrial fibrillation (AF) taking antplatelet drugs and explore differences between frail and non-frail patients.

Methods. We recruited inpatients with AF aged ≥65 years (mean 86±7) from Royal North Shore Hospital, Sydney. Frailty was determined using the Reported Edmonton Frail Scale. Platelet aggregation studies were performed using Whole Blood Impedance Aggregometry (Multiplate). The platelet agonists arachidonic acid and adenosine diphosphate were used to evaluate the response to aspirin and clopidogrel respectively. Cut-off for response to aspirin is an area under the curve (AUC) < 40 units (U). The target AUC in response to clopidogrel is comprised between 20U-42U.

Results. Amongst participants taking aspirin, mean±SD AUC was 15±13U (n=33) overall and did not differ with frailty (18±15U frail, n=20; 11±8U non-frail, n=13; p=0.1); 2 had AUC>40U (both frail). Amongst participants taking clopidogrel AUC was 31±15U overall (n=14), 30±16U in frail (n=8) and 33±15U in non-frail (n=6), p=0.8; 4 had AUC<20U and 3 had AUC>42U. Correlation of frailty and arachidonic acid-induced platelet aggregation in participants taking aspirin showed an increased variability in response to aspirin with increased frailty score.

Discussion. Nearly all participants responded to aspirin and half were outside the therapeutic range for clopidogrel. Platelet response did not differ between frail and non-frail older participants, although there was a trend towards increased variability in response to aspirin with increased frailty score.

Clinical Review and Documentation of Rationale for Potentially Inappropriate Medication Prescribing in Older Hospital In-Patients
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Introduction: While older persons are particularly vulnerable to polypharmacy, adverse drug reactions, and may benefit from deprescribing, ‘potentially’ inappropriate medications (PIMs) may warrant continuation when clinical judgement is exerted on a tailored case-by-case basis.

Aim: (1) Using the STOPP (Screening Tool of Older Persons’ Potentially Inappropriate Prescriptions) criteria (Gallagher et al 2008), to investigate prevalence of PIMs at both admission and discharge, and (2) to explore the ongoing prescription of such PIMs had been reviewed and a clinical decision-making process for their continued use had (i) been documented in the hospital medical notes, and (ii) communicated to the General Practitioner (GP), who provide longterm community-based care.

Methods: 100 patients aged ≥65, admitted to our hospital January 2013, were randomly selected (computer-generated) for retrospective chart review. Hardcopy and electronic medical records and discharge letters (sent to GPs) were reviewed.

Results: In total, 80 PIMs, in 35 patients, were identified at admission, and 101 PIMs, in 47 patients, at discharge. The commonest PIM recorded was prolonged proton-pump inhibitor use (n=15 at admission, n=22 at discharge) (Table). Amongst the 101 PIMs identified at discharge, 82% (n=83) had a clinical reason for continued prescription documented. For 71% (59/83) of these, this rationale was documented in the GP discharge letter.

Discussion: While PIMs were common, the medication had been clinically reviewed in many cases, and a clinical reason for continuation documented. However, such decision-making was less optimally communicated to GPs. Prescribing for older patients requires an individually-tailored approach, and understanding of a complex interplay of comorbidities and therapeutic goals. Documentation and communication of reasoning for continued PIM use are important in ensuring best patient management. [Gallagher P, O’Mahony D (2008) Age Ageing 37:673–679]

Patterns of Deprescribing Amongst Geriatricians: The Influence of Cognition, Dependency and Pill Burden
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Introduction. Deprescribing habits amongst physicians managing older, frailer, cognitively-impaired patients have not been well-investigated.

Aims. To analyse deprescribing habits amongst geriatricians.

Methods. Australian/New Zealand geriatricians (N=930) were presented with a series of case vignettes, including a list of patient medications, describing a similar patient with progressively more cognitive impairment and dependency (with ischaemic heart disease, hypertension, and constipation), and asked which medications they would change, and why.

Results. Amongst 134 respondents (14.4% response rate), 52.6% were male, 48.9% aged 35-50, 74.8% Caucasian, 84.1% specialists (15.9% advanced trainees [ATs]). With increasing dependency and cognitive impairment described, physicians were more likely to stop donepezil, aspirin, atorvastatin, ramipril, and amlodipine, or either antihypertensive (all p<0.001 for trend). On multivariate analysis, males (OR1.4, p=0.05) and ATs (OR 2.3, p<0.001) were more likely to STOPP (Screening Tool of Older Persons’ Potentially Inappropriate Prescriptions) criteria (Gallagher et al 2008), to investigate prevalence of PIMs at both admission and discharge, and (2) to explore the ongoing prescription of such PIMs had been reviewed and a clinical decision-making process for their continued use had (i) been documented in the hospital medical notes, and (ii) communicated to the General Practitioner (GP), who provide longterm community-based care.

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Discussion: While PIMs were common, the medication had been clinically reviewed in many cases, and a clinical reason for continuation documented. However, such decision-making was less optimally communicated to GPs. Prescribing for older patients requires an individually-tailored approach, and understanding of a complex interplay of comorbidities and therapeutic goals. Documentation and communication of reasoning for continued PIM use are important in ensuring best patient management. [Gallagher P, O’Mahony D (2008) Age Ageing 37:673–679]

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Aims. To analyse deprescribing habits amongst geriatricians.

Methods. Australian/New Zealand geriatricians (N=930) were presented with a series of case vignettes, including a list of patient medications, describing a similar patient with progressively more cognitive impairment and dependency (with ischaemic heart disease, hypertension, and constipation), and asked which medications they would change, and why.

Results. Amongst 134 respondents (14.4% response rate), 52.6% were male, 48.9% aged 35-50, 74.8% Caucasian, 84.1% specialists (15.9% advanced trainees [ATs]). With increasing dependency and cognitive impairment described, physicians were more likely to stop donepezil, aspirin, atorvastatin, ramipril, and amlodipine, or either antihypertensive (all p<0.001 for trend). On multivariate analysis, males (OR1.4, p=0.05) and ATs (OR 2.3, p<0.001) were more likely to stop antihypertensives, and males (OR 1.6, p=0.05) and older respondents (p<0.001) were more likely to discontinue senna. The commonest reason cited for discontinuing donepezil (49.6% of deprescriptions), aspirin (93.6%), atorvastatin (72.8%), ramipril (94.1%), and amlodipine (40.8%) was ‘severity of dementia’, with pill burden the next commonest cited reason, except in the case of paracetamol, where pill burden was the commonest reason for deprescribing (at 98.3%).

Discussion. On analyses of case-based scenarios, geriatricians are more likely to deprescribe multiple medications in the setting of advancing dependency and cognitive impairment, and report severity of dementia and pill burden as the main influencing factors. Physician characteristics are also associated with deprescribing habits. Further exploration of factors influencing deprescribing patterns, and whether these are associated with patient outcomes, is needed.
Rates of Polypharmacy at Admission and Discharge: A Retrospective Hospital-Based Study

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Introduction. Polypharmacy is common, especially amongst older patients. Hospitalisation is associated with both medication initiation and cessation, but potentially affords an opportunity to rationalise medications.

Aims. To compare total numbers of medications at admission and discharge amongst older hospitalised patients.

Methods. Numbers of medications (regular, regular + PRN), at admission and discharge, were retrospectively calculated for all patients ≥65 years old admitted to our tertiary university hospital, January 2013.

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

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

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

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Introduction. Dapagliflozin (DPF) is a novel anti-diabetic drug that works via inhibition of renal sodium-glucose co-transporters. DPF is routinely used in combination with other anti-diabetic drugs (typically metformin) for the treatment of type-2 diabetes. DPF is primarily cleared via glucuronidation, a reaction that is catalysed by the enzyme superfamily UDP-glucuronosyltransferase (UGT). Despite the role of UGT in DPF clearance, the capacity of DPF to perpetrate metabolic drug interactions (DDIs) by inhibiting UGT is currently unknown. Given that DPF is frequently administered in combination with other anti-diabetic drugs to patients with multiple co-morbidities also requiring pharmacotherapy, the potential of DPF to perpetrate metabolic DDIs by inhibiting UGT requires urgent elucidation.

Aims. To assess the capacity of DPF to inhibit enzymes of the UGT1A and 2B sub-families, and where observed to characterise the mechanism and potency of inhibition.

Methods. The capacity of DPF (1, 10 and 100 μmol/L) to inhibit 4-methylumbelliferone (4MU) glucuronidation by recombinant UGT 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, 2B7, 2B10, 2B15 and 2B17, and to inhibit lamotrigine glucuronidation by UGT1A4 and codeine glucuronidation by UGT2B4 was assessed using validated incubation conditions. Metabolite formation was quantified by HPLC with UV detection. Subsequent experiments were performed to characterise the potency and mechanism of DPF inhibition of human liver microsomal UGT1A9, using the selective substrate propofol, in the presence and absence of albumin (BSA; 1%).

Results. DPF caused modest inhibition of UGT2B enzymes (<40% at 100 μmol/L), but did inhibit multiple UGT1A enzymes; IC_{50} for UGT 1A1, 1A8, 1A9, and 1A10 were 66, 75, 39, and 55 μmol/L, respectively. DPF inhibited microsomal PRO glucuronidation in a non-competitive manner with an unbound K_i of 410 μmol/L, the presence of BSA (1%) in incubations resulted in an 6-fold reduction in K_i (to 67 μmol/L) but did not alter the mechanism of inhibition.

Discussion. DPF inhibited multiple UGT1A enzymes to a substantial extent. Inhibition was greatest with UGT1A9, which is a key enzyme in the metabolism of many drugs. As such further consideration of the clinical relevance of this inhibition is warranted.
Emerging definition of deprescribing: implications for research and practice in older adults
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Introduction. The term ‘deprescribing’ first appeared in the English health literature on medication management in older people in 2003 with an exponential increase in use since then. Deprescribing however, has not been systematically defined and variations currently exist.

Aims. To conduct a systematic review of the definition of ‘deprescribing’.

Methods. Systematic literature searches were performed (earliest records to Feb 2014) in MEDLINE, EMBASE, CINAHL, Informit, Scopus and Google Scholar. The terms deprescrib* or de-prescrib* were employed as a keyword search in all fields. Articles were included if they were medication related and used the term in the title, abstract or body of the article. Two researchers independently reviewed each article for inclusion and then extracted and identified characteristics of the definition where available.

Results. Following removal of duplicates, 231 articles, conference abstracts and non-peer reviewed items (e.g. newsletters, blogs) were retrieved. Eighty-nine fulfilled the inclusion criteria, 37 of which included a definition. Eight characteristics of the definitions were identified: use of the term stop/withdraw/cease/discontinue (35 articles), aspect of prescribing included e.g. long term therapy/inappropriate medications (18), use of the term ‘process’ or ‘structured’ (13), withdrawal is planned/supervised/judicious (11), involving multiple steps (7), includes dose reduction/substitution (7), desired goals/outcomes described (5) and involves tapering (4).

Discussion. Our findings suggest there is lack of consensus on the definition of deprescribing. A consistent definition is necessary for future research into deprescribing (e.g. determining outcomes) and for clinical practice as it will inform guideline development and implementation. We propose the following definition: Deprescribing is the process of cessation, dose reduction or substitution of an inappropriate medication, which is supervised by a clinician with the goal of managing polypharmacy and improving outcomes.

In vivo evaluation of felodipine as an inhibitor of cytochrome P450 enzymes and P-glycoprotein
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Introduction. Felodipine is an antihypertensive drug with low oral bioavailability that is sensitive to CYP3A and P-gp inhibition. In vitro studies indicate that inhibition of CYP3A4 by felodipine may also be a clinically important source of pharmacokinetic drug-drug interactions (PK-DDIs).

Aims. To evaluate felodipine as an in vivo inhibitor of drug metabolising CYP enzymes and P-gp.

Methods. Felodipine extended-release 10 mg was administered daily to 6 healthy subjects for 7 days (days 1 – 7). Subjects were administered a modified Inje cocktail comprising the selective probe substrates caffeine 100 mg (CYP1A2), losartan 25 mg (CYP2C9), omeprazole 20 mg (CYP2C19), dextromethorphan 30 mg (CYP2D6), midazolam 2 mg (CYP3A) and digoxin 250 μg (P-gp) on day 0 (prior to felodipine exposure) and day 7 (after felodipine exposure). Plasma samples were collected over 24 hours and drug concentrations measured by UPLC-MS/MS.

Results. The geometric means of the area under the plasma concentration-time curve ratios (probe AUC after felodipine exposure / probe AUC prior to felodipine exposure) and 95% confidence intervals for each probe were: caffeine 0.91 (0.64-1.30); losartan 1.05 (0.95-1.15); omeprazole 1.17 (0.78-1.76); dextromethorphan 1.46 (1.00-2.12); midazolam 1.23 (0.99-1.52) and digoxin 1.01 (0.89-1.15).

Discussion. Felodipine is a weak in vivo inhibitor of CYP3A and CYP2D6 but is unlikely to act as a clinically significant perpetrator of PK-DDIs.
Patient autonomy in deprescribing: a qualitative study of the views, attitudes and beliefs of older adults and non-paid carers of older adults
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Introduction. The term deprescribing describes the complex process that is required for the safe and effective cessation of medications likely to cause more harm (maleficence) than benefit (beneficence). To conduct deprescribing ethically, there must also be respect for patient autonomy. However, little is known about how older adults and non-paid carers think and feel about deprescribing.

Aims. To explore attitudes, beliefs and views towards deprescribing in older adults and non-paid carers of older adults.

Methods. People were eligible to participate in focus groups if they or the person that they cared for were aged more than 65 years and taking at least one long term prescription medication. The focus groups were audiotaped and then transcribed in full. Results were analysed via line by line coding and grouped according to the themes identified in a systematic review of patient barriers and enablers of deprescribing (which included participants of all ages).

Results. Four focus groups were conducted: two with older adults and two with non-paid carers. The older adults (n=14) were 79.3 years old (±5.3) and were taking 3.8 (±2.5) regular medications. The non-paid carers (n=14) were caring for older adults aged 86.2 (±9.1) taking 5.6 (±4.5) regular medications. All five previously published themes arose in all four focus groups: appropriateness of cessation, process of withdrawal, influences, fear related to cessation and dislike of medications. Overall, participants initially reported dislike of medications and wanting to take as few as possible. However, when the discussion moved to actual cessation there was strong resistance expressed with the main verbalisation of ‘why?’

Discussion. This study highlights that the initial discussion between the health care professional and the older patient/carer should revolve around why deprescribing is appropriate. Knowledge of these attitudes may empower medical practitioners to negotiate patient preferences while still respecting autonomy.

Assay for Measurement of Etoposide (ETOP) and Selected Anti-Retrovirals (ART) in Dried Blood Spots (DBS)
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Introduction. DBS is an accepted method for sample collection in genetic, viral epidemiology, and pre-clinical animal studies. DBS use in pharmacokinetic (PK) studies is a more recent use of this approach and has not been used extensively in clinical cancer research.

Aims. To develop a DBS assay to quantify ETOP and ART to support a clinical study in a challenging environment. Methods. Standard samples were prepared using spiked whole blood, which was then spotted onto Whatman 903 paper (GE Healthcare), filling the pre-printed ring. These were air dried for 2-3 hours, then placed in desiccator to complete dryness. A 6 mm punch was taken, rehydrated in water, precipitated with acetonitrile (ACN), transferred, dried, then dissolved in mobile phase (ACN:ammonium acetate). Using HPLC MS/MS (API4000, ABI Biosystems) with a Inertsil ODS-3 150X4.6 mm 5 micron column (GL Sciences) Q1/Q3 were determined for ETOP, lamivudine (LAM), lopinavir (LOP), indinavir (IND), zidovudine (ZID), nevirapine (NEV), stavudine (STAV), efavirenz (EFA) and tenofovir (TEN), using amprenavir (AMP) and teniposide (TENIP) as internal standard.

Results. All compounds could be quantified in a single HPLC run with lower limits of quantification as follows: 1 ng/mL – IND, LAM, LOP; 3 ng/mL – ZID; 10 ng/mL – ETOP, STAV, NEV; and 100 ng/mL – EFA, TEN. All compounds could be quantitated with a haematocrit (HCT) of 45% within acceptable error (<20%). Interestingly, with a HCT 30%, only ETOP and IND remained acceptable, and with HCT 60% only STAV remained acceptable. Filter paper developed for specifically microsampling (DMPKA,B and C, GE Healthcare) did not perform as well as Whatman 903 paper.

Discussion. An assay to quantify ETOP and ART was developed using DBS as a collection method. Use of DBS facilitated sample collection for a PK study in a challenging research environment (rural Western Kenya), allowing quantitation of ETOP and detection of ART. Further work into sample stability and the effect of HCT on sample quantitation needs to be explored.
**Oxycodone and other analgesics prescribing on discharge from Christchurch Hospital**

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Introduction. There is a worldwide rise in prescription opioid use with oxycodone use reaching epidemic proportions in the USA¹. A similar trend has been reported in New Zealand and Australia². Concerns about the contribution of oxycodone prescribing at hospital discharge to the rising oxycodone use in the community have been raised. Establishing which analgesics are being prescribed on discharge from Hospital could contribute to understanding local factors affecting opioid use.

Aims. To describe the analgesics prescribed on discharge from Christchurch Hospital.

Methods. A retrospective cohort study of patients discharged from Christchurch Hospital was undertaken. The medical records of consecutive patients discharged over ten days in May 2014 were reviewed. Data collected included analgesic(s), dose, frequency prescribed and the documented duration/quantity of supply. The data were analysed using descriptive statistics.

Results. During the study period the Emergency Department (ED) discharged 1473 patients, 250 of whom (17%) were prescribed one or more analgesics. None of these received a discharge prescription for oxycodone (95% CI 0-0.25%). The Orthopaedic Department discharged 533 patients, 295 of which (55%) were prescribed one or more analgesics. Seven of these received a discharge prescription for oxycodone 95% CI x-y%. The other analgesics prescribed were paracetamol (454), tramadol or codeine (355), non-steroidal anti-inflammatory drugs (244), morphine (7).

Discussion. Currently few patients are being discharged from ED with prescriptions for oxycodone. The Orthopaedic Department discharged approximately a patient a day on oxycodone and this should be explored further.


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

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**Background.** The Kakadu Plum (KP) is wild-harvested in Northern Australia (NT.WA) and a revered Aboriginal remedy for many ailments. The fruit is rich in ascorbate, other anti-oxidants and contains potent anti-bacterials (1). Colloidal metallic silver (CMS) preparations have shown anti-*Helicobacter* activity (2).

**Procedures.** Gastric *Helicobacter pylori* (HP) infection was assessed with a MetAtron diagnostic monitor (IPP). Volunteers attending a naturopathic clinic took oral treatments with either dried KP extract (1 gm b.i.d.) or CMS (≤0.3 mg Ag b.i.d.) as Lunasol™ for five days only. Probability indices for HP infection were determined on days zero, 7, 14 and 28 to assess a) efficacy and b) duration of response post-treatment. [Protocols for clinical studies were approved by an appropriate Human Ethics Committee.]

**Results:** KP extract effectively reduced gastric HP burden >85% within one week. The KP was particularly valuable as second-line treatment in a few subjects (3/17) showing lesser responses (<40%) to CMS alone.

**Conclusion:** At present, supply constraints and significant harvesting costs may limit the usefulness of KP as a phytotherapy to treat gastric inflammation/ulcers associated with HP. Nevertheless it is a valuable synergist for ‘tandem therapy’ with other traditional remedies for ulcer disease eg Bismuth, licorice (2).

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

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Introduction. The fractional clearance of urate (FCU) is the ratio of urate renal clearance and creatinine clearance. It has been proposed as a marker for altered urate renal handling and as a means of identifying urate ‘under-excretors’ (Indraratna et al). FCU is therefore expected to predict tubular urate transporter genotype in gout patients.

Aim. To explore the relationship between FCU and renal transporter genotype.

Methods. Plasma and urine concentrations of urate (P_u and U_u) and creatinine (P_cr and U_cr) from 116 gout patents were available for analysis. FCU was calculated using the formula; \( \frac{U_u \times P_cr}{U_cr \times P_u} \). Renal transporter variants associated with raised plasma urate concentrations included; URAT1 (rs3825018 G allele), OAT4 (rs17300741 A allele), GLUT9 (rs11942223 T allele), NPT1 (rs1183201 T allele), and, ABCG2 (rs2231142 G allele). The mean natural log of FCU (lnFCU) was compared across renal transporter genotypes and to the genetic risk score for hyperuricaemia.

Results. The mean lnFCU was significantly lower in carriers of the hyperuricaemia risk T allele for SLC2A9 compared to the CC genotype (1.39 vs 1.96, p=0.039). No differences were observed in mean lnFCU between carriers of the hyperuricaemia risk allele and the urate-lowering genotype for URAT1 (1.38 vs 1.47, p=0.19), OAT4 (1.37 vs 1.57, p=0.06), NPT1 (1.41 vs 1.38, p=0.79), and, ABCG2 (1.46 vs 1.35, p=0.16). No significant relationship was found between FCU and hyperuricaemia genetic risk score.

Discussion. FCU was found to predict SLC2A9 genotype, though there were only 2 individuals with the urate-lowering CC genotype. The result may therefore be due to statistical error. We found a trend towards reduced FCU in carriers of hyperuricaemia risk alleles for URAT1 and OAT4 but differences were not statistically significant. Overall, our results do not provide strong support for the use of FCU as a phenotypic marker of urate tubular handling in patients with gout.

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

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Introduction. The high rates of *A. baumannii* resistance highlight the urgent need for alternative treatment options such as rationally optimised combination dosage regimens.

Aim. To identify and rationally optimise β-lactam plus aminoglycoside combinations via novel mechanism-based modelling that synergistically kill and prevent resistance of carbapenem-resistant *A. baumannii*.

Methods. We studied combinations of ten β-lactams and three aminoglycosides against four *A. baumannii* strains, including two imipenem-intermediate (MIC<sub>IPM</sub>: 4 mg/L) and one imipenem-resistant (MIC<sub>IPM</sub>: 32 mg/L) clinical isolate, using high inoculum static concentration time-kill studies. We present the first application of mechanism-based modelling for bacterial killing and resistance with Monte Carlo simulations of human pharmacokinetics to rationally optimise combination dosage regimens for immune-compromised, critically-ill patients. Population pharmacodynamic modelling was performed in the S-ADAPT software and Monte Carlo simulations in Berkeley Madonna.

Results. All monotherapies achieved limited killing (≤ 2.3 log₁₀) of *A. baumannii* ATCC 19606 followed by extensive regrowth for aminoglycosides. Imipenem 8 mg/L plus tobramycin yielded synergistic killing (>5 log₁₀) and prevented regrowth against all four strains. Modelling demonstrated imipenem likely killed the aminoglycoside-resistant population and *vice versa* and aminoglycosides enhanced the target site penetration of imipenem. Against carbapenem-resistant *A. baumannii* (MIC<sub>IPM</sub>: 32 mg/L), optimised combination regimens (imipenem 4 g/day as continuous infusion plus tobramycin 7 mg/kg every 24h) were predicted to achieve >5 log₁₀ killing without regrowth in 98.2% of patients.

Discussion. Bacterial killing by any β-lactam or aminoglycoside in monotherapy was limited against a high inoculum of wild-type *A. baumannii* ATCC 19606. Among all tested combinations, imipenem plus an aminoglycoside provided the most extensive killing without regrowth against high inocula of susceptible, carbapenem-intermediate and carbapenem-resistant strains. Mechanism-based modelling identified both subpopulation synergy and mechanistic synergy for imipenem plus aminoglycoside. Monte Carlo simulations predicted a 98.2% success rate for clinically relevant imipenem plus aminoglycoside combination dosage regimens against a carbapenem-resistant clinical *A. baumannii* isolate with an MIC of 32 mg/L. Imipenem plus aminoglycoside combination regimens are highly promising and warrant further evaluation.
**In-vivo in-vitro correlation (IVIVC) of topical salicylate esters**
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Introduction. In order to replace expensive, time-consuming and potentially harmful in vivo skin penetration trials with studies using excised skin, it is first necessary to demonstrate appropriate in vitro-in vivo correlation (IV-IVC).

Aims. Our goal was to examine the correlation between in vitro and in vivo skin penetration data and to assess the influence of the skin hydration state on in vitro topical delivery.

Methods. Salicylate esters were applied to human epidermal membranes in Franz diffusion cells for 24 hr., under either dehydrated or hydrated conditions. We then used a convolution approach to predict Wurster & Kramer’s (1961) in vivo data from our in vitro data.

Results. Our convoluted data showed good agreement with previous in vivo results for glycol salicylate (GS) (Fig 1) and ethyl salicylate but not methyl salicylate, probably due to its vasodilatory effects in vivo. Salicylate penetration was strongly dependent on hydration state.

Discussion. Our results emphasise that excised human skin is an excellent model for in vivo conditions, provided the experimental conditions are replicated. The in vitro approach augmented with mathematical modelling to simulate urine and plasma levels of topically applied drugs saves time and effort; it can also help in accurate monitoring of therapeutic levels of drugs with narrow therapeutic windows.


**Effect of Beta-endorphin fragments on interleukin-1beta release**
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Introduction: Stimulation of opioid peptide-containing leukocytes by local factors results in release of beta-endorphin (BE 1-31) within the inflamed tissue. Released BE 1-31, although most well known for pain modulation, can also modulate immune cells through the modulation of pro-inflammatory cytokines such as interleukin-1beta (IL-1β). However, BE 1-31 is unstable in the inflamed environment and is transformed to several smaller fragments. It is possible that some of these BE 1-31 fragments may also play a role in the modulation of inflammatory cytokines such as IL-1β.

Aim: To investigate the modulatory effects of a selection of N-terminal BE 1-31 fragments on IL-1β release in differentiated THP-1 cells.

Method: Lipopolysaccharide (LPS) was applied to differentiated THP-1 cells, a cell line that displays a human tissue macrophage phenotype upon differentiation, to induce the production of IL-1β. BE 1-31 and selected fragments (BE 1-9, BE 1-11, BE 1-13, BE 1-17, BE 1-20) at concentrations of 10 pmol/L, 1 nmol/L, , and 0.1 µmol/L were incubated with differentiated THP-1 cells induced by LPS for 24 h to study their effect on IL-1β release. The modulatory effect on IL-1β release was also examined in the presence of naloxone (10 µmol/L), a non-selective antagonist of opioid receptors.

Results: A significant decrease in IL-1β release was observed for shorter fragments of BE 1-31 (i.e. BE 1-9, BE 1-11, BE 1-13) at 0.1 µmol/L. In contrast, at 0.1 µmol/L BE 1-17, BE 1-20, and BE 1-31 increased the release of IL-1β in differentiated THP-1 cells-induced by LPS. The modulatory effect of BE 1-31 fragments was not inhibited by naloxone characterising the effect as non-opioid activity for BE 1-31 fragments.

Discussion: BE 1-31 fragments can modulate the release of IL-1β in a concentration dependent manner. This may be potentially important, since BE 1-31 fragments are produced, via enzymatic degradation at different concentrations and at different times post-release of BE 1-31 into the inflammatory milieu. In conclusion, the examined fragments of BE 1-31 may modulate inflammatory processes differently as a result of enzymatic effects at the site of inflammation.
Targeting oxidative stress reduces cigarette smoke-induced lung inflammation and wasting in mice

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Introduction. Oxidative stress and reactive oxygen species (ROS) have been implicated in chronic obstructive pulmonary disease (COPD) and the observed wasting associated with this debilitating disease. Therefore, targeting oxidative stress and ROS production may be a novel way to treat COPD and its comorbidities.

Aim. To determine whether inhibiting oxidative stress and ROS production reduces cigarette smoke (CS)-induced lung inflammation and wasting in mice.

Methods. Male Balb/C mice (7 weeks of age) were exposed to 3 cigarettes per day (CS) or air (sham) for 8 weeks. Sham mice were handled identically and exposed to room air. Mice were treated daily with apocynin (5mg/kg, 0.1% DMSO, i.p.) or vehicle (0.1% DMSO in PBS). Food intake was measured daily and body weights were recorded 3 times a week. On day 56, mice were culled and lung, liver, kidneys, spleen, tibialis anterior, soleus, gastrocnemius, testicular and retroperitoneal white adipose tissue (WAT) were collected from the 4 groups of mice (n=8).

Results. CS-exposed mice had significantly more BALF inflammatory cells than all other groups (n=6-8 per group, P<0.05), and dex treatment did not change BALF inflammatory cell numbers. While CS+flu mice did not have significantly reduced body weight, dex treatment of CS+flu mice reduced body weight by 12% vs. CS alone (n=8 per group, P<0.05). DEX treatment of CS+flu mice significantly reduced gastrocnemius muscle weight by 9%, compared to CS mice (P<0.05).

Discussion. This model of AECOPD showed evidence of an exacerbation of lung inflammation. Treatment with dex did not reduce levels of lung inflammatory cells, however it did induce skeletal muscle wasting. These data could be used to elucidate mechanisms of glucocorticoid-induced wasting in patients hospitalised with AECOPD.

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

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Introduction. Dynorphin A 1-17 (DYN A) is an endogenous opioid peptide that has been demonstrated previously to produce a variety of opioid and non-opioid fragments upon release in inflamed tissue. Prior studies have suggested that the nuclear factor-kappa beta (NF-κB) pro-inflammatory, transcription-mediated signalling pathway as one of the possible mechanisms involved in the immunomodulatory effects of opioids during inflammation.

Aims. To evaluate the effects of a selection of DYN A fragments on LPS-induced NF-κB nuclear translocation in the human THP-1 cell-derived macrophages.

Methods. Phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells were established as an in vitro model for macrophages in inflammation. The differentiated macrophages were stimulated with lipopolysaccharide (1 mg/L, 1 hour) followed by incubation with DYN A and a range of biotransformed DYN fragments discovered previously in inflamed tissue (DYN 1-6, 1-7, 1-9, 1-10, 1-11, 2-17, 6-12, 3-14, 7-17 and 8-17) (1 μmol/L and 10 nmol/L, for 1 hour). The nuclear localization of NF-κB p65 subunit in the treated macrophages was immunolabelled and nuclear translocation was assessed using the ImageXpress (Molecular Devices) high-content cellular imaging system.

Results. LPS markedly induced the translocation of NF-κB p65 subunits into the nuclei of the immunolabelled cells to that representing 89% of immunolabelled NF-κB, whilst minimal translocation was observed in unstimulated, THP-1 cells (p < 0.05). NF-κB p65 nuclear translocation was significantly attenuated following treatment with DYN A by 67.5 ± 7.0% at 10 nmol/L. All DYN A fragments reduced NF-κB translocation, with the greatest effect observed with DYN A (1-7), reducing translocation by 56.7 ± 7.0% at 10 nmol/L.

Discussion. These findings suggest that DYN A biotransformed fragments may be involved in the modulation of the cellular signal transduction pathway associated with inflammation by inhibiting the NF-κB signal and thereby limiting the exacerbation of the inflammatory response. Further investigation is required to elucidate the mechanisms involved in the inhibition of NF-κB by biotransformed DYN A fragments.
Extracts of Australian native stingless bee (*Tetragonula carbonaria*) cerumen modulate dermal fibroblast proliferation and myofibroblast differentiation *in vitro*

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Introduction. Cerumen is a resinous, plant-derived product of stingless bees. We previously identified extracts and fractions of Australian native stingless bee (*T. carbonaria*) cerumen with anti-oxidant and anti-inflammatory activities *in vitro*; however, their wound-healing potential are currently unknown.

Aims. To examine the effects of *T. carbonaria* cerumen extracts and active fractions on the proliferation of human fibroblasts obtained from normal dermis (NF) and chronic wounds (CWF), and on transforming growth factor (TGF)-β1-mediated myofibroblast differentiation.

Methods. *T. carbonaria* cerumen collected from hives in South-East Queensland was partitioned into methanol and methanol-water extracts. Two active fractions (Fractions 1 and 9) were obtained from the methanol-water extract by preparative reversed-phase HPLC fractionation. Cultured NFs and CWFs were incubated in the absence or presence of cerumen extracts and fractions (0.3-20 μg/mL). MTT dye-reduction assays measured cellular proliferation over 120 h. The effects of extracts and fractions on myofibroblast differentiation were also investigated in TGF-β1-stimulated NFs (10 ng/mL; 72 h), by examining α-smooth muscle actin (SMA) immunoreactivity.

Results. Increased proliferative activity was observed in NFs exposed to 5 μg/mL methanol extract (129.9±11.2%; 120 h), 5 μg/mL methanol-water extract (124.0±15.8%; 24 h), 1 μg/mL Fraction 1 (131.1±17.7%; 120 h) and 3 μg/mL Fraction 9 (214.6±26.4%; 120 h) (P<0.05). Proliferation was also enhanced in CWFs treated with 5 μg/mL methanol extract (134.6±10.0%; 120 h) and 3 μg/mL Fraction 9 (134.8±5.7%; 120 h) (P<0.05). Exposure of NFs to Fraction 9 (3 μg/mL; 72 h) inhibited TGF-β1-mediated α-SMA expression and myofibroblast differentiation.

Discussion. Extracts and fractions of *T. carbonaria* cerumen possess proliferative and anti-scarring properties that may promote the healing of both acute and chronic wounds. Studies are underway to examine their effects on fibroblast migration; and to elucidate the active constituents of Fractions 1 and 9.

**Nanoparticles as potential novel mast cell modulating agents**

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Introduction: Mast cells are well known for their pathophysiological role in allergic diseases such as allergic rhinitis. When activated, mast cells degranulate releasing pro-inflammatory mediators which produce allergic symptoms. A number of studies have shown mast cell modulation by nanoparticles (NPs). However, the use of acetylated-dextran nanoparticles (Ac-DEX NPs) in this setting has not been examined. Ac-DEX NPs offer a biologically compatible, tunable material that can be employed as a drug delivery vehicle whereby upon cellular uptake, drug payloads can be released in low pH environments such as that of mast cell granules.

Aims: To characterize the selective uptake, trafficking and actions of a range of Ac-DEX NPs in mast cells.

Methods. Fluorescently labelled Ac-DEX NPs were synthesised using established protocols and dynamic light scattering used to measure their size distribution. Immunofluorescence and confocal microscopy, alongside flow cytometry, was used to determine the binding, uptake trafficking behavior of Ac-DEX NPs in a range of cell types with a focus on the mast cell lines RBL and LAD2. Following nanoparticle treatment, β-hexosaminidase (β-Hex) release and resazurin-based assays were utilised to measure mast cell degranulation and assess cell viability respectively.

Results: Ac-DEX NPs of varying diameters were synthesised and were shown not to affect cell viability. Immunofluorescence and flow cytometric analysis using Ac-DEX NPs (average size 120 nm) demonstrated greater selectivity of interaction with mast cells than with a range other of relevant cells types. Higher resolution imaging using confocal microscopy with ‘lysotracker’ co-staining confirmed internalization and localization of NPs to acidic organelles. Ac-DEX NPs had no effect themselves on β-Hex release nor on IgE-dependent and IgE-independent degranulation in both RBL and LAD2 cell lines.

Conclusions: Ac-DEX NPs interact preferentially with mast cells and when internalized, localize to acidic organelles. This suggests that Ac-DEX NPs could be used to selectively deliver mast cell inhibitory compounds and act as novel anti-allergic therapies.
**Glucocorticoid gene regulation differs in airway epithelial cells compared to hepatocytes**

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Introduction. Glucocorticoids are the most effective anti-inflammatory agents available to treat chronic inflammatory diseases, including asthma. However, severe and sometimes irreversible adverse effects (e.g. diabetes, peptic ulcer, osteoporosis, psychosis, glaucoma, Cushing’s syndrome) present a major obstacle to effective and safe treatment. The generation of adverse effects has generally been attributed to induction of gene expression (in particular metabolic genes such as PDK4), whilst the anti-inflammatory effects are thought to be due to repression of pro-inflammatory transcription factors (e.g. NF-κB, AP-1). However, recent evidence suggests that in airway epithelium, induction of anti-inflammatory gene expression (e.g. the genes encoding MKP-1 and p57 Kip2) is an important anti-inflammatory mechanism. (Clark & Belvisi 2012; Newton & Holden 2007).

Aims. In this study, we test the hypothesis that glucocorticoid-induction of gene expression differs in airway epithelial cells compared to hepatocytes.

Methods. Human bronchial epithelial cells (BEAS-2B) and hepatocellular carcinoma-derived epithelial cells (HepG2) were used in this study. Cells were serum-starved for 24 h – 48 h prior to stimulation with dexamethasone (Dex: 1 – 100 nM) after which total RNA was extracted and RNA expression was quantified by RT-PCR. Cy3- and Cy5-conjugated SmartFlare™ probes (Millipore) were used to detect RNA expression in live cells.

Results. Dex treatment of BEAS-2B cells induced a 19-fold upregulation of the gene encoding p57 Kip2, whereas this gene was undetectable in HepG2 cells. This difference was confirmed by SmartFlare™ live RNA detection. The gene encoding MKP-1 was expressed at a similar level in both cell types, but was induced to a greater extent by Dex in BEAS-2B cells. In contrast, the gene encoding PDK4 was induced to a greater extent in HepG2 cells.

Discussion. These results indicate that there is significant heterogeneity in glucocorticoid-induction of gene expression in cells derived from different tissue types. These findings have implications for optimisation strategies to improve the balance of efficacy and safety of glucocorticoid-like drugs.


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

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Introduction. Relaxin (RLN) mediates anti-fibrotic actions in lung fibroblasts (Unemori et al., 1996) through multiple mechanisms including activation of the RXFP1 and PPARγ receptors and via generation of nitric oxide (Samuel et al., 2004; Chow et al., 2014). In the present study, we compared bronchodilator effects of RLN, rosiglitazone (RGZ, a PPARγ agonist that relaxes airways) (Bourke et al, 2014) and isoprenaline (ISO, a β-adrenoceptor agonist).

Aims. To test whether pretreatment with RLN and RGZ inhibits the development of airway contraction.

Methods. Isolated mouse and guinea pig tracheal segments mounted in a wire myograph were contracted with KPSS or acetylcholine (30μM) to generate standard contractions prior to treatment. Tissues were then preincubated with RLN (100nM), RGZ (10μM, 100μM), RLN/RGZ (100nM/10μM) or ISO (10μM) prior to construction of full or partial concentration-response curves to methacholine (MCh, 10nM-100μM) or endothelin-1 (ET-1, 1-100nM).

Results. MCh-induced contractions in both species were significantly inhibited by RGZ, but not RLN, with reduced maximal responses but no loss of potency. The maximum MCh contraction (%ACh response in the same tissue) was 249±76% in untreated mouse trachea, and decreased to 57±18% and 17±7% in the presence of 10μM and 100μM RGZ respectively (n=4-6, P<0.05, 0.01). In contrast, ISO only reduced MCh potency in guinea pig trachea (pEC50, untreated 6.3±0.1, +ISO 5.5±0.2, n=3-4, P<0.05). All treatments reduced contraction to 100 nMET-1 (%KPSS: untreated 151±21%, +RLN 83±5%, +ISO 48±18%, n=3-4 mouse tracheal segments, P<0.05).

Discussion. Results from different species using different contractile agonists suggest that RLN and RGZ inhibit contraction by alternative mechanisms to β-adrenoceptor agonists. Further investigation is required to define these mechanisms and the therapeutic potential of RLN and RGZ to reduce the extent of airway narrowing in asthma.

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

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Introduction. COPD patients are susceptible to bacterial colonization and viral infections that drive acute exacerbations (AECOPD). The presence of both bacterial and viral pathogens is associated with higher levels of systemic inflammation, as determined by measuring Serum Amyloid A (SAA). SAA has been identified as a candid mediator of airway inflammation in COPD through its interaction with the GPCR, ALX/FPR2. In addition to interacting with pro-inflammatory SAA, ALX/FPR2 can interact with pro-resolving mediators such as Resolvin D1 (RvD1) to initiate resolution of inflammation.

Aims. To assess the effects of RvD1 compared to dexamethasone on airway inflammation and bacterial burden in a co-infection mouse model.

Methods. C57BL/6 mice were treated with Streptococcus pneumonia (Spn) (EF3030, 10^5 CFU) on day 1; followed by HKx31 influenza A virus (10^4 PFU) (IAV) on day 2. Mice were treated with dexamethasone (DEX, 0.5mg/kg, i.p.) or RvD1 (4μg/kg, i.p.) on day 4-6 with the control group receiving vehicle (0.5% ethanol in PBS). BALF inflammation and bacterial burden was measured through cell counts and CFU analysis.

Results. Mice inoculated with Spn alone efficiently cleared the bacteria by Day 7. In contrast, mice inoculated with both Spn and IAV displayed a 100-fold increase in pneumococcal load in the lungs. Increased pneumococcal load was associated with a decrease in neutrophilic inflammation at Day 2. By Day 7, there was a significant increase in inflammatory leukocytes that was predominately driven by IAV infection. Therapeutic administration of DEX resulted in a 2-fold increase in bacterial burden and neutrophilic inflammation. In contrast, RvD1 markedly suppressed inflammation and bacterial burden at Day7 relative to Vehicle treated mice.

Discussion. Glucocorticosteroids are routinely used to control inflammation in COPD and AECOPD. In our model, DEX failed to reduce bacterial burden and inflammation in the lungs, whereas RvD1 potently suppressed inflammation by over 50% and improved bacterial clearance relative to vehicle. Pro-resolving mediators that target ALX/FPR2 may offer a novel therapeutic strategy to combat co-infection in chronic lung diseases such as COPD.

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

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Introduction. Interactions between cancer cells and stromal cells in the tumour microenvironment play a key role in the control of invasiveness, metastasis and angiogenesis. Macrophages display a range of activation states in pathological contexts and alternatively activated (M2) macrophages can promote tumour aggressiveness. Opioids are able to modulate tumour growth and metastasis and are often administered to cancer patients.

Aims. We tested whether morphine can modulate the activation of RAW264.7 macrophages induced by (i) interleukin-4 (IL-4), the prototypical M2 polarization-inducing cytokine, or (ii) coculture with 4T1 breast cancer cells to induce a tumour-associated macrophage (TAM) phenotype in vitro.

Methods. The level of extra-cellular matrix (ECM) degrading enzymes, matrix metalloproteinase-9 (MMP-9) and -2 (MMP-2) was measured in cell-conditioned media using in-gel zymography. The expression of MMP-9 and M2 markers was measured at mRNA level using real time reverse transcriptase polymerase chain reaction (RT-PCR).

Results. We showed that IL-4 causes increased MMP-9 production and expression of the alternative activation markers arginase-1 and MRC-1. MMP-2 was unaffected. Morphine (10-20 μM) prevented the IL-4-induced increase in MMP-9 in a naloxone- and methylnaltrexone-reversible fashion. Morphine further prevented IL-4-elicited alternative activation of RAW264.7 macrophages. Expression of MMP-9 and arginase-1 were also increased when RAW264.7 macrophages were subjected to paracrine activation by breast cancer cells, and this effect was prevented by morphine.
Poly I:C, a TLR3 ligand and a viral RNA mimic, and heat-inactivated respiratory syncytial virus (RSV) recapitulate the glucocorticoid insensitivity evoked by RSV infection
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Introduction. Although inhaled glucocorticoids are the cornerstone of asthma therapy, they are ineffective in virus-induced asthma exacerbations. Respiratory syncytial virus (RSV), the prime cause of severe paediatric bronchiolitis, induces glucocorticoid insensitivity in human airway epithelial cell line, BEAS-2B cells, by mechanisms still to be elucidated. Since airway epithelium is the major site for viral infection and replication, emergence of resistance may occur through epithelium activation by pathogen-associated molecular patterns (PAMPs) with release of cytokines, including transforming growth factor-β (TGF-β), a potent inducer of glucocorticoid resistance (Salem et al, 2012).

Aims. To ascertain whether PAMPs mediate the suppressive impact of the RSV infection on human bronchial epithelial cell responses to glucocorticoids via mobilization of TGF-β.

Methods. BEAS-2B cells were transiently transfected with a glucocorticoid response element (GRE)-controlled secretory alkaline phosphatase (SEAP) reporter construct and pGL3 as an internal control vector to assess GRE activity. The expression of TGF-β1 and plasminogen activator inhibitor-1 (PAI-1) was quantified by RT-qPCR. Smad 2/3 phosphorylation was analyzed by western blotting.

Results. Both poly I:C (10 μg/mL) and heat-inactivated RSV at a multiplicity of infection 1 (MOI 1) caused a marked suppression of the GRE activation mediated by dexamethasone (30nM). The selective ALK5 inhibitor, SB431542 (1 μM) fully prevented the attenuation of GRE activation, suggesting the mobilization of endogenous TGF-β. This observation was reinforced by the up regulation of the TGF-β1 and the TGF-β-regulated PAI-1.

Furthermore, smad 2/3 phosphorylation was detected after 24 hr of Poly I:C or HI-RSV exposure.

Discussion. Our data suggest Poly I:C and heat-inactivated RSV can potentially mimic RSV detrimental effects on glucocorticoid signalling in bronchial epithelium. This effect may be partly explained by mobilization of endogenous TGF-β.


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Guanine Deaminase: Significance, Assay, Identification of Inhibitors, and Evidence for Redox Regulation
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Introduction. Mammalian guanine deaminase (GDA; EC 3.5.4.3) regulates both the intracellular guanine nucleotide pool and the production of xanthine, a substrate for the redox-sensitive immune regulator xanthine oxidase. GDA activity is vital for normal purine homeostasis, neural development and innate and acquired immunity. Oxidative stress stimulates GDA expression and detection of GDA activity in serum or cerebrospinal fluid indicates neoplasia or tissue trauma. Although it is an attractive target for drug development, studies of GDA have been hampered because of difficulties associated with its assay, notably the poor aqueous solubility of both substrate and product.

Aim. To develop a simple, versatile, robust and sensitive high-throughput GDA assay suitable for both research and routine diagnostic applications.

Methods. An ultrasensitive microtitre plate-based fluorimetric assay for GDA activity was devised by using guanosine as a pro-substrate and “nesting” the GDA-catalyzed reaction in a set of coupled enzymatic reactions that ultimately generate hydrogen peroxide, which was detected by oxidation of Amplex Red. The assay was used to establish a reference range for serum GDA activity and to monitor fluctuations in serum GDA activity sera obtained from 85 individuals during the course of interferon treatment for chronic hepatitis C infection. It was also used to screen some potential GDA inhibitors. Inhibitory activity was confirmed using an HPLC-base assay.

Results. Serum GDA activity in HCV infected individuals was elevated, increased during interferon treatment, then decreased to an extent that correlated with longer-term viral clearance. Besides those already reported by Fernandez et al. [1], 6-aza-thymine and 8-oxo-deoxyguanosine (8-oxoGdR) were amongst the GDA inhibitors identified.

Discussion. Together with existing data, these observations provide evidence that GDA expression and activity are subject to redox regulation, being enhanced by pro-inflammatory cytokines and inhibited by 8-oxoGdR, a product of oxidative damage to DNA. They further emphasize the biological significance of GDA and broaden the scope for future investigation of its activities. [1] Fernández, J.R.et al. Bioorg Med Chem. 2010; 18:6748-55.
Elastase activates PAR2/TRPV4 signaling complex and causes inflammatory pain
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Introduction. Proteases such as mast cell tryptase cleave protease-activated receptor-2 (PAR2) at R36/S37 and reveal a tethered ligand that binds to and activates the cleaved receptor. Activated PAR2 then sensitizes transient receptor potential (TRP) ion channels and thereby induce neurogenic inflammation and pain. Proteases that cleave the receptor at non-canonical sites may trigger different signalling cascades thereby act as biased agonists. Neutrophil elastase is activated during inflammation and is a biased agonist of PAR2. However, whether elastase induces PAR- and TRP-dependnet neurogenic inflammatory pain is unknown.

Aims. To investigate whether elastase induces inflammatory pain via biased agonism of PAR2 and TRPV4.

Methods. Elastase activation of PAR2 was examined in HEK293 cells stably overexpressing human PAR2 by measuring Ca²⁺ mobilization, cAMP accumulation, ERK1/2 activation and β-arrestin recruitment. Elastase signaling of dorsal root ganglion neurons from wild type, PAR2 and TRPV4 knockout mice were determined using single cell Ca²⁺ imaging. Mechanical hyperalgesia were evaluated after intraplantar injection of elastase to wild-type and PAR2 knockout mice.

Results. In HEK-PAR2 cells, elastase stimulated ERK1/2 activation and cAMP formation, but not Ca²⁺ mobilization or β-arrestin recruitment. In primary neurons, elastase induced adenylyl cyclase-dependent Ca²⁺ influx via ion channels such as TRPV4. Intraplantar injection of elastase induced PAR2-dependent mechanical hyperalgesia and inflammation.

Discussion. Our results identify a novel mechanism of elastase-induced activation of PAR2, and expand the role of PAR2 as a mediator of protease-driven inflammation and pain.

GHB activates a subset of GABAARs expressed in Xenopus oocytes
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Introduction. γ-hydroxybutyrate (GHB) is a small molecule with complex pharmacology. Present in low concentrations in the mammalian brain, it acts as a neuromodulator. When taken exogenously, it is used to treat narcolepsy and to ameliorate the withdrawal effects of alcohol, and is used as a recreational drug at higher concentrations, sometimes used as a “date-rape” drug. GHB is known to activate the GABA B receptor at high concentrations, but ligand-binding studies identified “GHB receptors” that bind GHB with high affinity.

Aims. To determine the role of the interaction between GHB, NCS-382, THIP and GABAARs in thermoregulation, and the differences in subtype selectivity that underlies these roles.

Methods. Thermoregulation was measured using radiotelemetry in wild-type and knockout mice injected intraperitoneally with GHB, NCS-382 and THIP. The activity of GHB, THIP and NCS-382 at GABAARs was determined by injecting mRNA encoding the sequences of the δ subunits of GABAARs in various ratios and combinations into Xenopus oocytes and measuring currents by two-electrode voltage clamp.

Results. GHB, THIP and NCS-382 all induced hypothermia in wild-type mice, but only THIP-mediated hypothermia was abolished in δ-knockout mice. We then investigated the pharmacology of NCS-382 at δ-subunit GABAARs by measuring concentration-response curves of THIP, GHB and NCS-382 on Xenopus oocytes injected with different combinations of δ subunits of GABAARs. These currents were inhibited with co-application of GHB. Furthermore, GHB activated δ homomeric receptors injected with a 1:10 ratio significantly more potently than when injected with a 10:1 ratio.

Discussion. Taken together, these data demonstrate that THIP, but not GHB or NCS-382 induce hypothermia via the activation of δ-containing GABAARs. It is likely that NCS-382 and GHB activates GABAA receptors that are expressed in Xenopus oocytes but not readily found on the extracellular surface of native neurons, and these receptors are most likely to contain a β-δ interface. While NCS-382 has previously been reported as an antagonist of GHB receptors, the pharmacological profile of NCS-382 is considerably more complicated.
Effect of positive allosteric modulators of M1 muscarinic receptors on psychosis-like behaviours in mice
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Introduction: Allosteric enhancement of M1 muscarinic acetylcholine receptors (mAChR) has emerged as a potential therapeutic antipsychotic strategy, however its effect in animals presenting aspects of psychoses remain unclear.

Aim: To investigate the ability of the M1 modulators, benzyl quinolone carboxylic acid (BQCA) and benzoquinazolinone-12 (BQZ12), to reverse locomotor hyperactivity and prepulse inhibition (PPI) deficits induced by the NMDA receptor antagonist MK-801.

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

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

Discussion: We provide an evidence of potential antipsychotic efficacy of the positive allosteric modulator of the M1 mAChR, and also demonstrate its potential to broaden the therapeutic spectrum of current antipsychotics to improve the pharmacological treatment of the schizophrenic syndrome.

Activity of illicit synthetic cannabinoids, UR-144, XLR-11 and 5-hydroxypentyl-UR-144 at human cannabinoid receptors and cannabinoid-sensitive ion channels
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Introduction: Illicit synthetic cannabinoids (SC) are of major concern in part because of apparently high toxicity associated with their use. Whether this arises through actions at CB receptors or other targets remains an open question, largely because almost nothing is known about their pharmacological activity.

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

Methods: CB1 and CB2 receptor mediated K channel activation was measured in AtT 20 cells using a membrane potential sensitive dye, activity at hTRPA1 was measured in HEK 293 cells using a Ca-sensitive dye. Assays were performed using a Flexstation 3, with a minimum of 3 independent replicates.

Results: All 3 drugs were agonists at CB1 and CB2 receptors with pEC50s: UR-144, 6.4±0.05, 7.1±0.05; XLR-11, 7.0±0.5, 7.1±0.15; SB5035, 5.7±0.1, 8.2±0.1 respectively. Maximum effects were similar to those of WIN55212, with exception of SB5035, which produced a maximum hyperpolarization approximately 50% larger than WIN55212 at CB1. Compared to the prototypical cannabinoid Δ9-THC, the SCs were less potent at CB1, but more potent and efficacious at CB2. UR-144 (30µM) activated TRPA1 to 38±4 % of the prototypic agonist cinnamaldehyde. XLR-11 and SB5035 (pEC50 5.1±0.05 and 4.95±0.02 respectively) had similar efficacy to cinnamaldehyde.

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

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

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Introduction. The nicotinic agonist varenicline improves motor, cognitive and affective symptoms in the YAC128 transgenic mouse model of Huntington’s Disease (HD; D’Souza et al, unpublished). It remains unclear which mechanisms underlie this functional improvement. Increased pro-inflammatory cytokine expression in the brain, plasma and CSF of HD patients suggests that an altered innate immune response may play a role in HD pathology1.

Aims. To investigate whether reduced brain inflammation contributes to functional recovery in varenicline-treated, late stage YAC128 mice.

Methods. Thirteen month old YAC128 mice (n=10) and age-matched wild-type (WT) littermates (n=10) received varenicline (1mg/kg/day for 14 days) via subcutaneously implanted matrix-driven delivery pellet (IRA®). Mice were trained and tested in the accelerated test prior to and at 1, 2, 3, 4, 6, 8 and 10 weeks after varenicline treatment. Cytokine protein expression levels were assayed in striatum, hippocampus, cortex and cerebellum samples using a mouse cytokine/chemokine premixed 22plex Milliplex kit (Millipore).

Results. Varenicline significantly increased the fall latency of YAC128 mice in the accelerod test (pre-drug: 26.27±4.77s v post-drug: 46.48±5.80s, p<0.001). Increased fall latency remained evident at week 8 (43.45s ± 6.28, p=0.012) but not at week 10 (35.55s ± 6.12, p=0.329). Varenicline-treated YAC128 mice showed decreased expression of INFγ in all brain regions, and IL-9 in striatum and cerebellum relative to untreated animals. In contrast, MCP-1 and IL-1 expression levels were increased in the cortex and striatum and cortex and cerebellum respectively.

Discussion. Varenicline produced significant, long lasting improvements in motor function in late stage YAC128 transgenic HD mice. Complex changes in cytokine expression profiles within brain regions involved in motor coordination may underlie improvements in motor performance.

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

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Introduction. G-protein coupled receptors (GPCRs) are the largest class of membrane bound receptors and are involved in the majority of pathophysiological signalling pathways. Upon activation, most GPCRs traffic to endosomes. Although GPCRs can continue to signal from endosomes, the mechanisms of endosomal signalling and its importance in complex pathophysiological processes are unknown. The substance P (SP) neurokinin 1 receptor (NK1R) is a mediator of pain and inflammation and is rapidly internalized to, and signals from, endosomes. However, the role of endosomal signalling in NK1R mediated pain is not understood.

Aims. Determine the importance of internalization and endosomal signalling of NK1R on pain and inflammation.

Methods. Dynamin-1 and clathrin inhibitors and siRNA were used to block NK1R endocytosis and trafficking to endosomes in cell lines and intact rats and mice. NK1R internalization and trafficking were quantified using BRET and immunofluorescence. Endosomally-mediated ERK, PKC and cAMP signalling were measured in cell lines using FRET biosensors and by immunofluorescence in rats. Capsaicin-evoked mechanical hyperalgesia was evaluated following intrathecal injection of dynamin, clathrin, NK1R, or MEK inhibitors in mice.

Results. In HEK293 cells, dynamin and clathrin disruption blocked SP stimulated NK1R endocytosis and inhibited SP-induced activation of nuclear pERK, cytoplasmic PKC, and cytoplasmic cAMP. Intrathecal injection of clathrin and clathrin inhibitors and siRNA blocked capsaicin-evoked endocytosis of the NK1R and activation of ERK in spinal neurons, and suppressed capsaicin-evoked mechanical hyperalgesia. Dynamin inhibitors also blocked SP-induced excitation of spinal neurons.

Discussion. Our results demonstrate a critical role for the endosomal signalling of the NK1R in pain transmission. These results also open new therapeutic targets for the treatment of NK1R mediated pain and inflammation.

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

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Introduction. Neuroinflammation contributes to Parkinson’s disease (PD) pathology. Previously, we have demonstrated that type-1 interferon (IFN) signalling drives the deleterious neuroinflammatory response in rotenone treated primary cultured neurons, however the role of type-1 IFNs in the glial cell response in PD is unclear.

Aims. To investigate the contribution of type-1 IFNs to the glial cell response in in-vitro and in-vivo models of PD.

Methods. Primary cultured wildtype and IFN-α receptor-1 knockout (IFNAR1⁻/⁻) mixed glia were treated with 25nM rotenone (24-72h). Wildtype and IFNAR1⁻/⁻ mice were administered MPTP (4x15mg/kg, 2h intervals) and brains collected at 1, 3 and 21 days post-MPTP for analysis.

Results. QPCR analysis confirmed IFNAR1⁻/⁻ glia display reduced expression of IFNα and IFNβ following rotenone treatment compared to wildtype controls (n=6, p<0.05). IFNAR1⁻/⁻ glia also exhibit diminished type-1 IFN signalling, with reduced STAT-3 phosphorylation identified by western blot. Significantly, levels of pro-inflammatory cytokines IL-1β, TNF-α and IL-6 were reduced in IFNAR1⁻/⁻ glia (n=6, p<0.05), as measured by QPCR and ELISA. In MPTP treated mice, reduced microglial activation (Iba-1⁺) was identified in the substantia nigra (SN) of IFNAR1⁻/⁻ mice 3 days post-MPTP. Additionally, IFNAR1⁻/⁻ mice display reduced levels of M1 pro-inflammatory markers (CD11b, CD16, CD32, CD86) and increased M2 anti-inflammatory genes (YM1, IL-10) compared to wild type mice (n=6, p<0.05). This reduced neuroinflammatory response correlated with increased survival of SN neurons in IFNAR1⁻/⁻ mice compared to wildtype mice (3907±64.61 versus 3079±70.59, n=10, p<0.001), identified by Tyrosine Hydroxylase quantification 21 days post-MPTP.

Discussion. These results confirm a role for type-1 IFN signalling in the neuroinflammatory glial cell response in neurotoxin models of PD. Targeting type-1 IFN signalling may provide a novel therapy to reduce neuroinflammation via modulating the glial cell phenotype, and limiting cell death in PD.
**Varenicline improves motor, cognitive and psychiatric symptoms in the YAC128 mouse model of Huntington’s Disease**

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Introduction. Huntington’s Disease (HD) is a fatal, inherited neurodegenerative disorder characterised by progressive movement, cognitive and psychiatric symptoms. Post mortem and genetic mouse model studies report a significant loss of acetylcholine and choline acetyl transferase activity in the HD brain, but no change in the number of nicotinic receptors1. This suggests impaired cholinergic neurotransmission may contribute to HD pathology.

Aims. To determine whether chronic treatment with the nicotinic agonist varenicline reduces motor, cognitive and affective symptoms in the YAC128 transgenic mouse model of HD.

Methods. The performance of 15 month old YAC128 mice (n=14) and age-matched wild type littermates (n=13) was assessed in the rotarod, T maze, novelty suppressed feeding (NSF) and forced swim test (FST) before and after treatment with varenicline for 4 weeks (5mg/kg/day). Thymidine analogues were used to assess progenitor cell proliferation and survival. DARPP32 immunohistochemistry was performed to visualise medium spiny neurons in the striatum, hippocampus and cortex.

Results. Chronic varenicline treatment significantly increased fall latency in the rotarod (14±3s vs 50 ±7s, p=0.004) and increased rewarded alternation in the T maze in YAC128 mice (65±2% vs 79±3%, p=0.045). Varenicline also, decreased latency to feed in the NSF test and reduced floating time in the FST in both YAC128 and wild type animals. Immunohistochemical analysis revealed increased progenitor cell proliferation and survival in addition to increased DARPP32 immunoreactivity in the striatum and cortex of varenicline treated animals.

Discussion. Chronic treatment with varenicline significantly improved motor coordination and spatial memory in late stage YAC128 mice. Varenicline also produced genotype-independent improvements in recognition memory, anxiety and depressive-like behaviour. Improved performance in YAC128 mice may be attributed to increased striatal and cortical neurogenesis.


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

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Introduction. In vitro studies have implicated the Toll-like receptor 4 (TLR4) signaling pathway in the unwanted effects of opioids such as reward and dependence. This raises the possibility that the benzylisoquinoline and promorphinan opiate alkaloid backbones possess molecular patterns detectable by the TLR4 innate immune pattern recognition systems. Furthermore, a broader class of non-opiate benzylisoquinoline and promorphinan alkaloids may possess previously uncharacterised TLR4 signalling (agonist or antagonist) activity.

Aims.1: Determine the potential of benzylisoquinoline and promorphinan alkaloids to bind to the TLR4 chaperone MD2. 2. Characterise the action of naturally occurring opioid alkaloids at TLR4. 3: Interrogate and characterise the biological activity of naturally occurring non-opioid alkaloids at TLR4.

Methods. In silico docking simulations of ligands were conducted to the crystal structure of MD2. Additionally, the molecular signalling pathways activated by opioid alkaloid-dependent activation of TLR4 will be examined and detailed pharmacological profiles will be generated using HEK293 cell lines overexpressing TLR4 and MD2 utilising an inflammatory cytokine readout. The biological activity of non-opioid benzylisoquinoline and promorphinan alkaloids at TLR4 will be established by conducting functional assays on recombinant cell systems over-expressing TLR4 in agonist and antagonist screens.

Results. We have undertaken preliminary analyses conducting in silico MD2 docking simulations of benzylisoquinoline and promorphinan alkaloids. Data shown in the figure displays the preferred binding conformations of each alkaloid to MD2 (morphine binding is designated in the grey cloud). The docking of many of the alkaloids overlaps entirely or substantially with this established pharmacologically active site. In vitro assays are ongoing.

Discussion. Amongst these alkaloids may exist compounds that have beneficial TLR4 antagonist or detrimental agonist pharmacologies. Thus, understanding the TLR4 activity of these alkaloids, either its presence or its absence, will aid the development of novel potent drugs with reduced side effects owing to the greater understanding of the structure activity relationship at TLR4.
Generic substitution of antiepileptic drugs: patient attitudes and perceptions
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Introduction. In recent years, the number of generic medicines of important prescription drugs has increased significantly. As generic medicines may be substantially lower in price than the innovator brands, they are perceived to provide major savings in healthcare cost, because they provide savings in terms of the purchase price of medications. Whether they produce other costs, such as increased doctor visits, serum monitoring, and hospitalizations, is in question. Moreover, generics are approved to be bioequivalent via comparison against the innovator, potentially there is greater disparity between two generic products than between any generic and the innovator.

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

Methods. A self-administered anonymous survey will be sent to patients with epilepsy in Australia by postal mail through Epilepsy Australia. This organization provides services to Australian patients with epilepsy. The survey is composed of multi-response and short-answer questions. The questionnaire is divided into two sections. Section 1 is designed to collect information on the patients’ demographics, including age, gender, type of epilepsy, ethnicity, when did the last seizure occur, self-rated health status, and the patients’ current medications. Section 2 collects information on the safety and efficacy of generic antiepileptic drugs and the patients’ willingness to use generic medicines for the treatment of epilepsy. The questionnaire will be evaluated with a sample (n = 10-20) of patients with epilepsy before the study commences. Invitations and information packages will be sent to patients diagnosed with epilepsy as self-reported. About 300 invitations will be sent out to patients in each of the 6 states of Australia. Data collection will be 3 months after the invitations being posted. Approval for conducting the research and the study protocol will be obtained from The Human Research Ethics Committee of the Research Ethics and Compliance Unit, Research Branch, The University of Adelaide.

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


Mechanism of α4β2 nicotinic acetylcholine receptor modulation unraveled by x-ray crystallographic and functional studies
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(introduced by Thomas Balle, Faculty of Pharmacy, The University of Sydney, NSW, Australia)

Introduction. Allosteric modulators of nAChRs are interesting in a drug discovery perspective but the mechanisms underlying modulation are poorly understood. To aid this understanding we have explored the binding mode of the stoichiometry selective modulator NS9283. It modulates through a binding site in the a4a4 interface of (α4)₃(β2)₂ receptor with a pharmacological profile similar to that of a benzodiazepine acting at GABAₐ receptors.

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

Results. Based on a co-crystal structure with Ls-AChBP, homology modeling, quantum mechanical calculations and extensive mutational mapping the binding mode and the selectivity determinants of NS9283 was determined. In Ls-AChBP, NS9283 bridges the subunit interface and forms close contacts to residues known to govern agonist potency and efficacy. It even forms favorable, albeit non-classical contacts to Trp143 (Ls-AChBP numbering) which in the a4 subunit is involved in cation-π interactions and is key to agonist activity.

Discussion. We have previously shown that the a4a4 interface nests a third binding site for the endogenous transmitter acetylcholine. NS9283 competes for this site and has an agonist-like mechanism of action. To confirm the agonist-like mechanism we show that NS9283 on a receptor engineered to contain more than one NS9283 compatible binding site is capable of producing a partial agonist response. An interesting consequence of this study is that the term “allosteric modulator” does not apply to NS9283.
**CYP2C8 genotype significantly alters imatinib metabolism in Chronic Myeloid Leukaemia patients.**
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Introduction: Between 25 and 49% of patients treated with imatinib for Chronic Myeloid Leukaemia (CML) withdraw from imatinib due to poor disease control or drug toxicity; large inter-individual variability in plasma concentration is a significant contributor (Gotta et al., 2014). Imatinib is metabolised to N-desmethyl imatinib by CYPs 3A4 and 2C8, with evidence for CYP3A4 autoinhibition and increased CYP2C8 contribution at steady-state (Filppula et al., 2013). We hypothesise that CYP2C8 polymorphisms (CYP2C8*3 and *4) alter steady-state plasma imatinib concentrations and metabolic ratio (N-desmethyl imatinib:imatinib, MR) in CML patients.

**Aim:** Determine CYP2C8 genotype, age-, sex-, time- and dose-effects on imatinib metabolism in CML patients.

**Method:** Retrospective analysis of 210 CML patients from the TIDEL II trial (imatinib 600 mg/day with dose changes based on response). Trough plasma imatinib and N-desmethyl imatinib concentrations, dose, age and sex data on days 8, 22 and 90 of treatment were available. Patients were genotyped for CYP2C8*3 (rs11572080, rs10509681) and *4 (rs1058930). Imatinib concentration and MR were compared between genotypes for patients on 600 mg/day at day 90 only (Kruskal-Wallis, Dunn’s post-hoc). Linear mixed effects (LME) modeling was used to investigate dose, time, age, sex and genotype effects on imatinib concentration and MR using day 8, 22 and 90 data.

**Results:** At 600 mg/day on day 90, genotype differences were significant (P=0.02) for MR [median±SD (n) *1/*1=0.22±0.09 (111); *2 carrier=0.26±0.06 (23); *1/*3=0.23±0.08 (15). Post-hoc P<0.05 *1/*1 vs *3 carrier], but not imatinib concentrations (P=0.9). LME showed only dose predicted imatinib concentration (P=3x10^-9), but dose, time and genotype predicted MR (nested ANOVA P=0.003, 2x10^-5, 8x10^-5 respectively). Controlling for dose and time, *3 carriers had 12% higher, and *1/*4 14% lower, MR compared to *1/*1.

**Discussion:** CYP2C8 genotype alters imatinib metabolism, but not trough plasma imatinib concentrations, in CML patients. Clinical impact will thus depend on presently unclear contributions of active metabolite(s) to response.


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**Impact of CYP2C8*1/*3 polymorphism on in vitro metabolism of imatinib to its active metabolite N-desmethyl imatinib**
Muhammad Suleman Khan, Daniel T Barratt, Andrew A Somogyi. Disc of Pharmacology, School of Medical Sciences, Univ of Adelaide, Adelaide SA.

Introduction: Imatinib is metabolised to its active metabolite N-desmethyl imatinib by CYP3A4 and CYP2C8. In *vitro* using human liver microsomes (HLMs) imatinib was found to be an autoinhibitor of its own CYP3A4 metabolism suggesting a more significant role of CYP2C8, especially on chronic dosing (Filppula, 2013). Functional effects of the major polymorphism CYP2C8*3 on N-desmethyl imatinib formation are unknown.

**Aim:** To investigate the impact of CYP2C8*3 polymorphism on imatinib N-demethylation in HLMs.

**Methods:** Kinetics of imatinib metabolism were determined using CYP2C8*1/*1 (n=5) and *1/*3 (n=4) HLMs and 20 imatinib concentrations (1-200 μM). Eadie-Hofstee plots directed the choice of kinetic model. Analysis of the data was compared between models for one enzyme Michaelis-Menten (MM), one enzyme substrate inhibition, two enzymes MM, and one enzyme MM with one enzyme substrate inhibition.

**Results:** One enzyme substrate inhibition was detected in all *CYP2C8*1/*1 HLMs, but not in three out of four *CYP2C8*1/*3 HLMs with Km values significantly lower than the wild type (Table). Substrate inhibition was found in one CYP2C8*1/*3 HLM with Km, Vmax and Ks values of 28 μM, 183 pmol/min/mg and 30 μM respectively.

**Discussion:** Our kinetic data could only find the involvement of one enzyme and that CYP2C8*3 appears to lead to enhanced functional activity suggesting that autoinhibition might be mediated via CYP2C8 and not CYP3A4.

<table>
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<tr>
<th>HLMs</th>
<th>Vmax (pmol/min/mg)</th>
<th>Km (μM)</th>
<th>Ks (μM)</th>
<th>Clint (μL/min/mg)</th>
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<td><em>CYP2C8</em>1/*1</td>
<td>130 ± 38</td>
<td>9.1 ± 2</td>
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<td>157 ± 69</td>
</tr>
<tr>
<td><em>CYP2C8</em>1/*3</td>
<td>107 ± 31</td>
<td>6.2 ± 0.75*</td>
<td></td>
<td>18±4</td>
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* Mann-Whitney U test P=0.03 versus *1/*1. Values are median ± SD.

Forensic Pharmacogenetics, Psychiatry and Public Health

Yolande Lucire PhD MBBS DPM (Forensic Psychiatrist)

Introduction: we wished to assess the forensic utility of CYP450 testing in serious adverse drug reactions to mental health drugs and combinations.

Aims: To investigate drug-drug, drug-gene and drug-drug-gene interactions that caused over 100 people with no previous history of mental illness, suicidality or violence to become disabled with akathisia, aggression and suicidality from drugs metabolized by CYP450.

Setting: A community where prescribers are educated by clinical practice guidelines. Prescribing did not accord with advice in Product Information or with Public Health Advisories from the United States Food and Drug Administration.

Patients and methods: Over 130 subjects were referred author’s medicolegal practice largely because of new and continuing disability. Adverse drug reactions, drug-drug and drug-gene interactions slowing metabolism and were documented in conjunction with their genotypes at CYP450 2D6, 2C9 and 2C19.

Results: Compared with primary care patients tested at the same facility, variant, diminishing and multiple polymorphisms at CYP450 were significantly higher in subjects with adverse drug reactions. Only four had wild type genes throughout and they were over medicated. Only nine started on a single drug at a standard dose. The rest were on multiple doses, interacting drugs, herbs, illicit substances and prescribing cascades followed. Some withdrew medication slowly and recovered but 50 percent were lost to follow up. Four developed delayed post-withdrawal akathisia which was intractable.

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

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

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Introduction. Pharmacogenomic tests are not used often in public psychiatry inpatient care and this study explored the effect of introducing an 6 gene multi-gene pharmacogenomic test into inpatient care together with the introduction of an electronic support system to order tests and retrieve the results.

Aims. To demonstrate the effect of introduction of a multi-gene pharmacogenomic test and an electronic support system into aged persons mental health in 6 different hospitals.

Methods. A secure intranet- based ordering and results retrieval system was introduced to support a 6 gene pharmacogenomic test into two wards in acute hospitals and 4 residential units. 177 patients were compared with 86 patients in a third ward where the pharmacogenomics testing was not offered. Also a further 82 patients were studied in residential units.

Results. Some subsets of the tested patients were found to have significantly increased length of stay. Patients found to be CYP2C19 ultrarapid and heterozygous ultrarapid metabolisers had a 12 day increase in median length of stay. For the intervention group the mean number of psychotropic medications fell by 31.4% however in the control group the number of psychotropic medications increased by 10%. The previous year’s medication use was studied in all 3 hospitals and in these control groups the medications increased by 26%. The number of patients with at least one fall in the intervention group was reduced (18.6 per 100 versus 34.2 per 100 patients). The decision support system was widely used and 90% of the clinicians surveyed acknowledged that having the electronic support system had increased their confidence in interpreting pharmacogenomic results.

Discussion. The pharmacogenomic decision support software was successfully used to introduce a multi-gene pharmacogenomic test into acute and residential aged persons mental health care. Some of the results have important economic implications such as increased stay in certain groups recognised by the test and reduced number and drugs and falls. This has the potential to save the health system millions of dollars.
Assessment of Janus protein tyrosine kinases as potential regulators of epithelial-mesenchymal transition using a model of epidermal growth factor induced breast cancer epithelial-mesenchymal transition

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

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

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

Results. Both JAK inhibitor I and PP2 significantly decreased EGF-induced STAT3 phosphorylation. JAK inhibitor I also appeared to decrease vimentin protein and mRNA expression at 24 hours. Discussion. Using the MDA-MB-468 model of EGF-induced EMT we have identified a potential role for Janus protein tyrosine kinases in the upstream regulation of STAT3 phosphorylation and vimentin expression. Further studies will be undertaken to identify the specific JAK isoforms involved and the intricacies of the relationship with calcium transporters.
Assessment of the consequences of silencing of the secretory pathway calcium ATPase 1 in MDA-MB-231 breast cancer cells.
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Introduction. Secretory pathway calcium ATPases actively transport Ca\textsuperscript{2+} into the Golgi. Secretory pathway calcium ATPase isoform 1 (SPCA1) is elevated in the poor prognosis, basal-like breast cancer molecular subtype. The silencing of SPCA1 reduces the proliferation of basal-like MDA-MB-231 breast cancer cells. However, the mechanism for this effect is not fully understood. We have recently used 2D-DIGE and MS/MS to identify proteins sensitive to SPCA1 silencing. These studies identified downregulation of heat shock protein 60 (HSP60) protein and mRNA with SPCA1 silencing in MDA-MB-231 cells.

Aims. To compare mRNA levels of proteins whose levels were identified using 2D-DIGE as potentially altered upon SPCA1 silencing in MDA-MB-231 cells.

Methods. MDA-MB-231 cells were treated with SPCA1 siRNA or non-targeting siRNA (control), 24 h after plating. RNA was isolated 48 h post siRNA treatment and levels of mRNA were assessed using real time RT-PCR. Thirteen targets were assessed (including HSP60).

Results. SPCA1 silencing significantly reduced the level of HSP60 mRNA (~60\%, \(P < 0.05\)). Junction plakoglobin/gamma-catenin mRNA levels significantly increased (~80\%, \(P < 0.05\)) with SPCA1 silencing, as did mRNA levels of tubulin beta-4B chain (22\%, \(P < 0.05\)).

Discussion. These studies have identified that silencing of SPCA1 produces a significant increase in the mRNA levels of junction plakoglobin/gamma-catenin and tubulin beta-4B chain in MDA-MB-231 breast cancer cells. Further studies are required to define the mechanism and significance of these changes when SPCA1 is silenced in MDA-MB-231 cells.

Expression of copper transporters in colorectal cancer cells and its relation to oxaliplatin
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Introduction. Copper transporters are important membrane-bound proteins to maintain mammalian copper homeostasis, including copper transporter 1 (CTR1), copper\textsuperscript{2+}-transporting P-type ATPase 7A (ATP7A) and 7B (ATP7B). Recent preclinical studies suggest their roles in regulating the uptake and export of platinum-based anticancer drugs. Resistance in colorectal cancer cells is attributable to reduced cellular concentration of oxaliplatin. We hypothesize that copper transporters may play important roles in the transport and sensitivity of oxaliplatin in colorectal cancer cells.

Aims. To characterize the expression of copper transporters in colorectal cancer cells and determine the effect of Cu chelator, varying levels of Cu and oxaliplatin on their expression pattern and to measure the sensitivity of colorectal cancer cells to oxaliplatin.

Methods. Cytotoxicity was measured for oxaliplatin in human colorectal cancer cells (COLO205, SW620, HCT-15 and DLD-1) using a MTT assay. Single and double fluorescence immunocytochemistry were carried out using anti-CTR1, ATP7A, ATP7B and Na/K ATPase primary antibodies and Alexa Fluor 488 or 494-conjugated secondary antibodies on cells treated with different levels of CuCl\textsubscript{2} and oxaliplatin. Digital images were acquired and processed using confocal microscope and ImageJ software.

Results. The sensitivity to oxaliplatin differed by 15-fold among these cell lines, with IC\textsubscript{50} values ranging from 0.82\textpm0.22 to 15.4\textpm2.2 \textmu M. Cellular immunoreactivity to CTR1, ATP7A and ATP7B was observed in all cell lines. CTR1 displayed stronger membrane staining in SW620 cells than other cell lines. Compared to CTR1, ATP7A and ATP7B expression were more abundant across all cell lines with apparent plasma membrane staining. Treatment of cells with Cu, Cu chelator and oxaliplatin did not cause significant changes in the expression pattern of transporters.

Discussions. Our data have demonstrated that copper transporter CTR1, ATP7A and ATP7B are expressed by these human colorectal cancer cells with stronger membrane presence for efflux transporters under basal culture conditions. The effect of extracellular Cu conditions and oxaliplatin on their expression pattern is minimal, but it needs to be investigated further at mRNA and protein levels. This work is supported by Cancer Council Tasmania.
Comparative anti-proliferative and pro-apoptotic actions of a novel ω-3 fatty acid analogue in human breast cancer cell lines.
Adam Hraiki1, Tristan Rawling2, Michael Murray1. Discipline of Pharmacology, University of Sydney1, Sydney, NSW; Graduate School of Health, University of Technology2, Sydney, NSW

Introduction. The CYP-derived epoxide formed at the ω-3 olefinic bond in ω-3 PUFAs inhibits tumour cell viability. However, because epoxides are metabolically unstable, we have developed a series of urea isosteres suitable for in vivo evaluation of anticancer activity. One agent - termed CTU - rapidly and effectively kills MDA-MB-231 cells in vitro and in vivo. It is well documented that breast cancer heterogeneity is an important factor in the in vivo efficacy of anticancer drugs. Cancer cell profiling could help identify tumour characteristics that promote susceptibility to new agents such as CTU in order to direct their optimal clinical use.

Aims. This study evaluated the comparative cytotoxic properties of CTU in four representative breast cancer cell lines: MDA-MB 231 (231; ER-), MDA-MB 468 (468; ER-), MCF-7 (ER+) and T-47D (ER+).

Methods. Cell proliferation was estimated by mitochondrial reduction of MTT, viability by ATP production, cell cycle progression by flow cytometry, apoptosis by caspase-3/7 activity and mitochondrial targeting with JC-1.

Results. CTU decreased MTT reduction in 231 cells after 24 and 48 hr of treatment to 58±2% and 25±1% of control at 10 μM (mean±SEM). ATP production in 231 cells was decreased to 38±3% and 29±4% of control after 24 and 48 hr of treatment, respectively (10 μM CTU). 231 cells were found to accumulate in G0/G1 phase when treated with 5 μM CTU, and fewer cells completed mitosis. Caspase-3 activity was strongly activated in 231 cells to 4.3±0.4-fold of control after 24 hr treatment at 10 μM. Using fluorescence microscopy, JC-1 staining indicated that the mitochondrial membrane potential in 231 cells was disrupted after only 4 hr of CTU treatment. In comparison, 468 cells and, in particular, MCF-7 and T-47D cells were less responsive to CTU.

Discussion. CTU was effective against the aggressive 231 breast cancer cells, exhibited intermediate activity against 468 cells and had lower activity in the MCF-7 and T-47D cell lines. Mitochondrial targeting by CTU, as reflected by JC-1 staining, decreased ATP production and increased caspase-3 activity, was most pronounced in 231 cells.
**Cruciferous vegetables and cancer prevention - A critical review**

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**Introduction.** Breast cancer is the second leading cause of cancer death in Australia. Nutrition, particularly intake of cruciferous vegetables, has been shown to reduce the risk of developing different types of cancers, including breast cancer. The anticancer effect of cruciferous vegetables has been attributed to their rich source of isothiocyanates (ITCs) and research over the last three decades has highlighted extensive scientific evidence for the efficacy of cruciferous vegetables in reducing the risk of developing breast cancer. The anticancer effect of cruciferous vegetables has been attributed to their rich source of isothiocyanates (ITCs) and research over the last three decades has highlighted extensive scientific evidence for the efficacy of cruciferous vegetables in reducing the risk of developing breast cancer.

**Methods.** The Medline and PubMed databases were searched for studies published in English, from January 1994 to May 2014. These studies examined the effects of cruciferous vegetables, such as broccoli, watercress, and their ITC constituents on breast cancer. The search terms used were: cruciferous vegetables, *Cruciferae*, Brassica vegetables, isothiocyanate, allyl isothiocyanate, phenethyl isothiocyanate, benzyl isothiocyanate, sulforaphane, mammmary, breast, cancer, cancers, prevention, chemoprevention, human, animal, randomised controlled trials, controlled clinical trial, random allocation, clinical trials, case control, cohort, in vivo, and in vitro studies.

**Results.** A total of 58 studies were reviewed, of which 8 animal and 50 in vitro studies were identified. Literature evidence demonstrates the anti-breast cancer effects of benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PITC), and sulforaphane (SFN). These are the most extensively investigated compounds. Allyl isothiocyanate, erucin, and iberin have been reported to inhibit breast cancer cell proliferation in one in vitro study. No symptoms of ITC toxicity have been reported in human studies. Of the 8 animal studies identified, 5 studies demonstrated a significant reduction in the incidence and/or multiplicity of mammary tumours by SFN, BITC and PITC. No published human clinical studies on cruciferous vegetables and breast cancers were identified in the last two decades. Description of the identified studies were summarised in Tables (1 and 2), which can be provided on request.

**Discussion.** There is consistent scientific evidence derived from animal and cell culture work demonstrating protective effects of SFN, BITC, PITC on breast cancer. The reported mammary cancer protective properties are dose related, and there is substantial evidence which highlighted the importance of ER in the development of breast cancer. In summary, the findings suggested that the different molecular targets modulated by SFN, BITC, PITC compounds are useful indicators of success in future human breast cancer prevention trials.

Comparative Investigation of Plasma Glucose Lowering Activity and Effect on Liver, Kidney and Antioxidant Enzymes Parameters of Palmatine and Palmatine-like alkaloid Isolated from Stem of Coscinium Fenestratum Plant on STZ Induced Diabetic Rat Model.

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

The expression of plasma membrane Ca²⁺ ATPase isofom, PMCA2, in breast tissue with lactational change and in breast cancer cells and the consequences of its silencing in MDA-MB-231 breast cancer cells.

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Introduction. The plasma membrane Ca²⁺ ATPase isofom, PMCA2, extrudes Ca²⁺ from the cytosol to the extracellular space. The expression of PMCA2 is mostly limited to cerebellar Purkinje cells and cochlear hair cells. In mouse and rat mammary glands, PMCA2 is highly expressed during lactation and is involved in the transport of Ca²⁺ into milk; however PMCA2 expression has not been assessed in human mammary glands during lactational change. PMCA2 mRNA levels are elevated in some breast cancer cells. Aims. To assess PMCA2 expression in human breast tissue with lactational change and in breast cancer tissue. To investigate PMCA2 as a potential therapeutic target in breast cancer. Methods. PMCA2 expression was assessed in breast tissues by immunohistochemistry. PMCA2 was silenced in the MDA-MB-231 breast cancer cell line using siRNA. The percentage of S-phase positive cells and cell number were evaluated by staining the siRNA treated cells with EdU (Click-iT EdU kit) and DAPI and using high-content imaging (ImageXpress Micro). The proliferation of cells treated with siRNA and doxorubicin was assessed by measuring the cell area using a kinetic imaging system (IncuCyte). Results. PMCA2 expression was observed in the luminal epithelium of breast tissue exhibiting lactational change. Approximately 10% of breast tumours (9/96) exhibited PMCA2 staining on the plasma membrane. No correlation was seen with PMCA2 expression and the expression of estrogen, progesterone or HER2 receptor. Silencing of PMCA2 in MDA-MB-231 cells reduced total cell number and the percentage of cells in S-phase. PMCA2 silencing also promoted the anti-proliferative effects of doxorubicin in MDA-MB-231 breast cancer cells. Discussion. Our findings suggest that PMCA2 protein expression is increased in human mammary glands during lactation and is a feature of some breast cancers. Inhibitors of PMCA2 may increase the effectiveness of therapeutics currently used to treat basal breast cancers and represent a new therapeutic approach for some breast cancers.
Exploring the structure activity relationships of a series of agonists targeting the α4β2 nicotinic acetylcholine receptor
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Introduction. The nicotinic acetylcholine receptor α4β2 is the most abundant receptor subtype of the excitatory Cys-loop family of receptors in the mammalian central nervous system. Among other things, it is implicated in attentive and learning processes, and there is ample evidence of pro-cognitive effects of drugs targeting α4β2 in preclinical as well as clinical studies. The α4β2 receptor is known to express in two different stoichiometries, (α4)2(β2)3 and (α4)3(β2)2, which show distinct pharmacology. The underlying structural reason for the stoichiometry-specific pharmacology was recently resolved with the discovery that the (α4)3(β2)2 stoichiometry holds a third orthosteric binding site for ACh in the α4-α4 subunit interface. Hence, full activation of a (α4)3(β2)2 receptor is achieved by ACh binding in three interfaces (two at α-β and one at α-α interfaces) whereas the (α4)2(β2)3 receptor is fully activated by binding to only two interfaces (the two α-β interfaces).

Aims. In the present study, a series of closely related agonists ranging from partial to full agonists are tested for their activities at both stoichiometries of α4β2 receptors. In combination with computational docking studies the overall aim was to broaden the structure-activity understanding of how agonists interact with the two types of interfaces.

Methods. Functional activities of the agonists were measured in electrophysiological measurements using two-electrode voltage-clamp electrophysiology in Xenopus laevis oocytes. Docking studies were used to suggest binding modes and interpret the biological results.

Results. Concentration-response relationships for the agonists will be presented along with docking results.

Discussion. The results reveal that seemingly small chemical differences in a highly related series of compounds can significantly alter how compounds interact with α-β and vs. α-α interfaces. The implication of this is that the structure-activity understanding at α4β2 receptors seen as comprehensive just few years ago is in fact incomplete. This underscores the importance of determining all parameters of novel compounds in future drug-discovery programs.

Allosteric interactions of indole and benzimidazole derivatives at the human translocator protein
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Introduction. The 18 kDa translocator protein (TSPO) has become a target for the development of drug treatments for anxiety and cancer. Limited knowledge of TSPO binding sites has hindered development of these drugs, however recent work suggests pyrazolo [1,5-a] pyrimidine acetamides may be a promising class of TSPO ligands (Scarf et al, 2009).

Aims. We aim to examine the effect of altering the number and position of nitrogen atoms around the pyrazolo [1,5-a] pyrimidine acetamide heterocyclic core on affinity and efficacy at the human TSPO.

Methods. Affinity was examined by competitive radioligand binding on human T98G glioblastoma cells using [3H]-PK 11195, while a bromodeoxyuridine ELISA was used to examine anti-proliferative action.

Results. When nitrogen atoms were reduced to either 1 in the case of indoles or 2 in the case of benzimidazole, complex binding was displayed (n_H = -2.11±0.05 & -0.49±0.15 respectively, p<0.05 for n_H = 1). Compounds displaying complex binding also positively modulated the anti-proliferative ability of an IC50 concentration of PK 11195 without themselves affecting proliferation (Fig1). This behavior was absent in the pyrazolopyrimidine, imidazopyridine and purine heterocyclic cores.

Discussion. We found evidence that changing the number and position of nitrogens in the heterocyclic core of pyrazolo [1,5-a] pyrimidine acetamide influences the ability of these derivative compounds to interact allosterically at the TSPO. This is the first evidence of allosteric behavior at the human TSPO and opens an avenue for the production of novel allosteric anxiolytics, anti-cancer and neuroprotective drugs.

High antioxidant Australian plant extracts: anti-proliferative activities against CaCo2 and HeLa cell lines
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Introduction. High levels of antioxidant phytochemicals have been linked to the treatment and prevention of several cancers. Recent reports have identified a number of native Australian fruits as having high antioxidant capacities. Despite this, several of these species are yet to be tested for anticancer activity.

Methods. Solvent extracts were prepared from high antioxidant native Australian plants and their antioxidant capacities were determined by the DPPH free radical scavenging assay. Anti-proliferative activities against CaCo2 and HeLa cancer cell lines were determined by an MTS based cell proliferation assay. Toxicity was determined using the Artemia franciscana nauplii bioassay.

Results. The methanolic and aqueous extracts of all plant species displayed high antioxidant contents (equivalent to 16-95 mg of vitamin C per gram of plant material extracted). In contrast, the ethyl acetate extracts for all species had relatively low antioxidant contents (generally below 5 mg of vitamin C equivalents per gram of plant material extracted). The antioxidant contents correlated with the ability of the extracts to inhibit proliferation of CaCo2 and HeLa cancer cell lines. The high antioxidant methanolic and aqueous extracts of all species were potent inhibitors of cell proliferation, with IC50 values generally below 1000 μg/mL. The aqueous S. australe fruit extracts were particularly effective, with IC50 values of 27 and 172 μg/mL against CaCo2 and HeLa cells respectively. In contrast, the lower antioxidant content ethyl acetate extracts generally did not inhibit cancer cell proliferation. Indeed, exposure of the cancer cells to most of the ethyl acetate extracts induced potent cell proliferation. The methanolic and aqueous Syzygium extracts displayed significant toxicity in the Artemia franciscana bioassay, with LC50 values below 1000 μg/mL. All other extracts were nontoxic.

Discussion. The antiproliferative activity of the high antioxidant plant extracts against HeLa and CaCo2 cancer cell lines indicates their potential in the treatment and prevention of some cancers.
**Macadamia nut as an anti-Giardial agent**
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**Introduction**
Macadamia integriflora (family Proteaceae) is an endemic Australian plant that has been used for thousands of years as a food. It is known to contain high levels of medium chain saturated fatty acids, raising the possibility that they may be useful in the treatment of Giardiasis.

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

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

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

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**Biopharming *Carica papaya* compounds with anti-Proteus activity: The potential for the treatment and prevention of rheumatoid arthritis**
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**Introduction.** Previous studies have reported field grown *Carica papaya* leaves to have antibacterial activity. However, *Carica papaya* has not been studied for the ability to block the bacterial triggers of autoimmune inflammatory diseases. Furthermore, studies into the bioactivity of the roots are lacking.

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

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

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

**Discussion.** The growth inhibitory bioactivity of *Carica papaya* root extracts against Proteus spp. indicates their potential in blocking the onset of rheumatoid arthritis.
**Tasmannia lanceolata extracts: anti-Proteus activity and potential for the treatment and prevention of rheumatoid arthritis**

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Introduction. A wide variety of herbal medicines are used in indigenous Australian traditional medicinal systems to treat RA and inflammation. *Tasmannia lanceolata* (Tasmanian pepper) has received recent attention as a potential medicine due to its high antioxidant content (Cock 2013) and antibacterial activity (Winnet et al 2014).

Aims The current study was undertaken to test the ability of a panel of Tasmanian pepper extracts for the ability to block the microbial trigger of RA and to use metabolomics fingerprint analysis to detect anti-inflammatory compounds.

Methods. Tasmanian pepper berry and leaf were extracted with solvents of varying polarity and investigated for the ability to inhibit the growth of the bacterial trigger of RA (*P. mirabilis*). The extracts were tested for toxicity in the Artemia nauplii bioassay. The most potent inhibitor of *P. mirabilis* growth was further analysed by RP-HPLC coupled to high accuracy TOF mass spectroscopy.

Results. The Tasmanian pepper berry extracts were determined to be the most effective inhibitors of *P. mirabilis* growth, with MIC values as low as 11 and 126 μg/ml for the methanolic and aqueous extracts respectively. Subsequent analysis of the *T. lanceolata* fruit extracts by RP-HPLC coupled to high resolution TOF mass spectroscopy detected the resveratrol glycoside piceid and 2 combretastatin stilbenes in both *T. lanceolata* fruit extracts. All extracts were also shown to be non-toxic in the Artemia nauplii bioassay.

Discussion. The low toxicity of these extracts and their inhibitory bioactivity against Proteus spp. indicate their potential in blocking the onset of rheumatoid arthritis.


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

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Introduction. Immobilised metal affinity chromatography (IMAC) is an aqueous-compatible separation technique. In previous studies (Braich et al, 2008; Gu et al, 2012), we demonstrated the utility of single-column IMAC for purifying metal-binding metabolites with important clinical applications, including the anticancer drug bleomycin and the siderophore desferrioxamine B (DFOB, used in iron chelation therapy).

Aims The aim of this study was to improve upon IMAC for capturing metabolites so that multiple classes of metal-binding metabolites could be purified simultaneously. We have called this new approach 'multi-dimensional IMAC' (MD-IMAC). MD-IMAC involves joining in series two single IMAC columns, each containing resin charged with a different metal ion, with the goal of simultaneously separating and capturing two classes of bacterial metabolites. This has the potential to increase the efficiency of current methods of metabolite purification and discovery.

Methods. The first part of this study involved the development of the MD-IMAC setup to find the optimal resin conditions (chelator and metal ion) that would maximise the capture of each metabolite. The second part of this study involved cultivating *Streptomyces verticillus* and using MD-IMAC to capture native bleomycin and DFOB.

Results. The optimised MD-IMAC setup consisted of a Yb(III)-charged upper resin and a Cu(II)-charged lower resin. Using this setup, two distinct metabolites (bleomycin and DFOB) were successfully captured from *S. verticillus* bacterial culture: DFOB on the Yb(III)-resin and bleomycin on the Cu(II) resin.

Discussion. Selectivity of the IMAC resin for a metal-binding metabolite is affected by the metal ion on the resin and the nature of the covalently-bound chelator attached to the resin beads. By varying these factors, we were able to tune each resin in the MD-IMAC setup to capture a different metabolite. We have been the first to develop MD-IMAC for the simultaneous capture of multiple metabolites. MD-IMAC could be used to purify useful metal-binding metabolites other than bleomycin and siderophores. This work has implications for green chemistry, streamlining pharmaceutics processing and metabolite discovery.

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

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**Introduction.** Doxorubicin is an important anticancer agent isolated from the culture of *Streptomyces peucetius* var. *caesius* (Arcamone et al, 1969). Current methods for the purification of this agent consists of a labour-intensive, multi-step process involving the use of large volumes of organic solvents (Arcamone et al, 1974). Due to the metal binding properties of doxorubicin (Beraldo et al, 1985, Guin et al, 2012), it was considered a potential candidate for capture using immobilised metal ion affinity chromatography (IMAC) – traditionally used in the isolation of recombinant proteins.

**Aims.** To capture doxorubicin from bacterial culture using IMAC.

**Methods.** The first part of this study involved developing optimum conditions on an IMAC column for capturing doxorubicin using a standard. The second part consisted of culturing *S. peucetius* on a solid agar plate, lysing the colonies and running the solution through an IMAC column. The presence of doxorubicin was determined using UV-Vis absorption spectroscopy (490 nm) and liquid chromatography/mass spectrometry.

**Results.** The optimised conditions for doxorubicin capture used a Ni(II)-charged resin with binding and elution buffers at pH values of 7.5 and 5.5, respectively. The binding capacity of doxorubicin was about 4.5 μmol/mL of resin. Doxorubicin was successfully isolated from bacterial culture using the IMAC methods in a yield of approximately 70%.

**Discussion.** This work was the first to use IMAC as a way of isolating doxorubicin, which is in restricted supply worldwide. This method has potential in industry, as it uses less steps and aqueous buffers, complying with the need for green chemistry.


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

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**Introduction.** Recently, treatment for type 2 diabetes is focused to incretin hormone but this hormone is metabolized by Dipeptidyl Peptidase IV (DPP-IV) excessively into inactive form. The inhibition of DPP-IV can enhance incretin bioavailability and regulate blood glucose level, therefore it would be beneficial in the treatment of type 2 diabetes. In the other hand, synthetic drugs of DPP-IV inhibitor for long term use has not been obtained complete data especially the safety. It induces the search of DPP-IV inhibitor compounds from herbs having less side effect. *Urena lobata* is plant growing in Indonesia and has been used to cure many diseases due to their biology activity.

**Aims.** To determine anti diabetic effect of *U. lobata* leaf extract on DPP-IV inhibitor activity.

**Methods.** In vitro study use Gly-pro-p-nitroanilide (GPPN) as substrate and Vildagliptin as a reference standard. Both ethanolic and water of *U. lobata* leaf extract were tested the activity of DPP-IV inhibitor. p-nitroanilida as product of reaction was observed with microplatereader wavelength 407 nm. All data are expressed as the mean ± SD and the IC-50 value was determined by linear regression curve.

**Results.** The ethanolic extract of *U. lobata* showed DPP-IV inhibitor activity stronger than water extract with an Inhibitory Concentration-50 (IC-50) value of 2355.43 and 7361.89 μg/ml respectively. Vildagliptin used as reference standart of DPP-IV inhibitor activity have IC-50 value 57.44 μg/ml.

**Discussion.** Water extract of *U. lobata* more applicable in use but the activity of DPP-IV inhibitor lower than ethanol extract. *U. lobata* leaf extract have inhibitory effect on DPP-IV related to active compound in this extract such as fitosterol and flavonoid groups. The inhibition of DPP-IV will increase bioavailability of incretin hormone that contribute to regulate blood glucose level through stimulation of insulin secretion, β cells proliferation and inhibition of glucagon. It might be potential to be an candidate of fitotherapy for the diabetes mellitus type 2.
New histone deacetylase inhibitors from innovations in chemical biology
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Introduction. Inhibitors of the histone deacetylase enzyme family are an emerging class of anticancer drugs that act by increasing the transcription of genes associated with cell differentiation and apoptosis (Marks et al, 2009). This project aims to source new drug leads as inhibitors of Zn(II)-containing histone deacetylase from the parent compound desferrioxamine B (DFOB). Selected bacteria that produce DFOB for iron acquisition, including Streptomyces pilosus, co-exist with Azospirillum irakense, which is able to catabolise DFOB as a carbon source (Winkelmann et al, 1999). While DFOB itself does not display any inhibition of histone deacetylase, we have identified catabolic fragments including N-5-aminopentyl-N-(hydroxy)-succinamic acid (AHS) and N-5-aminopentyl-N-(hydroxy)-acetamide (AHA), as potential candidates for histone deacetylase inhibitors due to structural similarity to currently used inhibitors.

Aims. To biochemically source new histone deacetylase inhibitors by exploiting the catabolic potential of A. irakense.

Methods. Cultures of A. irakense were supplemented with 10 mg DFOB as the sole carbon source, with catabolism of DFOB confirmed by the diminution of absorbance values characteristic of Fe-DFOB and the metabolites of DFOB characterised by analytical HPLC-MS. Semi-preparative HPLC was used with a 6-20% ACN:H2O gradient over 30 min and a 0.5 mL min⁻¹ flow rate or purification.

Results. AHS and AHA were detected in the A. irakense culture supernatant after supplementation of 10 mg DFOB, with close-to-maximal concentrations of each at 42 hours. A. irakense cultures supplemented with 20 mg or 50 mg DFOB generated greater quantities of AHS and AHA, indicating potential to upscale this system for a greater yield. AHS and AHA were successfully purified from the A. irakense supernatant. These fragments were then assessed for the in vitro inhibition of HDAC group I enzymes.

Discussion. Overall, this study developed a biochemical method for the production of AHS and AHA that can be upscaled in future to generate greater yields of these potential histone deacetylase inhibitors.


The potential role of nonulosonic acid analogues in drug discovery: The road to new antibacterial agents
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Bacterial infections by multi-drug resistant pathogenic bacteria are a global issue that not only affect people of all socio-economic backgrounds and age, but also place a major burden on the health systems of all countries1. Pseudaminic acid and legionaminic acid are 5,7-diamino-3,5,7,9-tetraideoxy-nonulosonic acids that play an essential role in the pathogenic capabilities of numerous clinically relevant drug resistant Gram-negative bacteria. The enzymes that process these important carbohydrates are attractive potential drug targets as they are found only within micro-organisms.

Currently, research findings within the literature clearly show that interfering with the biosynthesis of these carbohydrates is a potential opportunity for developing new anti-bacterials2,3.

Therefore our research group has been actively investigating novel pathways for the synthesis of such compounds, which includes the synthesis of selectively functionalised analogues of pseudaminic acid and legionaminic acid which are intended for use are small molecule biological probes to gain a more thorough understanding of how Gram-negative pathogenic bacterium utilise these important endogenous compounds in order to become pathogenic.

**Differential regulation of the μ-opioid receptor by distinct agonists**

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**Introduction.** The μ-opioid receptor (MOPr) has been extensively studied due to its importance as the main target of opiate analgesics. Recent data from our group shows differential regulation of the MOPr in a ligand-dependent manner and suggests that opioid agonists have distinct effects on the dynamics and signalling of this receptor. The spatiotemporal distribution of both receptor and signalling components is key for the generation of distinct and highly specialized GPCR-mediated responses.

**Aims.** It has recently been reported that morphine-activated MOPr is retained within a defined membrane microdomain resulting in a sustained increase in cytosolic extracellular signal-regulated kinase (ERK). In contrast, activation of MOPr by a prototypical high-efficacy agonist DAMGO ([D-Ala2,N-Me-Phe4,Gly5-ol]-enkephalin) causes receptor redistribution within the plasma membrane, transient increases in cytosolic and nuclear ERK, and receptor internalization. However, it remains unknown whether these effects are also observed when stimulating MOPr with endogenous opioids such as endomorphin or other opiate analgesics such as oxymorphone.

**Methods.** Biophysical techniques ( Förster Resonance Energy Transfer (FRET) and Bioluminescence Resonance Energy Transfer (BRET)) and imaging approaches were used to investigate the trafficking and spatiotemporal signalling of MOPr upon activation by different agonists.

**Results.** Endomorphin-1 displays signalling and regulation profiles similar to DAMGO responses, promoting receptor internalization. In contrast, oxymorphone follows a pattern similar to morphine, restraining the receptor within the plasma membrane and inducing a similar ERK activation profile.

**Discussion.** Our results suggest that the ability of MOPr to translocate across the plasma membrane is ligand-dependent and linked to distinct signalling outcomes. Further studies to determine the protein complexes controlling differential MOPr signalling and regulation are required.

**Comparative pharmacology of allosteric modulators at the M₅ muscarinic acetylcholine receptor**

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**Introduction.** Studies analysing M₅ muscarinic acetylcholine receptor (M₅R) knockout mice have indicated that this protein can regulate brain reward circuitry. As such, the M₅R may be an attractive target for drug dependence. However, the discovery of suitably selective orthosteric ligands has been limited to date by the high homology between muscarinic receptor subtypes.

**Aims.** The aim of the study was to characterise the pharmacology of recently identified positive (ML129 and ML380; Bridges et al., 2009; Gentry et al., 2014) and negative (ML375; Gentry et al., 2013) allosteric modulators (PAM and NAM) of the M₅R using radioligand binding and cell based functional assays.

**Methods.** ML129, ML380 and ML375 were assessed alone and in combination with orthosteric agonists in assays of inositol phosphate (IP) accumulation and [³H]-N-methyl scopolamine ([³H]-NMS) binding in whole FlpIn-CHO cells stably expressing the human M₅R (hM₅R). Data were analysed according to an allosteric ternary complex model (binding) and operational model of allosterism (functional assays; Leach et al., 2007).

**Results.** M₅R PAMs caused a concentration-dependent, saturable increase in the affinity of the orthosteric agonists ACh, carbachol or oxotremorine-M for the hM₅R (Logᵢ values range 0.4 ± 0.2-1.3 ± 0.2). Such effects accounted for their ability to potentiate functional IP responses to the same agonists. Additionally, ML129 and ML380 displayed partial or full allosteric agonism, respectively, in their own right. Conversely, both compounds displayed neutral cooperativity with respect to pilocarpine affinity, but caused a 20-fold increase in pilocarpine signalling efficacy. ML375 acted as a NAM with respect to orthosteric agonist affinities and functional potencies in the IP assay. Despite increasing agonist affinity, allosteric modulators inhibited binding of the inverse agonist, [³H]-NMS.

**Discussion.** ML129 and ML380 exert probe dependent potentiation of agonist-mediated hM₅R function, consistent with a two-state model. However, ML375 inhibits the binding of both agonists and inverse agonists suggesting a more complex mode of interaction.


Leach et al. (2007) TiPS. 28: 382-89
Identifying novel small molecules that enhance the bronchodilation and anti-inflammatory effect of Vasoactive Intestinal Peptide

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Introduction. Chronic Obstructive Pulmonary Disease (COPD) is a lung disease characterised by chronically poor airflow and is caused by persistent inflammatory responses to inhaled irritants. It is forecast to be the third leading cause of death by 2030 and current treatments are ineffective. Vasoactive intestinal peptide (VIP) is a widely distributed neuropeptide that has potent bronchodilatory and anti-inflammatory effects. It is hypothesised that an agonist or positive allosteric modulator (PAM) of the VIP receptor (VPAC\textsubscript{1}-R) could lead to an increase in bronchodilation and reduction of inflammation in COPD patients.

Aims. To screen a focused set of 2,000 proprietary compounds supplied by Alchemia that were selected based on prior knowledge from previous exploratory research, to identify positive allosteric modulators of the VPAC\textsubscript{1}-R that will be triaged through a screening cascade, to test the efficacy of lead compounds in an \textit{ex vivo} guinea pig trachea model and an \textit{in vivo} cell-based inflammation model.

Methods. Cell-based high throughput cAMP accumulation assays (PerkinElmer Ultra LANCE, LANCE and AlphaScreen kits) were developed to screen compounds in VPAC-1R expressing CHO FlpIn cell lines. A kinetic CAMYEL biosensor assay was used as an orthologous screen to confirm hits, before the lead compounds were tested in an \textit{ex vivo} guinea pig trachea relaxation model and an \textit{in vivo} macrophage inflammation model measuring TNF\textalpha release.

Results. We identified a number of compounds that positively modulate the VPAC\textsubscript{1}-R using cAMP accumulation assays that were further ranked using a more sensitive kinetic assay. Positive modulation of the VIP response was confirmed using an \textit{ex vivo} guinea pig trachea relaxation model measuring bronchodilation, and an anti-inflammatory model measuring inhibition of TNF\textalpha release.

Discussion. We have identified the first known positive allosteric modulators of VPAC\textsubscript{1}-R. The compounds positively modulate VIP relaxation in an \textit{ex vivo} guinea pig bronchodilation model and enhance the inhibitory effect of VIP on TNF\textalpha release in an \textit{in vitro} model, indicating anti-inflammatory effects. Further medicinal chemistry SAR studies are underway to identify more potent analogues.

Phase I metabolism study of bacterial NorA efflux pump inhibitors

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

Aims. Seven promising EPIs have been further developed, by studying in vitro their phase I metabolism.

Methods. Metabolic stability on Human liver microsomes was assessed through in vitro experiments. For those compounds with low half-life we also identified the major metabolites.

Results. The seven compounds showed different metabolic profile, from stable (P07 and P09) to intermediate (P02, P06, P10) and even largely unstable (P04, P05). Various metabolites have been identified for those that are unstable.

Discussion. Metabolite identification (MetID) was carried out both manually and with the use of new recent software, that is supposed to speed up the MetID process on pharmaceutical industry. The comparison between MassMetasite (Molecular Discovery Ltd) and UNIFI (Waters) is provided.

Rosignitazone induction of mouse brite adipocytes derived from subcutaneous white adipose tissue
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Introduction. Whereas classical white adipose tissue (WAT) stores chemical energy, brown adipose tissue (BAT) releases energy as heat. BAT thermogenesis thus represents a target for therapies aimed at reducing weight gain. Numerous studies have revealed functional BAT in adult humans, including inducible “brite” (brown in white) adipocytes that express uncoupling protein UCP1 and the BAT transcriptional regulator Prdm16, but also the WAT marker Hoxc9. Brite differentiation can be induced by the PPARγ activator rosiglitazone (Petrovic et al, 2010).

Aims. We determined the effect of rosiglitazone (Rosi) on gene expression profiles and UCP1 protein distribution in primary mouse adipocytes derived from the stromal vascular fraction of interscapular BAT, subcutaneous inguinal WAT (iWAT) and epididymal WAT (eWAT).

Methods. Adipocytes were cultured for 7 days in the presence of 2.4 nM insulin, plus or minus 1 μM Rosi. Gene expression was measured using a custom qPCR array and UCP1 protein was detected by immunocytochemistry.

Results. UCP1 mRNA was 375-fold higher in Rosi versus control BAT cultures (98 vs 0.3% relative to Actb), and 1200-fold higher in Rosi-treated iWAT cultures (4.6 vs 0.04% relative to Actb). Immunocytochemistry indicated that Rosi-treated iWAT cultures consist of a mixed cell population with varying levels of UCP1 protein. eWAT cultures displayed much lower induction of UCP1 mRNA or protein. Despite the heterogeneity of Rosi-treated iWAT cultures with respect to UCP1, both iWAT and BAT cultures displayed similar expression of key adipocyte and thermogenic markers, including Fabp3, Fabp4, Cpt1b, Pgc-1α, PPARγ, Acadl, Sirt3 and Trib3. Overall, Rosi-induced changes in iWAT gene expression were biased toward genes encoding mitochondrial proteins and transcription factors critical to thermogenesis, fatty acid uptake and metabolism, and energy production.

Discussion. Our data indicate that cells from subcutaneous iWAT undergo Rosi-induced brite differentiation, but that high-level, homogeneous UCP1 expression requires further activating stimuli.


Discovery and optimization of novel, highly selective M₅ mAChR allosteric modulators
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Introduction. The muscarinic acetylcholine receptors (mAChR) are a family of class A G protein-coupled receptors comprised of five subtypes (M₁-₅) expressed throughout the CNS. These receptors play a vital role in regulating a wide range of physiological functions. The design of M₅ mAChR selective ligands is challenging due to the conserved nature of the orthosteric pocket across mAChR subtypes. Activation of this receptor has been particularly implicated as a potential target in the treatment of chronic cerebrovascular diseases, whereas inhibition of the M₅ mAChR may provide novel therapies for the treatment of addictive behavior. Aims. The objective of this project was to obtain potency optimized, highly selective allosteric modulators for the M₅ mAChR in order to provide novel chemical probes for the elucidation of this receptor’s role in the CNS. Methods. A high throughput screen for M₅ mAChR-selective ligands was performed on the Molecular Libraries Probe Center Network screening deck. This effort revealed a weakly active M₅ mAChR positive allosteric modulator (PAM) scaffold along with a scaffold identified as an M₅ mAChR inhibitor. The structure-activity relationships (SAR) of the lead chemical scaffolds were subsequently optimized using iterative parallel synthesis techniques. The activity of the synthesized compounds was tested via Ca²⁺ mobilization assays. Results. SAR optimization of the PAM scaffold, VU0472882 (hM₅ EC₅₀ > 10 μM), yielded an M₅ mAChR-prefering PAM with a >50-fold improvement in potency, ML380 (hM₅ EC₅₀ = 190 nM, hM₅ EC₅₀ = 2.1 μM, hM₁,4 EC₅₀ > 30 μM). The inhibitor scaffold, VU0352221 (hM₅ IC₅₀ = 3.5 μM) was similarly optimized, resulting in ML375 (hM₅ IC₅₀ = 300 nM, hM₁,4 IC₅₀ > 30 μM). Furthermore, radioligand binding experiments have revealed ML375 to possess an allosteric mode of pharmacology; thus, ML375 represents the first M₅ mAChR-selective negative allosteric modulator (NAM). Discussion. The M₅ mAChR PAM, ML380, and NAM, ML375, represent novel, highly potent, and selective probes for the study of this receptor. The selectivity and unique modes of pharmacology of these probes make them invaluable for a variety of in vitro studies into M₅ mAChR structure and function. At present, further chemical optimization efforts are underway for both scaffolds with the goal of producing a second generation of M₅ mAChR-selective allosteric modulators with optimized PK properties in order to furnish tool compounds for in vivo studies.
Probing the structure and dynamics of a class A G protein-coupled receptor using small-angle X-ray scattering.

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Introduction. A novel GPCR drug screening approach using a nanofluidic device is currently being developed. This approach involves the use of a nanofluidic device to measure translocation of target molecules (GPCRs) through a nanopore approximately 10-50 nm diameter. The translocation time of the molecule through the nanopore is determined by its size, charge and shape, thus it is imperative that the size and shape of the target molecule is known. The neurotensin 1 receptor (NTS₁) has been engineered to be stable in detergent, making it ideal for structural studies. X-ray crystallography and NMR spectroscopy yield high-resolution information for the receptor, but structural information of the receptor/detergent complex as a whole is lost. For use in nanofluidic devices as proposed here, this information is crucial. Small-angle X-ray scattering (SAXS) is an ideal technique to investigate the size and shape of the NTS₁ solubilised in detergent as the whole receptor/detergent complex can be observed.

Aims. To characterize the protein-detergent complex (PDC) formed when GPCRs are extracted from the cell membrane and solubilized in detergent. Different detergents produce varying PDC shapes and sizes, and understanding the size and shape of PDC is crucial when introducing GPCRs into the nanopore.

Methods. NTS₁ solubilised in n-decyl-β-D-maltopyranoside has been purified using affinity chromatography and size-exclusion chromatography. Affinity chromatography coupled to SAXS at the Australian Synchrotron was then used to investigate the size and shape of the PDC. Coupling affinity chromatography directly to a SAXS beam line is completely new and is the perfect technique for the NTS₁ system.

Results. The scattering profile of the PDC showed a large micellar species with a characteristic peak at 0.18 Å⁻¹, indicative of a DM micelle. Additionally, SAXS has shown that micelles are stable at acidic and basic pH, but unstable in high salt.

Discussion. Further studies in this area will extend to the use of other detergents such as n-octyl-β-D-glucoside, which will be important for crystallisation experiments concurrently underway in the lab.
A novel role for mammalian target of rapamycin complex 2 (mTORC2) in brown adipose tissue (BAT) glucose uptake mediated by β-adrenoceptors (AR).
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Introduction: BAT is the primary site for thermogenesis and can consume, in addition to free fatty acids, a high amount of glucose from the blood that can both acutely and chronically affect glucose homeostasis. Therefore BAT may be a potential therapeutic target for a number of metabolic disorders dependent on glucose homeostasis, such as type 2 diabetes. Recent data indicates a role of mTORC2 in glucose homeostasis, with adipose-specific ablation of rictor, a mTORC2 component, depressing insulin-stimulated glucose uptake in vitro and impairing glucose tolerance in vivo (1). Mice with adipose specific deletion of raptor, a mTORC1 component, are resistant to diet-induced obesity and are insulin sensitive (2), indicating vastly different roles for mTORC1 and mTORC2 in adipose tissues.

Aims: To investigate the role of mTOR on sympathetic stimulation of glucose uptake in BAT.

Methods: Mouse brown adipocytes or human multipotent adipose-derived stem cells were used. The role of mTOR in β-AR mediated increases in glucose uptake, GLUT1 transcription and translocation was assessed by ablation of rictor or raptor using siRNA or pharmacological inhibition of mTOR by specific mTOR inhibitors.

Results: β-ARs increase glucose uptake in BAT via two different signaling pathways: one part is dependent upon cAMP mediated increases in GLUT1 transcription and translation, and another part is dependent on mTORC2 stimulated translocation of GLUT1 to the plasma membrane, leading to increased glucose uptake. Both parts are essential for β-AR-stimulated glucose uptake. Importantly, β-AR effects via mTORC2 are separate from the classical insulin-phosphoinositide 3-kinase-Akt pathway, highlighting a novel mechanism of mTORC2 activation.

(2) Polak et al Cell Metab 8:399-410 (2008)

Monitoring angiotensin II receptor complexes using bioluminescence resonance energy transfer
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Introduction. The renin-angiotensin system is involved in the regulation of blood pressure and fluid balance. Its major effector, the hormone angiotensin II, acts upon the angiotensin II type 1 receptor (AT1 receptor) and the angiotensin II type 2 receptor (AT2 receptor). The AT1 receptor is a well-characterised G protein-coupled receptor that mediates most of the well-known biological effects of angiotensin II such as hypertension and antinatriuresis. In contrast, the molecular and physiological functions of the AT2 receptor remain poorly understood, though it is often believed to counteract many AT1 receptor-mediated effects. In order to understand the molecular and physiological properties of these receptors, it is necessary to understand how they interact, and the functioning of the resultant complex, within the cellular environment.

Aims. The aim of this study was to investigate the interactions of the AT1 and AT2 receptors, and elucidate the properties of the resultant multi-component complex.

Methods. Bioluminescence resonance energy transfer (BRET) was used to investigate interactions. BRET is a widely used technique for monitoring very close proximity in live cells in real time. Receptors and their interactors were tagged with either BRET donor luciferase enzymes or BRET acceptor fluorophores and transiently transfected in HEK293FT cells.

Results & Discussion. This study has enabled elucidation of the AT1-AT2 receptor complex and its potential role in the context of broader functional interactions with various partners.
Expression and Purification of Human Calcitonin Receptor
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Introduction. G protein-coupled receptors (GPCRs) play vital roles in a range of biological processes and are attractive targets for therapeutic drugs. In recent years, there have been significant advances in both structural and biophysical techniques that have led to high-resolution structures of an increasing number of GPCRs. Despite these recent advances, structural studies of GPCRs remain a significant challenge due to difficulties in producing high levels of stable receptor. To date, there are no full length Family B GPCR structures and only two family B GPCR transmembrane domain structures have been reported. The human calcitonin receptor (hCTR) is a family B GPCR that mediates responses to the peptide hormone calcitonin. To advance our understanding of the mechanism of family B GPCRs, we have optimised conditions to overexpress and purify hCTR for structural studies.

Aims. To overexpress and purify hCTR for structural studies

Methods. A hCTR construct bearing an N-terminal FLAG-tag and a C-terminal His-tag was cloned into a baculovirus vector for insect cell expression. Receptor expression was determined by radioligand binding. The receptor was purified by immobilised metal affinity chromatography followed by FLAG antibody affinity chromatography in the presence of dodecyl-d-maltopyranoside and cholesterol. Receptor purity was analysed by SDS-PAGE and receptor monodispersity was assayed by size exclusion chromatography.

Results. hCTR expression in insect cell was optimised with yields of approximately 10 nmol/L. hCTR can be purified to near homogeneity using affinity chromatography followed by size exclusion chromatography. Purity was found to be >95% assessed by SDS-PAGE. Preliminary results revealed purified hCTR was able to couple to Gαs heterotrimer the presence of peptide agonist.

Conclusion. The ability to produce high amount of receptor and G protein complex for structural studies will aid in understanding family B GPCR function.
Pharmacological characterization of a muscarinic acetylcholine M₁ receptor positive allosteric modulator MIPS1463

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Introduction. Activation of the muscarinic acetylcholine M₁ receptor (M₁R) is a potential approach for the treatment of cognitive impairment, such as in Alzheimer’s disease. Due to the high homology in the orthosteric binding site of muscarinic receptor subtypes, selective M₁R activation can be obtained more easily by targeting less conserved allosteric binding sites.

Aims. This study aimed to characterize the in vitro pharmacology of a novel M₁R positive allosteric modulator, MIPS1463.

Methods. [³H]N-methylscopolamine binding and inositol phosphate accumulation assays were utilized to study the pharmacology of MIPS1463 at M₁R expressed heterologously in CHO cells, or endogenously in murine primary neuronal cells.

Results. MIPS1463 displayed binding affinity with a pKᵦ value of 5.56±0.10 and selectively potentiated the binding affinity of acetylcholine at M₁R (binding cooperativity with a log  rãi value of 2.84±0.13) as well the potency of acetylcholine in inducing the accumulation of inositol phosphate. Furthermore, it displayed direct allosteric agonism in both recombinant and neuronal cells, in addition to modulating the cognate agonist.

Discussion. MIPS1463 is a new selective M₁R allosteric positive modulator (PAM) that is more potent than the current exemplar M₁R PAM, benzyl quinolone carboxylic acid (BQCA). Having shown activity in murine neuronal cells, MIPS1463 represents a potentially useful pharmacological tool for further M₁R in vivo studies.

Investigating the molecular determinants of allosteric modulation at the adenosine A₁ receptor.

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Introduction. The adenosine A₁ receptor (A₁AR) is an important therapeutic target for a number of cardiovascular and neuronal conditions (Jacobson et al, 2006). However, effective targeting has remained elusive due to its widespread distribution and high conservation of the orthosteric adenosine-binding pocket. A₁AR allosteric modulators interact with a topographically distinct binding site from that of adenosine and offer a number of theoretical advantages, including increased subtype selectivity and preservation of the spatial and temporal pattern of endogenous agonist signalling. To facilitate the rational design of more selective and efficacious A₁AR allosteric ligands, greater structural knowledge of the allosteric binding site is required.

Aims. To probe the key residues involved in conferring A₁AR allosteric ligand affinity and cooperativity.

Methods. Mutant A₁ARs containing single alanine substitutions were stably expressed in FlpINCHO cells. Radioligand binding interaction studies between the orthosteric agonist, NECA, and the allosteric ligand, PD81723, quantified the influence of mutations on allosteric ligand affinity and cooperativity. Molecular dynamic simulations were performed using a 3D model of the human A₁AR based on an active A₂AAR structure (PDB ID: 3QAK).


Discussion. A₁AR mutagenesis and molecular modelling suggest that V87 3.32, Q92 3.37, N184 5.42 and H251 6.52 have an important role in conferring conformational rearrangements upon activation through the formation of hydrophobic and hydrogen bonds with NECA, while E172 5.42 forms a hydrogen bond and E171 4.12 forms an aromatic stacking interaction with PD81723. The extracellular residues, K168 5.42, E170 5.42, E172 5.42, K173 5.42, form hydrogen interactions with one other, which may explain their role on the transmission of allosteric cooperativity.

**Fluorescently labelled ligands targeting the dopamine D2 receptor**

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Introduction. Fluorescently labelled ligands (FLLs) are useful tools for exploring G Protein-Coupled Receptor (GPCR) function such as receptor-ligand interactions, receptor internalization, compartmentalization and trafficking (Vernal et al, 2014). FLLs can be adapted towards competition-based binding assays that may be used for screening for new compounds and scaffolds (Stoddart et al, 2012). The dopamine D2 receptor (D2R) is implicated in multiple CNS disorders. However, there is a lack of fluorescent ligand tools with which to investigate the function of this important therapeutic target.

Aims. To functionalize a range of ligands of differing pharmacology with organic fluorophores and pharmacologically evaluate them at the D2R.

Methods. Chemical synthesis of FLLs. Pharmacological evaluation in radioligand binding assays and confocal microscopy using FlpIn CHO cells stably expressing dopamine D2R receptors.

Results. The 2-MPP-SB269252 FFLs maintained high binding affinity for the D2R (best analogue pKi = 7.88 ± 0.20 (13 nM)). Three FFLs showed selective binding to D2L receptors using confocal microscopy.

Discussion. We identified three novel FFLs with high binding affinities that could be utilised in applications such as competition based binding assays as an alternative to radioligand binding assays for the D2R.


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**Potent non-peptide agonists for human C3a receptors**

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Introduction. C3a is a product of complement activation and has been implicated in the pathogenesis and progression of numerous inflammatory conditions such as asthma, allergies, arthritis and others¹. To date, there is no potent and selective non-peptide agonist or antagonist of C3a receptor (C3aR), which can be used as a pharmacological probe for interrogating the roles of human C3a in physiology and disease.

Aims. To explore new compounds containing an oxazole ring that can potentially impart and stabilise a turn structural motif by replacing an amino acid and the N-terminus of this scaffold.

Methods. Human monocytes were isolated using Ficoll-paque density centrifugation from buffy coat of human blood. CD14⁺ monocytes were positively selected using CD14⁺ and differentiated to macrophages (HMDMs) using M-CSF. Compound activity was assayed by radioligand binding for affinity and selectivity and by intracellular calcium release for function.

Results. A study of N-acyl amino acid-oxazole-arginine compounds showed that the optimal amino acid side chain for attachment to the oxazol scaffold was leucine (56) or isoleucine with only slightly different binding affinity (pIC50=7.71±0.08 versus 7.53±0.10) and potency (pEC50=7.72±0.27 versus 7.57±0.11). Further modification of the leucine terminus (56) with an indole ring gave the greatest improvement in affinity and potency (pIC50=8.03±0.05; pEC50 =8.15±0.10) in calcium mobilisation assay. In addition, one of the most potent C3a agonists had an isoleucine side chain and a bromine substituent on the pyridine ring. This significantly improved binding affinity and was one of the most potent agonists in this series with pIC50 = 8.30±0.10 and pEC50 = 7.76±0.36.

Discussion. Introduction of a rigid turn-like conformation enforced by an oxazole heterocycle has produced multiple potent and selective C3aR non-peptide agonists (EC50 < 20 nM, HMDMs). These compounds could be useful for probing the physiological roles of C3a, which is highly unstable in vivo losing its C-terminal arginine and no longer binding to C3aR. By contrast the small molecule agonists reported here are extremely stable.

**Designed multiple ligands targeting the dopamine D2 and muscarinic M1 receptors.**

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**Introduction.** Atypical antipsychotics display marked polypharmacology. Although this is thought to confer favourable efficacy, it was achieved through serendipitous discovery rather than by a rational drug design process. Activity at dopamine D2 and serotonin 5-HT2A receptors has been implicated as useful for antipsychotic efficacy. Additionally, the M1 muscarinic acetylcholine receptor (mAChR) is highlighted as important for the cognitive deficits in key CNS disorders. The concept of designed multiple ligands (DMLs) takes two pharmacophores and integrates the two into a single molecule (Morphy et al, 2009). Therefore this approach can be used as a rational approach to achieving desirable polypharmacology.

**Aims.** To use D2 privileged structures derived from putative and clinically relevant antipsychotics and hybridize with a M1 scaffold to develop a series of DMLs.

**Methods.** Chemical synthesis and pharmacological evaluation of ligands in both functional (D2 and M1) and radioligand binding assays (D2, M1, and 5-HT2A).

**Results.** The most promising DML displayed an activity profile at all three receptors; M1 (pEC50 = 5.98 ± 0.25 (1 μM), Emax = 64 ± 10), D2 (pK_i = 7.75 ± 0.10 (18 nM)) and 5-HT2A (pK_i = 8.24 ± 0.37 (5.8 nM)).

**Discussion.** We designed and characterized a novel DML using a privileged structure derived from the antipsychotic ziprasidone, that retained strong binding and functional activity at the D2R, weak partial agonism at the M1 mAChR and a high affinity for the 5-HT2AR. This approach highlights the utility of privileged structures in drug discovery.


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

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**Introduction:** Opioid receptors are G protein-coupled receptors that modulate cAMP through action on adenylyl cyclase. Beta-endorphin (BE1-31) is a major endogenous opioid peptide with affinity for both mu and delta opioid receptors (MOP and DOP). This peptide is biotransformed rapidly in-situ producing an array of bioactive fragments. Beta-endorphin 1-11 (BE1-11) is one of the major peptide fragments produced from this biotransformation and may itself be involved in an array of biological actions. Opioid peptide fragments of BE1-31 have been shown to provide less selectivity for MOPs, potentially indicative of different pharmacological effects dependent upon the nature of the specific receptor interactions. This study examined the effect of BE1-11 on the inhibition of cAMP production in Human Embryonic Kidney 293 (HEK) cells over expressing MOP and DOP, (HEK-MOP, HEK-DOP) and a native cell line (retinoic acid differentiated SHSY5Y) with phenotypically neuronal properties.

**Aim:** To investigate the inhibitory action of BE1-11 through MOP and DOP action in HEK-MOP, HEK-DOP and differentiated SHSY5Y cells on the forskolin induced cAMP accumulation assay.

**Method:** Changes in cAMP accumulation were assessed at a range of concentrations of BE1-11 (0.1nmol/L – 1μmol/L). Forskolin was used to stimulate cAMP accumulation, assessed using the Alpha-screen cAMP assay (Perkin Elmer23). The antagonism effects of the opioid antagonists, CTAP (MOP antagonist) and naltrindole (DOP antagonist) were examined for confirmation of cAMP modulatory effect of BE1-11 on MOP and DOP.

**Results:** A concentration-dependent decrease in cAMP accumulation was observed with increasing BE 1-11 concentrations in HEK-MOP, HEK-DOP and differentiated SHSY5Y cells. The EC50 values of BE1-11 in HEK-MOP and HEK-DOP cells were determined to be 18 and 21 nmol/L respectively. In addition, the EC80 for BE1-11 in differentiated SHSY5Y cells was 1μmol/L; cAMP inhibition action of BE 1-11 was antagonised by CTAP and naltrindole independently.

**Discussion:** In this study we have shown that BE1-11 not only has equal efficacy in modulating cAMP in HEK-MOP and HEK-DOP cells, but also reduces cAMP accumulation in a native cell line, differentiated SHSY5Y cells, through both receptors.
Modulation of the M4 Muscarinic Acetylcholine Receptor Regulation by Allosteric Ligands
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Introduction. The M4 muscarinic acetylcholine receptor (mAChR) is predominantly expressed within the CNS. Importantly, M4 mAChRs represents a potential pharmacological target for drug therapies in schizophrenia and Alzheimer’s disease (Langmead, 2008). More recently, M4 mAChR drug discovery has focused on the development of allosteric modulators of this receptor as means to achieve more selective drugs.

Aims. To investigate the effects of typical orthosteric ligands and novel allosteric ligands on M4 mAChR regulatory processes.

Methods. β-Arrestin-YFP recruitment upon incubation with different orthosteric and allosteric ligands to a c-terminus tagged Renilla Luciferase (RLuc) M4 mAChR will be measured by BRET in transfected CHO and NG-108 cells treated with and without pertussis toxin (PTx).

Results. Preliminary results show that while canonical M4 mAChR signalling pathways are dependent on Gai proteins, β-Arrestin-1 and -2 recruitment, is independent of Gai protein in both CHO and NG108-15. This finding indicates that β-Arrestins recruitment does not require activation of Gai proteins. Furthermore, the allosteric M4 mAChR ligand (LY2033298) has the capacity to potentiate the potency of ACh for these assays in a dose-dependent manner.

Discussion. Given the therapeutic potential of M4 mAChR in locomotion, cognition and psychosis-associated disorders, understanding the mechanisms behind the regulation of this receptor upon orthosteric and allosteric ligands is of key importance. Our results demonstrate that β-Arrestin recruitment to M4 mAChR, which traditionally precedes receptor internalization, is independent of Gai protein activation. Future studies will focus on a detailed characterisation of the role of β-Arrestin in M4 mAChR signalling.


Mechanistic Insights into Allosteric Structure-Function Relationships at the M1 Muscarinic Acetylcholine Receptor
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Introduction. The M1 muscarinic acetylcholine receptor (mACHr) is an attractive therapeutic target for CNS disorders such as Alzheimer’s disease and schizophrenia. Benzylquinolone carboxylic acid (BQCA) is the first highly selective positive allosteric modulator (PAM) for the M1 mAChR, but possesses low affinity for the allosteric site of the receptor. Recent drug discovery efforts identified benzoquinazolinone 12 as a more potent M1 mAChR PAM that is structurally related to BQCA.

Aims. To characterise the pharmacology of benzoquinazolinone 12 and define the molecular basis for its improved affinity at the M1 mAChR.

Methods. Radioligand binding and Inositol-1-phosphate accumulation assays on cells expressing either WT or mutant M1 mAChRs in addition to molecular modelling studies.

Results. The improved potency of benzoquinazolinone 12 is derived from a 50-fold increase in affinity for the allosteric site, as compared with BQCA, while it retains a similar level of positive cooperativity with acetylcholine (ACh). Our results validate the allosteric binding pocket previously described for BQCA as a shared site for benzoquinazolinone 12, and provide a molecular basis for its improved activity at the M1 mAChR. This includes a key role for hydrophobic and polar interactions with residues Tyr179, in the second extracellular loop (ECL2), and Trp400 in transmembrane (TM) domain 7, and additional interactions of benzoquinazolinone 12 with TM2 residues Tyr822 and Tyr852.

Discussion. This study highlights how the properties of affinity and cooperativity can be differentially modified on a common structural scaffold, and identifies molecular features that can be exploited to tailor the development of M1 mAChR-targeting PAMs.
Signal Transduction Pathways Activated by Insulin-like peptide 5 at Relaxin Family Peptide Receptor 4
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Introduction. Insulin-like peptide 5 (INSL5) is a two-chain, three-disulphide bonded peptide belonging to the insulin/relaxin superfamily that is uniquely expressed in the enteroendocrine L-cells of human and mouse colon (Grosse et al, 2014). It is the cognate ligand for the G protein-coupled relaxin family peptide receptor 4 (RXFP4) that is mainly expressed in the colorectum and the enteric nervous system. Wild-type mice injected with INSL5 show an increase in food intake, an effect that was abolished in RXFP4 knockout mice, demonstrating that INSL5-RXFP4 forms a novel gut-hormone axis that regulates appetite (Grosse et al, 2014). However, currently there is little known regarding the signal transduction pathways activated by RXFP4.

Aims. This study examined intracellular signalling pathways activated by human INSL5 (hINSL5) and mouse INSL5 (mINSL5) acting at the human RXFP4 stably expressed in CHO cells.

Methods. The effect of hINSL5 and mINSL5 on cyclic AMP (cAMP), mitogen-activated protein kinase (MAPK) and protein kinase B (PKB/Akt) pathways was investigated using AlphaScreen® proximity assays. Intracellular Ca2+ flux was monitored in a Flexstation® using X-rhod-1AM.

Results. mINSL5 inhibited forskolin-stimulated cAMP accumulation and activated ERK1/2, p38MAPK, Akt-Ser473 and S6 ribosomal protein (S6RP) more potently than hINSL5; however both peptides were equipotent for Akt-Thr308. No JNK1/3 activity or intracellular Ca2+ mobilisation was observed.

Discussion. INSL5 negatively regulates cAMP production and activates multiple signalling pathways important for diverse cellular functions including growth, differentiation and proliferation (ERK1/2, p38MAPK, Akt) and protein synthesis (S6RP). Information on signalling pathways activated by INSL5 at RXFP4 is essential for understanding the biological roles of this novel gut hormone.


Investigating the signalling bias of GLP-1(9-36)amide at the GLP-1 receptor
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Introduction. Glucagon-like peptide 1 (GLP-1), an incretin synthesised in the intestine, is released in response to food ingestion to stimulate insulin secretion via binding to the GLP-1 receptor (GLP-1R). Secreted GLP-1(7-36)amide, is rapidly cleaved by the serine protease, dipeptidyl peptidase-4 (DPP-4), to produce GLP-1(9-36)amide, the predominant form of GLP-1 in postprandial plasma. Despite GLP-1(9-36)amide being at a concentration 5-to 10-fold greater than GLP-1(7-36)amide, no consensus on its biological role has been identified in humans.

Aims. The GLP-1R has been reported to signal via several, stimulatory and inhibitory G proteins and we have previously shown that different ligands influence this coupling. Here, we aim to address the effect of GLP-1(9-36)amide on GLP-1R signalling through the inhibitory Gi and Gq subunits.

Methods. To compare the effect of GLP-1(9-36)amide on Gi and Gq signalling, we expressed the GLP-1R in a yeast reporter assay, allowing the study of individual G protein subunit activation. We then validated our results through the use of pertussis toxin (PTX) on GLP-1R transfected cells lines.

Results. Consistent with previous findings reported in the yeast system [1] results indicated that GLP-1(9-36)amide is a partial agonist of the GLP-1R-Gi response (pEC50= 7.4±0.2 and EMax =48±1.4), and a full agonist of Gq (pEC50 =7.7±0.3). Further, we demonstrated that GLP-1(9-36)amide is a full agonist of GLP-1R coupled Gz, but with a much lower potency (pEC50 =6.5±0.2), when compared to Gq. Using GLP-1R expressing HEK 293T cells treated with PTX (200ng/ml for 16 h), we confirmed that the GLP-1R is coupled to inhibitory G proteins in mammalian cells. GLP-1R stimulation with GLP-1(9-36)amide showed a more pronounced effect when compared to GLP-1(7-36)amide.

Discussion. Our data provide further evidence that GLP-1(9-36)amide is an active metabolite and we suggest that it may act to modulate the GLP-1(7-36)amide activated signalling pathway, through inhibitory G proteins. We are currently investigating the Gq signalling bias in mammalian cells to clinically prescribed GLP-1 mimetics.

Inhibition of human neuronal Ca\textsubscript{v2.3} channels via \(\mu\), \(\delta\) and \(\kappa\)-opioid receptor activation
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Introduction. G protein-coupled (GPC) opioid receptors mediate the pain relief offered by opioid analgesics such as morphine. Presynaptic voltage-gated Ca\textsubscript{v2.1} and Ca\textsubscript{v2.2} calcium channels are efficiently inhibited by GPC opioid receptor activation in mammalian neurons and heterologous expression systems (Altier and Zamponi, 2008). This mechanism is at least partly responsible for the analgesic effects of opioids. Neuronal voltage-gated Ca\textsubscript{v2.3} channels are widely expressed in the central and peripheral nervous system where they contribute to neurotransmission and pain sensation. However, modulation of the Ca\textsubscript{v2.3} channel through \(\mu\)- and \(\delta\)-opioid receptors is poorly defined and has not previously been reported for \(\kappa\)-opioid receptors.

Aims. We hypothesised that activation of human \(\mu\)-, \(\delta\)- or \(\kappa\)-opioid receptors modulate human Ca\textsubscript{v2.3} channels via G protein signalling.

Methods. Whole-cell Ba\textsuperscript{2+} currents were recorded in human embryonic kidney (HEK293T) cells co-expressing human Ca\textsubscript{v2.2} or Ca\textsubscript{v2.3} channels and \(\mu\)-, \(\delta\)- or \(\kappa\)-opioid receptors. The voltage dependence of Ca\textsubscript{v2.2} or Ca\textsubscript{v2.3} channel inhibition was investigated with depolarizing paired-pulse protocols, whereby depolarizing pre-pulses to +80 mV or +120 mV were used to test relief from inhibition. Selective opioid receptor agonists and antagonists were used to study receptor modulation. The involvement of intracellular signalling pathways was investigated using specific inhibitors of GPC receptor-G protein coupling.

Results. Activation of \(\mu\)-, \(\delta\)- or \(\kappa\)-opioid receptors inhibited Ca\textsubscript{v2.3} or Ca\textsubscript{v2.2} channel current amplitude by ~45% and ~60%, respectively. Inhibition of the Ca\textsubscript{v2.3} channel was not dependent on the type of calcium channel \(\beta\) subunit co-expressed. Inhibition of the Ca\textsubscript{v2.3} channel was primarily voltage independent, as depolarizing pre-pulses could not relieve the inhibited current. This was in marked contrast to the primarily voltage dependent modulation of Ca\textsubscript{v2.2} channels that showed classical characteristics of G protein \(\beta\gamma\) subunit interaction with the channel and nearly complete recovery of the current by depolarizing pre-pulses. For all three types of opioid receptors, the pathway leading to Ca\textsubscript{v2.3} channel inhibition was sensitive to pertussis toxin and to intracellular application of non-hydrolysable GDP analogue GDP-\(\beta\)-S. Similarly, the overexpression of a G protein \(\beta\gamma\) subunit scavenger, myristoylated-phosducin, significantly reduced the magnitude of Ca\textsubscript{v2.3} channel inhibition.

Discussion. It is generally believed that Ca\textsubscript{v2.3} channels are relatively insensitive to modulation by GPC receptors. We demonstrate that Ca\textsubscript{v2.3} channels are efficiently inhibited by activation of \(\mu\)-, \(\delta\)- or \(\kappa\)-opioid receptors. Inhibition occurs via voltage-independent G protein signalling mechanisms. These results suggest opioid receptors control specific members of the Ca\textsubscript{v2} channel family via differential signalling pathways. Neuronal Ca\textsubscript{v2.3} channels are therefore potential targets for opioid analgesics.

**Functional analysis of the N-terminal residues of human amylin**
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**Introduction.** Amylin is a 37-amino acid peptide hormone involved in metabolism, glucoregulation and satiety. Accordingly, amylin has been investigated as a therapy for diabetes and obesity. Amylin is a Family B G protein-coupled receptor (GPCR) peptide ligand, proposed to activate its receptors via a two-domain model of binding whereby the peptide N-terminus is important for receptor activation. The calcitonin family, of which amylin is a member, all possess a highly conserved N-terminal disulphide loop which is critically important for receptor activation. Nevertheless, very little is currently known about the significance of the individual N-terminal residues of amylin in receptor activation.

**Aims.** To investigate the role of the N-terminal loop residues of human amylin in receptor activation by completing an alanine scan of amino acids in the loop.

**Methods.** Fmoc solid-phase peptide synthesis was used to make peptide analogues, followed by cleavage, precipitation, oxidation and purification of the peptides. All were amidated with the disulphide bond. Biological functional assays were carried out in COS7 cells transiently transfected with the human calcitonin receptor hCT\(^{(a)}\) or the amylin 1 receptor hAMY\(^{(1a)}\) consisting of hCT\(^{(a)}\) with human RAMP1. Alpha Screen detection of cyclic AMP formation upon peptide stimulation was measured as an indicator of receptor activation.

**Results.** Alanine substitution of lysine at position 1 or asparagine at position 3 did not significantly alter human amylin activity at either receptor hCT\(^{(a)}\) or hAMY\(^{(1a)}\) suggesting that these residues are not critical for receptor activation. The substitution of threonine to alanine at position 4 significantly decreased pEC\(_{50}\) at hCT\(^{(a)}\) but not hAMY\(^{(1a)}\) with no changes in E\(_{\text{max}}\). Threonine substitution with alanine at position 6 caused a significant reduction in E\(_{\text{max}}\) at both receptors with no change in pEC\(_{50}\).

**Discussion.** It appears that the N-terminal residues which encompass the peptides “activation loop” have disproportionate and differential impacts on receptor activation. The strongly conserved threonine at position 6 is clearly a key residue in the N-terminal loop with threonine 4 showing slightly more importance in activation of hCT\(^{(a)}\) over hAMY\(^{(1a)}\).

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**The third intracellular loop of the human L-amino acid receptor GPRC6A controls cell surface expression and receptor function.**
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**Introduction.** Human G protein-coupled receptor, family C, group 6, subtype A (GPRC6A) was cloned by us 10 years ago (Wellendorph et al. Gene. 2004) and great effort has since been dedicated to characterize the receptor. One challenge has been to understand why the two close orthologs (human vs. mouse, 80% seq. identity) are differently expressed on the cell surface when expressed recombinantly (Wellendorph et al. Mol. Pharmacol. 2005). Previous work with chimera constructs between human and goldfish GPRC6A has shown that the transmembrane and/or C-terminal part of human GPRC6A prevent surface expression (Wellendorph et al 2005).

**Aims.** The aim of the study was to identify the amino acid motif responsible for the intracellular retention and lack of functional response of the human GPRC6A receptor.

**Methods.** Chimeric receptors where parts of the 7-transmembrane domain in human GPRC6A had been replaced by the corresponding mouse sequence were generated by PCR. The cDNA encoding the bonobo chimpanzee GPRC6A sequence was synthesized using Genscript. Mutations were generated using the QuikChange protocol. Cell surface expression was determined by ELISA of a N-terminal c-myc epitope and functional responses were determined by measurement of inositol monophosphate using the IP-ONE time-resolved FRET assay (Cisbio).

**Results & Discussion.** The chimeric human/mouse receptors demonstrated that the third intracellular loop (ICL3) controls cell surface expression and function given that the human receptor containing the mouse ICL3 was trafficked to the surface and could be activated by the GPRC6A receptor agonists L-arginine and L-ornithine. Next we showed that the GPRC6A receptor from bonobo, which is 99% identical to the human receptor, trafficks to the cell surface. This allowed us to narrow down the motif controlling surface trafficking to a “RKLP” motif in the bonobo ICL3, which is shortened and changed to “K--Y” in the human sequence. Subsequent introduction of the RKLP motif in the human GPRC6A receptor generated a fully functional receptor trafficking to the cell surface.

**Conclusion.** We have identified the motif in the human GPRC6A receptor causing intracellular retention and lack of function. Our current efforts focus on delineating the impact of the ICL3 variation on human physiology.
Deciphering the complex mode of relaxin receptor (RXFP1) activation
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Introduction. The receptor for the peptide hormone relaxin, RXFP1, and its related receptor RXFP2 are unique among GPCRs in that they contain a large ectodomain consisting of 10 leucine-rich repeats (LRRs) and an N-terminal low-density lipoprotein type A (LDLa) module. Activation of RXFP1 by relaxin requires coordination of ligand binding contacts with multiple receptor domains with the LDLa module driving the final receptor active conformation(s) via interactions with other domains by an unknown mechanism.

Aims. In this study, we explore the role of the linker between the LDLa and LRR domain in binding and activation utilizing functional analysis of receptor mutants and nuclear magnetic resonance (NMR) experiments on recombinant proteins of the LDLa module with or without linker.

Methods. Protein constructs were expressed using fermentation and purified on an IgG sepharose column. 15N-labeled LDLa proteins were analysed by HSQC NMR with varying concentrations of H2 relaxin as well as a synthetic H2 relaxin analog with a paramagnetic label on the N-terminus of the A-chain. Site-directed mutagenesis was performed on RXFP1 and once verified, mutants were transfected transiently into HEK cells and submitted to relaxin binding, cAMP signalling and cell-surface expression assays.

Results. Mutations of residues in the linker region showed a marked loss of function as well as reduced binding, despite cell surface expression being equivalent to wild-type levels. Chemical shifts in residues seen in NMR data suggested that relaxin binds to the linker with μM affinity, and experiments with paramagnetically labelled relaxin implicated the A-chain of relaxin in this interaction. Furthermore, the broadening of resonances in the NMR spectrum is non-sequential, indicating a specific, binding-induced conformational change is occurring.

Discussion. Taken together, these experiments implicate the linker region between the LDLa module and LRRs in the activation process of RXFP1. The linker is hypothesized to be a secondary binding site, and by undergoing a binding-related structural change, the LDLa and linker together interact with the TM domain to induce activation. The understanding of the mechanics of this activation will help in the discovery of alternative means to control RXFP1 activity as well as enhancing the knowledge of this interesting and unusual GPCR.
Characterising the Conformational Diversity of the Neurotensin Receptor 1
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Introduction. The neurotensin receptor 1 (NTS1) is a GPCR for the tridecapeptide neurotensin (NT). Activation of NTS1 by NT has been implicated in schizophrenia, Parkinson’s disease and cancer cell growth. Crystal structures of NTS1 bound to fragments of NT, as well as mutagenesis data, have defined intermediate states of NTS1 along with the high affinity binding site for NT. It is desirable to complement these achievements by probing conformational dynamics and identifying conformations mediating particular signaling responses. Furthermore, little is known about transient interactions that may underlie NT binding. Cellular high-throughput encapsulation, solubilisation and screening (CHESS)-based directed evolution, yielded stable mutants of NTS1 suitable for solution NMR studies.

Aims. We aim to assess the dynamic landscape of site-specific 13CH3-methionine labeled NTS1 and to characterise low affinity binding sites for NT using solution NMR techniques.

Methods. NTS1 mutants are expressed to the E. coli inner membrane and reconstituted in detergent micelles. Isotopic labeling using 13CH3-methionine facilitates NMR based dynamics studies of NTS1 mutants that may reveal motions at the ns scale. Saturation transfer difference NMR (STD NMR), a technique commonly used for fragment screening against soluble proteins is applied to detect low affinity peptide interactions to samples of NTS1 mutants.

Results. The high stability of these receptors in detergent allowed us to repeatedly measure the binding of NT fragments over 24 h periods at 25°C without significant loss of the STD signal. Regions of NT involved in receptor interactions could be mapped, exhibiting agreement with the crystal structures. We could further show that signals originating from low affinity ligands are abolished in the presence of a higher affinity competitor. Using 1H-13C-HMQC NMR we identified the 9 13CH3-methionine resonances that are well dispersed across the whole sequence of one of our labeled NTS1 mutants. These 13CH3 groups will act as local probes in NMR based dynamics studies.

Discussion. This work will help to understand the dynamics of NTS1 upon ligand interaction and to uncover binding pathways of NT fragments to high affinity binding sites in NTS1. This is the first time, to our knowledge, that STD NMR was successfully applied to show interaction of a GPCR with its ligand.

Identifying novel sites of ligand interaction on the ã1 adrenoceptors.
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Introduction. The homobilvalent bisacridines (see figure, “Cn” bisacridine) are ligands for the ã1 adrenoceptors (ARs) with steep binding curves and the potential to interact bitopically with the ã1 ARs. C4 is >16X selective for the ã1A AR and C2 is >8X selective for the ã1B AR over the other ã1 ARs. Aims. To identify novel binding sites on the ã1 ARs.

Methods. The bisacridines were characterised using competition and dissociation radioligand binding assays. Homology models and in silico docking were used to identify potentially novel interactions between the bisacridines and the ã1 ARs.

Results. The bisacridines increase the dissociation rate of [3H]prazosin from the ã1A and ã1B ARs showing increasing effect with increasing linker length (ã1A: r2: 0.27, p<0.001, ã1B: r2:0.22, p<0.001). 100 µM C9 can increase [3H]prazosin dissociation 17.6 ± 2.5X from the ã1A AR (p<0.001) and 62.3 ± 19.3X from the ã1B AR (p<0.001). Docking revealed interactions with the second extracellular loop (ECL2) and a small cluster of residues at the extracellular end of transmembrane helix II (TMII). Mutation of ECL2 residues in the ã1A AR (Q177G.I178V.N179T) reverses the selectivity of C4, but does not alter affinity of C2. Functional effects of bisacridines and kinetic effects of mutants are being characterised.

Discussion. The ã1A AR is of particular clinical interest as it is considered an efficacious target in the treatment of benign prostatic hyperplasia (Lepor, 2007), a common disease in older men, as well as a potentially useful target in the use of seizure disorders (Hillman et al. 2009). Current, clinically used antagonists do not possess high selectivity over both the ã1B and ã1D ARs. Identification of novel sites of interaction of the receptor may yield alternative, selective sites of interaction, or non-orthosteric modes of antagonism allowing for the design of drugs with higher selectivity or improved side effect profiles.

Signalling of indole-2-carboxamides as allosteric modulators of the cannabinoid receptors.
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Introduction. The GPCR cannabinoid receptor type 1 (CB₁) has an allosteric binding site. One of the most studied of the CB₁ allosteric modulators is ORG27569 which we have recently shown to produce a complex, concentration and time-dependent modulation of agonist-mediated regulation of cAMP levels, as well as an increased rate of desensitisation of CB₁-mediated cellular hyperpolarisation and a decrease in agonist-induced receptor internalisation (Cawston et al, 2012).

Aims. To characterise the cAMP signalling response of indole-2-carboxamide compounds structurally correlated to ORG27569 and previously characterised for ability to modulate binding of [3H]CP55,940 to hCB₁ (Piscitelli et al., 2012).

Methods. Using the real-time kinetic BRET CAMYEL assay we assessed the forskolin stimulated cAMP response of 10 μM indole-2-carboxamide compounds in both the presence and absence of EC90 CP55,940 in HEK 3HA-hCB₁ cells and Flp-In 293 HA-3TCS-hCB₂ cells. Compounds that displayed altered cAMP responses were then characterised further with regards to potency and cAMP signalling characteristics.

Results and Discussion. The initial screening of forskolin stimulated cAMP responses of 14 indole-2-carboxamide compounds identified three compounds that antagonized the CP55,940 mediated inhibition of cAMP more rapidly than ORG27569, suggestive of higher potency in HEK 3HA-hCB₁ cells. More detailed analysis confirmed this enhanced potency, and demonstrated that these compounds all displayed equivalent inverse agonism to ORG27569.

Three further compounds displayed allosteric antagonism of the CP55,940 mediated effect with no inverse agonism. Four compounds displayed agonist activity at the hCB₂ receptor. As these compounds were previously described to decrease [3H]CP55,940 binding, further studies are required to determine if this effect is allosteric or orthosteric.

Data from this study will be used to further define the appropriate chemical structures for targeting therapeutically useful pathways in relation to CB₁ and CB₂.


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

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

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

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

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

Architecture and function of signalling complexes assembled by β2-adrenergic and M3 muscarinic receptors
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Introduction. Protein complexes have diverse structure and function within cells. Recently, a newly discovered signalling complex (signalosome) built around RXFP1 conferred some unique properties to this GPCR. Assembly of the RXFP1 signalosome, comprising several scaffolding and signalling proteins, enabled ultrasensitive responses to sub-picomolar relaxin (Halls et al, 2010). Although the closely related receptor RXFP2 does not form a signalosome, we have confirmed this phenomenon for other GPCRs, namely the β2-adrenoceptor (β2AR) and M3 muscarinic receptor (M3R).

Aims. To investigate the architecture of β2AR and M3R signalosomes, to identify domains that are important for protein-protein interactions, and to investigate the cellular function of these novel complexes.

Methods. FRET biosensors transiently expressed in HEK293 cells were used to measure responses to sub-picomolar concentrations of isoprenaline/carbachol, mediated by endogenous β2AR or M3R respectively. GST immunoprecipitation (IP) and co-IP were used to determine the architecture and interacting domains of these complexes. Quantitative proteomics was employed to decipher the function of the signalosomes.

Results. We show that β2AR signalosome activation involves Gs/Gi stimulation of adenylyl cyclase (AC). Scaffolding is provided by Gravin and β-arrestins and negative regulation of the cAMP signal is mediated by Gip3, PKA and PDE4D5. M3R signalosome activation involves Gi/o stimulation of PKC that leads to increased AC activity. The complex is scaffolded by AKAP79 and β-arrestins. Negative regulation of cAMP signalling occurs by PKA and PDE4D3. The C-terminal fragment (Helix 8) of β2AR is important for these interactions, whilst M3R binds its interacting protein partners via the intracellular loop 3. Activation of either the β2AR or M3R signalosome results in compartmentalised downstream signalling (nuclear ERK or cytosolic PKC, respectively), and quantitative proteomics revealed both up- and down-regulation of sets of proteins unique for each receptor signalosome.

Discussion. We show that β2AR and M3R can form signalosomes, sense sub-picomolar concentrations of ligand and activate nuclear ERK or cytosolic PKC, respectively. This leads to up/down-regulation of distinct subsets of the proteome. Future experiments will aim to elucidate a physiological role for these unique GPCR complexes.


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

Aims. To investigate the pharmacology of ATF936, the most recently described calcilytic to enter clinical trials (John et al., 2013), in both heterologous and endogenous CaSR expression systems using both endogenous (Ca2+)o and exogenous (Sr2+)o orthosteric agonists.

Methods. The ability of ATF936 and NPS-2143 to modulate Ca2+o and Sr2+o-induced Ca2+ mobilisation, ERK1/2 phosphorylation and IP1 accumulation was investigated in HEK293-CaSR cells and human thyroid carcinoma (TT) cells. CaSR expression was confirmed with western blotting and RT-qPCR.

Results. ATF936 acts as a NAM of Ca2+o and Sr2+o-induced signalling, with similar estimated affinity and cooperativity values for each endpoint evaluated. These data suggest that, unlike NPS-2143, ATF-936 is not biased in its modulation of Ca2+o versus ERK1/2 phosphorylation.

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

Thomsen ARB et al. (2012) JPET 343:638–649
Cook AE et al. (2014) Br J Pharmacol In Press
Discovery of novel selective C5a2 ligands that modulate IL-6 release from macrophages

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Introduction. G Protein-Coupled Receptors (GPCRs) are a superfamily of transmembrane proteins that mediate cellular responses to their environment upon binding of an effector to their extracellular-facing binding pocket. With over 800 human GPCRs playing key roles in modulating tissue/cell physiology and homeostasis, they represent a major target for pharmaceutical intervention. Recently, an increasing number of GPCR X-ray crystal structures were solved; Computer-Aided Drug Discovery (CADD) methods such as Virtual Screening (VS) leverage that growing structural information. VS by docking attempts to identify new drug leads, ranking libraries of small molecules based on the predicted complementarity between small molecules and the target GPCR binding pocket. The success of VS is highly dependent on the conformation of the target binding pocket, therefore, a key step of CADD is to refine the binding pocket within a protein structure.

Methods. Here we present a new computationally efficient Ligand Directed Modelling (LDM) method for GPCR binding pocket refinement. This method aims to establish the global energy minimum of a GPCR binding pocket in complex with a single known active ligand for that GPCR. Our LDM method uses protein sampling, ligand docking and scoring of ligand/binding pocket complexes in recursive steps.

Results. We benchmarked the method in a range of different scenarios for which there is an available GPCR X-ray crystal structure bound to the ligand that was used for LDM refinement. The LDM refined structures were compared to both the starting and final X-ray crystal structures for their capacity to distinguish known ligands from decoys in a small scale VS.

Discussion. This benchmark provides a guideline for the application of this LDM method in future CADD projects.
**Functional expression of modified calcitonin receptor in nanodics for biophysical measurements of family B GPCR conformational changes.**

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Introduction. The complex mechanism of family B GPCR activation that allows ligand binding to the extracellular N terminal domain to activate signalling effectors within the cell is currently not understood at a structural level. Although this mechanism is not completely understood for family A GPCRs, there is much more information available for this class due to recent methodological advances. These include the crystallisation of receptors in inactive, partially and fully active conformations, the incorporation of GPCRs into nanodiscs, that allow biophysical studies in a near native environment and biophysical measurements using environmentally sensitive probes introduced at various positions in the GPCR to measure conformational changes.

Aim. This project aims to identify a model system to investigate conformational changes that occur in family B GPCRs upon activation by ligands.

Methods. Cysteine reactive probes will be used to measure conformational changes in the calcitonin receptor (CTR) expressed in nanodiscs. We will use (i) mass spectrometry: to identify reactive cysteine residues in the native CTR. (ii) Molecular modelling: to identify potential residues in the CTR that may undergo conformational changes. (iii) Mutagenesis: to mutate reactive cysteine to serine, and to introduce cysteine residues in positions that may undergo conformational changes, that can then be coupled with cysteine reactive probes. (iv) cAMP AlphaScreen and whole cell binding assays: to assess the pharmacology and cell surface receptor’s expression of native and mutant CTRs in COS7 cells. (v) CTR purification and nanodisc production: to assess CTR function in nanodiscs.

Results. Mutation to serine of solvent accessible cysteine residues and introduction of cysteines in the positions predicted by the molecular model did not alter the CTR pharmacology. Conditions for assembling nanodiscs containing functional CTR were identified.

Conclusions. We have successfully inserted CTR into nanodiscs to create a system to perform biophysical measurements that may provide insights into the activation mechanism of family B GPCRs.

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

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Introduction: The calcium-sensing receptor (CaSR) is activated by many divalent cations including the cognate ligand, Ca\(^{2+}\) and Sr\(^{2+}\), which is used in the treatment of osteoporosis. Calcimimetics, including cinacalcet, BTU compound-13 (Deprez et al., 2013) and AC-265347 (Ma et al., 2011), are all allosteric modulators of the CaSR. As such, they exert allosteric effects to positively modulate activation the actions of Ca\(^{2+}\). However, characterisation of their effects on Sr\(^{2+}\)-mediated CaSR activation is yet to be investigated.

Aim: This study compares the pharmacology of the structurally distinct calcimimetics BTU compound-13, AC-265347 and cinacalcet to modulate Ca\(^{2+}\) and Sr\(^{2+}\)-dependent CaSR activation.

Methods: Compounds were characterised for their modulation of Ca\(^{2+}\) and Sr\(^{2+}\)-mediated activation of well-characterised CaSR signalling pathways including phosphorylation of ERK1/2, intracellular Ca\(^{2+}\) mobilisation and IP\(_3\) accumulation in HEK293 cells stably transfected and induced to express the human CaSR.

Results: Comparative analysis of the effect of each of the calcimimetics on Ca\(^{2+}\) and Sr\(^{2+}\) signalling revealed no probe dependent modulation within the intracellular Ca\(^{2+}\) mobilisation assay, with similar affinity and cooperativity estimates in combination with both Ca\(^{2+}\) and Sr\(^{2+}\) (see Figure).

Discussion: Initial data suggests that calcimimetics exert similar effects on both Ca\(^{2+}\) and Sr\(^{2+}\) induced CaSR signalling.

In silico analysis and cardiac gene expression data provide novel insights into human and mouse taste receptor gene regulation
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Introduction. G protein-coupled receptors (GPCRs) are important mediators of sweet, umami, bitter and fat taste qualities that serve as important discriminators of food intake and rejection. Taste receptors are also expressed outside of the oral cavity, including in the gut, airways, brain and heart, where they have additional functions and contribute to disease. However, there is little known about the mechanisms governing the transcriptional regulation of taste receptor genes.

Aims. Following our recent delineation of taste receptors in the heart, we sought to investigate the regulatory mechanisms that drive their cardiac expression.

Methods. Gene expression analyses of healthy and diseased human and mouse hearts were performed, in conjunction with in silico analyses of taste receptor cis-regulatory regions.

Results. We observed coordinated expression for a subset of chromosomally clustered taste receptors in heart tissues, consistent with a common gene regulatory control of the taste receptor locus. We also identified unique regulatory domains with strong evidence for regulatory potential in the vicinity of taste receptor genes. In addition, we identified several novel over-represented DNA-motifs in cardiac taste receptor gene promoters corresponding to ubiquitous and cardiac-specific transcription factor binding sites.

Discussion. This study represents the first investigation of the cis-regulatory landscape for taste GPCRs and opens a new area of research in this field. Given that GPCRs represent such tractable therapeutic targets, unravelling the upstream mechanism of taste receptor regulation, specifically in the heart, might reveal other means of targeting and controlling the expression of these receptors.
Understanding the nature of efficacy at a model family B GPCR
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Introduction. Efficacy is a well-understood pharmacological term, however there is little data that addresses the biochemical mechanisms by which differential efficacy can occur. In the case of canonical G protein-coupled receptor (GPCR) signalling via heterotrimeric G proteins, the role of the GPCR may be considered as a guanine nucleotide exchange factor (GEF). The extension of this model is that the role of ligands is to allosterically modulate this (GEF) activity. β2-adrenoceptors, bound with ligands of different efficacy show differential sampling of conformational space (Nygaaard et al., 2013). Others have proposed that efficacy might be defined by relative contributions of entropy and enthalpy to binding energy, however this is not supported by data for the β2-adrenoceptor and histamine H1 receptors (e.g. Strasser & Wittmann, 2012). Ultimately, efficacy differences must translate into differential GEF activity.

Aims. Our aim was to define how 2 ligands, possessing differential efficacy, achieve differential GEF activity using a model family B GPCR, the calcitonin receptor.

Methods. We used cAMP accumulation and fluorescent ligand binding to pharmacologically determine efficacy. GEF activity was defined using native PAGE, fluorescence anisotropy, BRET, TIRF and GSD super-resolution microscopy.

Results. Differential GEF activity was defined in terms of GTP affinity, GTP on rate and G protein residency.

Discussion. Defining the biophysical nature of efficacy is an important step forward in understanding the way in which GPCRs function.


Hypotonic stress indirectly activates TRPV4 via intracellular signalling
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Introduction. The ability of cells to sense changes in osmolarity is fundamental to the body’s ability to respond to its internal environment. The Transient Receptor Potential Vanilloid 4 (TRPV4) ion channel has been identified as an important mammalian osmosensor; however, the mechanism(s) of TRPV4 activation are not well understood.

Aims. To investigate the mechanisms of activation of TRPV4 by hypotonic stress (HTS).

Methods. The response of primary human and bovine cells, and hTRPV4-transfected HEK293 cells to HTS (200 mOsm) were pharmacologically investigated using the calcium fluorescent dye Fura2.

Results. All cells responded to a TRPV4 agonist, but not all cells responded to HTS. hTRPV4-HEK cells showed a two-phase calcium response to HTS: phase one involving Gq-dependent intracellular calcium release; and phase two extracellular calcium influx through TRPV4 (Figure). PLA2 signalling and Y110 phosphorylation were found to modulate both phases of the HTS response. The two phases of the response were independent, but both required the presence of the TRPV4 protein, as non-transfected HEK cells did not respond to a TRPV4 agonist or HTS.

Discussion. Not all TRPV4-responsive cells also responded to HTS, indicating that TRPV4 responsiveness to cell stretch is not an intrinsic property of the channel. Part of the hTRPV4-HEK cell response is mediated by a Gq coupled GPCR which requires the presence of, but is not dependent on the opening of TRPV4. Further research is required to elucidate the relative importance of the biphasic TRPV4 response to hypotonic stress in generating cell depolarisation, or initiating further intra- or inter-cellular signalling cascades that allow the cells to respond appropriately to their local environment.
Species-dependence of Cannabinoid Receptor 2 functional selectivity

Introduction. Cannabinoid Receptor 2 (CB2) is a promising therapeutic target in immune-related diseases. Recently Atwood et al. (2012) compared the propensity of cannabinoid ligands to stimulate CB2 internalisation and reported functional selectivity for two potent agonists: CP55,940 (CP) internalised CB2, whereas WIN55,212-2 (WIN) did not. Despite the wealth of structure-activity relationships and mutagenesis studies on CB2 receptors, much remains to be understood regarding the dynamic ligand-receptor interactions that govern allosteric modulation.

Aims. We aimed to generate clickable photoactivatable allosteric modulators for CB2.

Results. Based on well-characterised allosteric modulator scaffolds, compounds were modified to contain a photoreactive moiety, to covalently modify the receptor, and a click chemistry handle to allow attachment of a fluorescent moiety for visualisation. We report on three probes that retain sub-micromolar affinity for CB2 and retain negative cooperativity with glutamate in an intracellular Ca²⁺ mobilization assay. RVDU-3-130 contains a photoreactive benzophenone and has 4-fold lower affinity than the parent compound (MTEP). RVDU-3-154, also based on the MTEP scaffold, contains a photoactivatable azide and has ~10-fold lower affinity than parent. RVDU-3-185 is based on the MPEP scaffold, contains a photoreactive azide and has 10-fold higher affinity than MPEP. RVDU-3-130 and RVDU-3-185 exhibit slow binding kinetics resulting in low affinity estimates derived from Ca²⁺ mobilization assays due to non-equilibrium conditions. All three CB2 negative allosteric modulator probes irreversibly label the receptor following UV exposure. Furthermore, we demonstrate successful conjugation of fluorescent dyes using click chemistry onto the irreversibly bound photoprobes with in-gel fluorescence.

Discussion. Clickable photoprobes represent an innovative strategy to identify unknown binding sites and the work here is the first application of this approach to a G protein-coupled receptor. The common allosteric site photoprobes disclosed here represent novel tool compounds to study the structural basis of allosteric modulation of CB2.
Do relaxin receptors function as obligatory dimers?
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Introduction. Relaxin is a peptide hormone that has recently passed a Phase III clinical trial for treatment of acute heart failure. RXFP1 is the cognate receptor of relaxin, and is a G protein coupled receptor (GPCR) with a complex mechanism of activation. RXFP1 forms constitutive dimers at the cell surface; however it is not known whether dimerisation is necessary for activation or if monomeric RXFP1 could be activated. A better understanding of the mechanism of RXFP1 activation will help drug design efforts to target this receptor.

Aims. To elucidate the functional importance of RXFP1 dimerization by disrupting the constitutive dimerisation of RXFP1 at the cell surface.

Methods. A BRET (Bioluminescence resonance energy transfer) assay was established as a measure of RXFP1 dimerisation. Receptor function was assessed using a cAMP reporter gene assay. DNA sequences representing single RXFP1 transmembrane (TM) domains and relevant control TM proteins were cloned into the pcDNA3.1 expression vector for co-expression assays. Synthetic RXFP1 TM peptides were also used to disrupt dimerization.

Results. Synthetic TM peptides were designed to block RXFP1 TM interactions, however they demonstrated no effect when tested in functional assays. In a parallel approach, single RXFP1 TM domain sequences have been cloned into pcDNA3.1 and will be tested for effects on dimerization and RXFP1 activation. A BRET assay was successfully established to show RXFP1 homodimerisation, using RXFP1-Venus and –Rluc8 pairing. Functional assays demonstrated the constructs retained activity and BRET saturation assays showed a BRET MAX of ~170 mBU for RXFP1 homodimers, in contrast with the TRHR1-Rluc8 control which demonstrated non-saturable BRET.

Discussion. Initial attempts to block RXFP1 dimerization with TM peptides did not affect RXFP1 function, probably due to poor solubility of the peptides. TM peptides have since been redesigned, containing poly-lysine tails to improve solubility. The established BRET assay will be used to assess disruption of RXFP1 homodimers by these peptides or by co-transfection of single RXFP1 TM domains in parallel to functional assays. Other means of disrupting RXFP1 dimerisation are also being investigated, and it is hoped that this work will lead to a greater understanding of RXFP1 activation and hence better drug targeting of this receptor.

Biphasic Ca²⁺-dependent control of 25-hydroxyvitamin D-1α-hydroxylase expression mediated by the calcium-sensing receptor
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Introduction. Elevated extracellular Ca²⁺ concentration (Ca²⁺₀) suppresses expression of 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) in the renal proximal tubule but promotes it in the parathyroid, thus exerting opposing effects on 1,25-dihydroxyvitamin D synthesis in these tissues. However, the underlying mechanisms are unknown.

Aims. In the present study, we investigated the mechanisms of Ca²⁺₀-dependent modulation of CYP27B1 expression.

Methods. CYP27B1-luciferase constructs were transfected into HEK-293 cells stably expressing the CaSR (HEK-CaSR cells), as well as control HEK-293 cells. The cells were then exposed to different Ca²⁺₀ concentrations in the presence or absence of allosteric modulators for 24 h.

Results. At lower Ca²⁺₀ (0.5-3.0 mM), there was a Ca²⁺₀-dependent increase in CYP27B1-luciferase expression in HEK-CaSR cells, which was absent in control HEK-293 cells. Interestingly, as Ca²⁺₀ concentrations increased further (5.0-6.5 mM), suppression was observed, to yield an overall biphasic Ca²⁺₀-dependent response. The positive modulator cinacalcet (1.0 μM) that binds in the heptahelical domain of the CaSR enhanced both these responses, shifting the peak response from 3.0 mM to 0.5 mM, whereas the negative modulator NPS 2143 (1.0 μM) abrogated the Ca²⁺₀-dependent response, indicating that both stimulatory and inhibitory effects are CaSR-mediated. However, the γ-glutamyl peptide S-methylglutathione (30 μM) that binds in the receptor’s Venus flytrap domain had no effect.

Discussion. The results demonstrate the existence of ligand-biased, CaSR-mediated signalling control of gene expression. Experiments that identify the promoter response elements and upstream signalling pathways that support the stimulatory and inhibitory responses may provide a mechanistic basis for the activation of 1,25-dihydroxyvitamin D synthesis in the parathyroid, and inhibition of 1,25-dihydroxyvitamin D synthesis in renal proximal tubule cells.
Identification of cAMP signalling phenotypes in subpopulations of cells using a flow cytometer
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Introduction. Mixed cell populations, containing a range of transgene expression levels, may be a more realistic model of GPCR function in neuronal cells. Traditional cAMP assays rely on averaging the cAMP in populations of cells (often >30,000 cells per sample), which can obscure interesting phenotypes within a mixed population. Fluorescence-based biosensors allow imaging of cAMP changes in single cells; however microscopy remains a labour-intensive way of collecting data when the population of interest occurs at a low frequency.

Aims. In order to further investigate a cell signalling phenomenon observed in a mixed population of cells, we aimed to develop a high through-put method of measuring cAMP which was suitable for looking at small subpopulations within mixed cultures.

Methods. The “cAMP sensor using YFP-Epac-Rluc” (CAMYEL) biosensor (Jiang et al., 2007) was modified to increase the biosensor signal by substituting the bioluminescence resonance energy transfer (BRET) pair to Venus and Rluc8; this construct was named “V8 CAMYEL”. V8 CAMYEL was stably transfected into HEK293 cells, followed by transient transfection of the human cannabinoid CB1 receptor. Cells were stimulated using forskolin and CB1 agonist, then analysed using a Becton Dickinson FACSVantage cell sorter and a VictorXLight platereader.

Results. V8 CAMYEL provided a significantly improved bioluminescent output, allowing BRET detection from small samples of cells. Under different transfection conditions, CB1 signalling was seen as either “Gi-like”, “Gs-like” or as giving no apparent cAMP signalling response, as measured by population average. The flow cytometry results further characterise this phenomenon in terms of surface CB1 expression.

Discussion. Mixed cell populations are a valuable source of physiologically-relevant data. This approach allows multiple cell phenotypes to be identified and characterised in the same assay. In the example of CB1 transient transfection, we have shown that a reading from a mixed population may not best reflect the phenotypes of the constituent subpopulations.


Signalling pathway-selective consequences of the common μ-opioid receptor variants A6V and N40D
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Introduction. SNPs of GPCRs can affect receptor signalling. μ-Opioid receptor (MOPr) SNPs A6V and N40D are the most common variants, with allelic frequencies of up to 20% and 50% respectively within some populations. Considering their high prevalence, changes in MOPr signalling arising from these SNPs may explain some of the variability in clinical response to opioids.

Aims. We investigated agonist activation of signalling pathways by A6V and N40D MOPr variants compared to the wild-type (WT) MOPr. We used the endogenous opioid analogue DAMGO and the clinically important opioids morphine and buprenorphine (bup).

Methods. CHO and AtT20 cells were stably transfected with human WT, A6V or N40D MOP receptors. MOPr mediated adenylyl ciclase (AC) inhibition and ERK1/2 phosphorylation was assayed in CHO cells (Knapman et al, 2014). MOPr-induced K channel activation and desensitisation was assayed in AtT20 cells using membrane potential-sensitive dye in a Flexstation 3 and compared with agonist-induced MOPr phosphorylation at Ser377 determined by Western blot.

Results. At N40D-MOPr, bup efficacy was reduced by over 50% for AC inhibition (Emax (%) WT 35±6; N40D 16±4 (n=5, P<0.05)) and ERK1/2 phosphorylation (Emax (%) WT 35±7; N40D 14±6 (n=5, P<0.05)) with no effect on potency; while bup potency was reduced threefold for K channel activation (pEC50 WT 7.0±0.1; N40D 6.7±0.1 (n=6, P<0.05)) but efficacy was unchanged. At A6V-MOPr, bup inhibition of AC and stimulation of ERK1/2 phosphorylation was lost and DAMGO and morphine inhibition of AC and ERK 1/2 phosphorylation was significantly reduced; however activation of K channels was preserved. DAMGO induced phosphorylation of Ser377 at 30 min was significantly reduced at N40D (one-sample t-test 80±4% (n=5, P=0.008)), with morphine-induced phosphorylation unaffected while the modest bup-induced phosphorylation was increased at A6V.

Discussion. DAMGO and Morphine signalling through N40D MOP receptors was not changed, but buprenorphine signalled less effectively. At A6V, signalling to AC and ERK by all agonists was compromised but K channel activation was preserved. Agonist-induced MOPr phosphorylation at S377 was only modestly affected by the SNPs. Knapman et al. (2014) Br J Pharmacol 171:4273-4288
Signalling profile of CRIP1a: G-protein activation and signal transduction
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Introduction. Cannabinoid receptors are a family of GPCRs that are involved in a wide range of physiological functions and diseases. Key regulators unique to cannabinoid receptors are cannabinoid receptor interacting proteins (CRIPs). Among them, CRIP1a was found to decrease the constitutive activity of the cannabinoid type-1 receptor. Aims. To gain an understanding of how CRIP1a modulates agonist-induced CB1 receptor function. Methods. CB1R agonists, WIN55,212-2 (WIN), CP55,940 (CP), anandamide (AEA) and 2-arachydonylglycerol (2-AG) were used to investigate changes in signalling in the presence or absence of CRIP1a. Changes in K+ channel signalling were determined using a Membrane Potential Assay in AtT20-hCB1 cells inherently expressing CRIP1a. Changes in ERK1/2 phosphorylation and cAMP accumulation were determined using AlphaScreen assays in HEK293-hCB1±CRIP1a cells. These cells were also used to determine changes in G-protein activation using BRET technology following transient transfections with Rluc8- and Venus-tagged constructs. Results. K+ channel activation: CRIP1a protein knockdown was observed in cells treated with CRIP1a-siRNA (20nM), 48 to 72 hours post-transfection (p<0.001). This siRNA-induced CRIP1a knockdown significantly increased both Anandamide and 2-AG-induced K+ channel activation (p < 0.05) whilst no change was observed in response to WIN and CP. ERK1/2 phosphorylation and cAMP accumulation: Downstream signalling studies found no significant difference in adenyl cyclase or MAP kinase activity in response to WIN, CP, AEA and 2-AG in CRIP1a expressing HEK293-hCB1 cells compared with HEK293-hCB1 cells not expressing CRIP1a. G-protein activation: ERK1/2 phosphorylation assays, demonstrated that addition of Rluc8 to CB1 did not alter the potency or efficacy of G protein-coupling following activation by WIN and CP. BRET kinetic studies were used to show ability of the CB1R to signal via specific G proteins, including Gai1, 2 and 3, in response to WIN and CP. Ongoing BRET studies are looking at changes in agonist mediated Gi/o protein activation when CRIP1a is overexpressed. Discussion. Overall these results suggest that CRIP1a modulates CB1 receptor signalling in the ligand- and pathway-specific manner.

Novel agonist engagement and signalling bias by bitopic ligands at the M1 muscarinic ACh receptor
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Introduction. Selective activation of the M1 muscarinic ACh receptor (mAChR) may provide a therapeutic treatment of cognitive dysfunction (Langmead et al., 2008). The M1 mAChR selective agonist TBPB [1-(1’-2-methylbenzyl)-1,4’-bipiperidin-4-yl]-1H benzo[d][imidazol-2(3H)-one] has recently been described to utilise a bitopic mechanism of action, engaging both the ACh binding (orthosteric) site and a topographically distinct allosteric site (Keov et al., 2013). We adopted a mutagenesis approach to identify key interactions that TBPB makes with the receptor. Aims. To characterise structural determinants underlying the mode of engagement of TBPB with the M1 mAChR. Methods. TBPB and other bitopic ligands were pharmacologically characterised via radioligand binding, intracellular calcium mobilisation and extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation assays in cell lines expressing various M1 mAChR mutants. The binding and functional profiles were compared with those of orthosteric ligands at the same mutants. Computational modelling of TBPB binding to the receptor was also performed to complement these pharmacological data. Results. Compared to tested orthosteric ligands and consistent with a bitopic mode of binding, the binding affinities and signalling efficacies of bitopic ligands were similarly impaired at orthosteric site mutants. However, mutations outside of the orthosteric site discriminated between the two ligand classes. Strikingly, mutations in the second extracellular loop altered bitopic agonist bias profiles between the examined signalling assays. Computational modelling predicted a bitopic binding pose of TBPB that supports engagement with these identified regions. Discussion. These findings and the proposed mode of binding further support TBPB’s bitopic interaction with the orthosteric site and extracellular vestibule residues. We also identify a novel role for the second extracellular loop in dictating receptor activation states. This information can aid the development of bitopic and/or allosteric ligands selective for the M1 mAChR.

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

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Introduction. CB1 cannabinoid receptors (CB1Rs) are the most abundant G protein-coupled receptors in the brain and are potential targets for the treatment of numerous central nervous system disorders. Although a large number of CB1R ligands exist, their therapeutic applications are limited due to adverse on-target effects. Thus, selective activation of only receptor signalling events that mediate desired therapeutic effects would greatly improve CB1R-targeted therapies and may be achieved via a phenomenon referred to as ligand biased signalling (Kenakin and Christopoulos, 2013). There is growing evidence that CB1R agonists, and more strikingly, the CB1R allosteric modulator Org27569 (Price et al, 2005) may display pathway selective effects at CB1Rs.

Aims. The present study aimed to detect and quantify ligand-biased signalling from the CB1R and provide evidence for probe-dependence and biased allosterism by Org27569.

Methods. The level of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) was measured using zymography of cell conditioned media. Moreover, the expression of markers of M1 (iNOS) and M2 (Arginase-1 and MRC-1) phenotypes were measured using immunoblot analysis or real time reverse transcriptase polymerase chain reaction (RT-qPCR). MMP-9 mRNA was also measured using RT-qPCR.

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

Discussion. Our results indicate that morphine can modulate tumour aggressiveness and invasiveness by decreasing macrophage MMP-9 production and M2 polarization within the tumour microenvironment.
Genetic Encoding of Unnatural Amino Acids in the Glucagon-like Peptide-1 Receptor (GLP-1R)
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Introduction. The glucagon-like peptide-1 receptor (GLP-1R) has a critical role in metabolic homeostasis, principally through increasing insulin biosynthesis and secretion, and is a key therapeutic target for type II diabetes mellitus. The GLP-1R can be activated by both endogenous (GLP-1(1-36)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. The aim of this study is to elucidate direct interaction points between each peptide ligand and the GLP-1R to identify structural elements in the receptor core responsible for determining ligand-specific receptor activity.

Methods. We used amber codon suppression technology to site-specifically introduce unnatural amino acid residues (p-benzoyl-Phe, BzF) into select positions of the GLP-1R expressed in mammalian cells in culture. Functional BzF-labeled GLP-1R can subsequently be used in targeted UV photocrosslinking reactions to map sites of interaction between ligands and receptor. Sites chosen for introduction of BzF residues were determined based on existing functional data and molecular models of receptor-ligand complexes. The goal is to introduce BzF residues in such a way that ligand-dependent signaling is maintained in the BzF-receptor mutants, while placing the photoactivatable group close enough to the ligand to create a crosslink upon photolysis.

Results. A panel of site-specifically-labeled BzF GLP-1R mutants has been created and characterized pharmacologically. Functional BzF-GLP-1R mutants are being tested in crosslinking reactions with a series of fluorescently labeled agonist analogues.

Discussion. The differences in interactions between each peptide and the receptor that are established from application of this technology is beneficial in understanding the activation mechanisms involved in GLP-1R function, and will also provide valuable knowledge that will be advantageous to exploit in the design and application of superior therapeutics targeted to this receptor system.

Disruption of GLP-1 receptor dimersation results in liraglutide sensitivity
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Introduction. Glucagon-like-peptide 1 (GLP-1) acts via a 7 transmembrane receptor that couples, predominantly, to the Gₛ subunit stimulating insulin. Recently, treatment of obese or diabetic patients with GLP-1, or one of its longer lasting analogues has been of significant pharmaceutical interest although these drugs are not without severe adverse side effects.

Aims. Abolishing homodimerisation of the GLP-1 receptor has been demonstrated to significantly alter agonist dependent signalling [1]. Here, we determine the effects brought about by abolishing GLP-1 receptor dimerization on G protein selectivity, following stimulation with a range of GLP-1 mimetics (liraglutide and exenatide),.

Methods. The GLP-1 receptor couples to multiple G protein subunits including the stimulatory Gᵥᵥ, the inhibitory Gᵢᵢᵢᵢ families as well as Gₛ. Previously we have described the use of a yeast system developed by GSK expressing individual Ga chimeras to investigate the G protein signalling bias of the human GLP-1 receptor [2]. Here we use the same yeast platform to determine the effects, on individual G protein couplings when GLP-1 receptor dimerization is abolished.

Results. We report that the dimer disrupting mutant, upon stimulation with GLP-1 (7-36) amide, displays a 100-fold reduction in potency (compared to wild type) when coupled to either the GPA1/Gₛ (pEC50 =6.4±0.3) or GPA1/G₅q (5.2±0.9) subunits. Interestingly, no change in potency between the dimer mutant and wild type receptor was observed when coupled to Gₛ. In addition, abolishing GLP-1 receptor dimerization appears to significantly reduce the ability of liraglutide to activate Gₛ while potentiating the effects of exenatide.

Discussion. This study highlights the need to understand the molecular mechanisms modulating the GLP-1 receptor. We have demonstrated the importance of GLP-1 receptor dimerization in modulating signalling by the clinically used GLP-1 mimetics, liraglutide and exenatide. We are currently validating these results in mammalian cell lines.

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

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Introduction. The glucagon receptor (GCGR) is a family B G protein-coupled receptor (GPCR) that predominantly couples to the Gα subunit. Glucagon is its native ligand and is released from pancreatic α cells and serves to oppose the action of insulin thereby maintaining glucose homeostasis. GCGR like many other family B GPCRs has been suggested to couple through multiple Gα subunits although the physiological consequences remain to be determined. Recently, building upon previous data [1] we have demonstrated that the GCGR interacts with receptor activity-modifying protein 2 (RAMP2) leading to significant alterations in G protein coupling.

Aims. To determine the effects on G protein coupling when GCGR interacts with RAMP2.

Methods. The human GCGR was expressed, in the presence or absence of RAMP2, in yeast strains specifically engineered to couple to a growth reporter through the use of chimeric GPA1/Gα subunits [2]. This system facilitates the characterization of ligand-GPCR-G protein pharmacology in isolation. Stimulation of strains with glucagon enables pharmacological effects to be determined.

Results. We report that, analogous to Gαs, GCGR association with RAMP2 increases the potency of glucagon-stimulated signaling in the chimeric Gαq yeast strains without increasing the maximal response. Further we report that, unlike other family B GPCRs, GCGR does not appear to couple to Gαz, irrespective of the presence of RAMP2.

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


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

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Introduction. The calcitonin receptor-like receptor (CLR) is a member of the secretin-like family of G protein coupled receptors. It associates with each of the three receptor activity-modifying proteins (RAMPs) to define 3 receptors with distinct pharmacology to the three native ligands; calcitonin gene-related peptide (CGRP), adrenomedullin (ADM1) and adrenomedullin2 (ADM2) [1]. Signaling of the CLR through Gαs and Gαi subunits has been well characterized, however RAMP-dependent signaling through the Gαq subunit has not been defined.

Aims. To investigate the Gαq-dependent signaling bias of the CGRP, AM1 and AM2 receptors.

Methods. The CLR was co-expressed with each RAMP in Saccharomyces cerevisiae yeast strains that facilitate coupling to the HIS3 growth reporter through the use of chimeric GPA1/Gα subunits [2]. The yeast strains were then stimulated with the three native peptides to determine their varying pharmacology.

Results. Expression of the CGRP receptor in the chimeric Gαq yeast strain, generated potencies of the three ligands CGRP>ADM1>ADM2 and is in broad agreement with that for Gαs subunit. In contrast to that observed with Gαs, where the maximal response of ADM2 was observed to be 27.8±7.04 (relative to CGRP), ADM2 displayed an elevated maximal response (Emax =137±14.11) in the Gαq expressing strain. For the AM1 receptor, the rank potencies of the ligands were ADM1>ADM2>CGRP in the Gαq strain (Gαs: ADM1>CGRP=AM2). For the AM2 receptor there was no difference between the relative potencies ADM1>ADM2>CGRP for the two Gα subunits.

Discussion. This study demonstrates the RAMP-dependent G protein signaling of the three receptors to the three ligands. Specifically we have demonstrated the ability of ADM2 to generate a high maximal response when used as the stimulating ligand for the CGRP receptor. This data may help to define the as yet undetermined biological role for ADM2. These data highlight the predictive nature of the yeast system and we are currently validating these experiments in mammalian cells.

Calcilytics and calcimimetics modulate calcium sensing receptor (CaSR) activity via overlapping but distinct allosteric binding sites
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Introduction. The CaSR is a class C G protein-coupled receptor (GPCR) whose primary role is to maintain Ca\(^{2+}\) homeostasis throughout the body. Calcilytics and calcimimetics are allosteric modulators of CaSR function (Davey et al., 2012; Leach et al., 2013). They have been trialled, or are currently in clinical use, for the treatment of osteoporosis, hyperparathyroidism or disorders of calcium homeostasis. These drugs show diverse pharmacologies (Cook et al., Br. J. Pharmacol., In Press) but how they bind to the CaSR and exert their unique actions is unknown.

Aims. To probe residues in the transmembrane (TM) domains of the human CaSR important for calcilytic and calcimimetic binding and function.

Methods. The effect of up to 40 amino acid substitutions on the functional affinity of cinacalcet, NPSR568, AC265347 and NPS2143 was determined using calcium mobilisation assays. To map the location of amino acid residues identified to be important for modulator activity, a homology model of the CaSR based on the recent crystal structure of the family C metabotropic glutamate receptor 1 (mGluR1) was generated.

Results. Amino acid residues that altered the functional affinity or allosteric modulation (cooperativity) of calcilytics or calcimimetics were generally homologous to residues that line the allosteric binding sites in mGluR1 or mGluR5. However, whereas the binding of the calcilytic, NPS2143, was predicted to extend from the TM domains up towards the extracellular loops, the small calcimimetic, AC265347, was predicted to bind deeper in the TM domain. The larger calcimimetics, cinacalcet and NPSR568, likely bind in an intermediary location.

Discussion. Family C GPCRs share a common allosteric binding site that extends from the top to the middle of the TM domains. Calcilytics and calcimimetics can occupy different portions of this large binding cavity, which may partly explain their diverse pharmacology.

Structurally distinct calcimimetics engender biased signalling from the human calcium sensing receptor (CaSR)
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Introduction. The clinical use of cinacalcet in hyperparathyroidism is complicated by hypocalcaemia, arising, at least in part, via activation of CaSRs in the thyroid and kidney and stimulation of calcitonin release and ion transport, respectively. Alternative calcimimetics that selectively bias signalling of the receptor towards pathways that mediate desired effects (e.g. PTH suppression) at the exclusion of those that mediate undesirable effects (e.g. hypocalcaemia), may offer superior therapies.

Aims. To determine the potential for structurally distinct calcimimetics to engender ligand-biased signalling from the CaSR.

Methods. The ligand-biased profile of calcimimetics (Figure 1) was characterised in HEK293 cells stably expressing the human CaSR and monitoring effects on Ca\(^{2+}\) mobilisation, IP\(_1\) accumulation, pERK1/2 and receptor expression.

Results. Phenylalkylamine calcimimetics (cinacalcet, calindol, NPS-R-568) were biased towards allosteric modulation of Ca\(^{2+}\) mobilisation and IP\(_1\) accumulation. S,R-calcimimetic B was biased only towards IP\(_1\) accumulation. R,R-calcimimetic B and AC-265347 were biased towards IP\(_1\) accumulation and pERK1/2. Nor-calcimimetic B was unbiased. In contrast to phenylalkylamines and calcimimetic B analogues, AC-265347 did not promote trafficking of a loss-of-expression naturally occurring CaSR mutation (G670E).

Discussion. The identification of allosteric modulators that bias CaSR signalling towards distinct intracellular pathways provides an opportunity to develop desirable biased signalling profiles in vivo for mediating selective physiological responses.

Figure 1. Structure of calcimimetics used in this study
**Desensitization of \( \alpha_{1A} \)-adrenoceptors by oxymetazoline**
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Introduction and Aims. The \( \alpha_{1A} \)-adrenoceptor has an important role in the regulation of smooth muscle contraction in the prostate and vasoconstrictor effects in resistance blood vessels. This receptor is also known to be less phosphorylated, desensitized and internalized upon exposure to endogenous catecholamines, including noradrenaline (NA) and adrenaline, than other adrenoceptor subtypes. Oxymetazoline (OXY) is a partial agonist at the \( \alpha_{1A} \)-adrenoceptor that was recently found to induce phosphorylation, followed by rapid desensitization and internalization (Akinaga et al., 2013). The aim of this study is to investigate whether OXY-promoted desensitization has differential effects on a range of signalling pathways activated by the \( \alpha_{1A} \)-AR.

Methods. Chinese Hamster Ovary (CHO) cells stably expressing the human \( \alpha_{1A} \)-adrenoceptor were pre-incubated for 5 min with OXY (10 \( \mu \)M) or NA (10 \( \mu \)M), followed by rapid washing. Cells were then assayed for NA-stimulated intracellular calcium release, ERK phosphorylation and extracellular acidification rate (ECAR).

Results and Discussion. A decrease in the \( E_{\text{max}} \) of cells pre-treated with 10\( \mu \)M OXY compared to the control concentration-response curve was used as an indicator of receptor desensitization. A statistically significant decrease in the \( E_{\text{max}} \) for NA was observed following pre-exposure to OXY (10 \( \mu \)M) in assays for intracellular calcium release, ECAR and ERK phosphorylation with reductions of 17\%, 37\% and 100\% respectively. No desensitisation was seen following exposure to NA (10 \( \mu \)M). These results indicate that desensitisation is closely linked with \( \alpha_{1A} \)-adrenoceptor stimulation by OXY, although each pathway shows differential sensitivity, perhaps associated with coupling efficiency. This suggests that OXY is a biased agonist selective for the desensitization pathway of the \( \alpha_{1A} \)-adrenoceptor, which may provide an alternative to the pharmacotherapeutic use of \( \alpha_{1} \)-adrenoceptor antagonists.


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**Lipid Conjugation for Targeting Endosomal Signalling**
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Introduction. G protein-coupled receptors (GPCRs) can be regulated through internalization to the endosomal recycling pathway or lysosomal degradative pathway. Mounting evidence suggests that GPCRs can stimulate specific cell signalling events upon entering the endosomal network and that these are distinct from plasma membrane GPCR signalling events. The neurokinin 1 receptor (NK\(_1\)R) is a mediator of pain and inflammation. Upon stimulation by the neuropeptide Substance P, NK\(_1\)R undergoes arrestin-mediated internalization to endosomes and promotes sustained signalling as a part of a signalling complex. These internalized signalling complexes are a potential therapeutic target and hence, the rational targeting of drugs to the endosomal network offers the potential to selectively manipulate NK\(_1\)R signalling.

Aims. To evaluate drug lipidation as a method for targeting soluble drugs to intracellular compartments.

Methods. Constructs comprised of cholestanol (sterol group) for membrane anchoring, a polyethylene glycol (PEG) chain and either an NK\(_1\)R antagonist (spantide), agonist (GR73632) or cyanine5 (cy5) fluorophore reporter. As a control, the same constructs were synthesized without the cholestanol anchor. Cholestanol dependent trafficking of the Cy5 conjugate was investigated using high-resolution confocal microscopy. Forster resonance energy transfer biosensors were used to investigate spatially and temporally distinct signalling pathways initiated from cell surface and internalized NK\(_1\)R.

Results. Unlike the ethyl ester control, Cholestanol-cy5 rapidly associated with the plasma membrane of cells and internalized to discrete compartments. Cholestanol-cy5 trafficked to Rab5-positive early endosomes within 2 hours and to Rab7-positive late endosomes after 4 hours. When comparing soluble or membrane-anchored antagonists and agonists, changes in the magnitude and duration of endosomal signalling were observed.

Discussion. lipid conjugation offers the potential for targeting GPCRs in specific intracellular locations and over extended periods, as a novel approach for selectively regulating spatially and temporally distinct signalling events.

Enhanced $\beta_3$-adrenoceptor expression and function in rosiglitazone-treated inguinal white adipocytes.
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Introduction: The high prevalence of obesity has provoked substantial interest in adipocyte thermogenesis as a means of expending excess energy. A potential therapeutic target is the $\beta_3$-adrenoceptor (AR), as sympathetic stimulation of brown adipose tissue (BAT) activates the $\beta_3$-AR to uncouple mitochondria via increased UCP1 expression and function. Recently functional BAT activity has been demonstrated in adult humans \cite{1}, but these cells may more closely resemble the brite (brown in white) adipocyte rather than classical BAT \cite{2}. Brite adipocytes are derived from the conversion of a fat-storing white adipocyte into a brown-like genetic phenotype by multiple stimuli including the PPAR\textgamma activator rosiglitazone (ROSI) \cite{3}, but little is known of their thermogenic capacity.

Aims: To determine the effect of rosiglitazone on $\beta_3$-AR expression and function in mouse brite adipocytes.

Methods: Primary inguinal white preadipocytes from FVB mice were differentiated in vitro, and brite adipocyte expression induced by 1$\mu$M ROSI treatment.

Results: ROSI treatment of white adipocytes increased $\beta_3$-AR expression and NE-stimulated cAMP production (control: max 5.7\%\pm0.6\% forskolin (100$\mu$M), pEC\textsubscript{50} 7.2\%\pm0.4; +ROSI: max 12.9\%\pm1.3\%, pEC\textsubscript{50} 7.0\%\pm.3, n=5, $p<0.05$). This coincided with an increase in UCP1 mRNA expression (1000-fold) and NE-stimulated (1$\mu$M) oxygen consumption (control: 127.7\%\pm12.5\% basal; +ROSI 234.8\%\pm27.6\% basal, n=12, $p<0.01$) and $[3H]$-2-deoxyglucose uptake responses (control: 124.3\%\pm11.6\% basal; +ROSI: 175.9\%\pm17.5\% basal, n=7, $p<0.05$).

Discussion: ROSI-induced brite adipocytes show an increased capacity for glucose uptake and mitochondrial uncoupling compared to classical white adipocytes, taking on a brown adipocyte metabolic phenotype.

\cite{1} Marken Lichtenbelt, WD van, et al. (2009) NEJM 360: 1500–8
\cite{3} Petrovic, N et al (2010) JBC 285: 7153-64

\begin{table}
\centering
| Binding pocket residue analysis of orphan G protein-coupled receptor, GPR37L1, a potential mediator of cardiovascular homeostasis: insights into ligand structural determinants |
| Tony Ngo\textsuperscript{1,2}, Irina Kufareva\textsuperscript{2}, James LJ Coleman\textsuperscript{1}, Robert M Graham\textsuperscript{1}, Ruben Abagyan\textsuperscript{2}, Nicola J Smith\textsuperscript{1}. Molecular Cardiology & Biophysics Division, VCCRI\textsuperscript{1}, Darlinghurst, NSW; Skaggs School of Pharm & Pharma Sciences, UCSD\textsuperscript{2}, La Jolla, CA. |
\end{table}

Introduction. Over 100 orphan Class A G protein-coupled receptors (GPCR) remain an unlocked source of novel therapeutic targets. No endogenous ligand has been identified for GPR37L1, classified in the peptide $\epsilon$-subgroup of Class A GPCRs, although neuropeptides prosaptide and prosaposin have been proposed (Meyer et al, 2013).

Aims. To gain insights into interaction pharmacophores of GPR37L1 ligands based on pocket residue analysis.

Methods. For each unique Pocketome GPCR entry (www.pocketome.org), binding pocket residues were propagated through an alignment from the entry sequence to GPR37L1 using ICM v3.8-1 (Molsoft LLC, San Diego). A GPR37L1 homology model was built based on the neurotensin 1 receptor (PDB: 4GRV; 20\% sequence identity).

Prosaptide was tested for GPR37L1 activation using various assays \textit{in vitro}.

Results. In at least 70\% of GPCR Pocketome entries, conserved ligand-receptor interactions were seen with residues at positions 3.32, 5.43, 6.51, 6.52 and 7.39 (Ballesteros and Weinstein numbering). This indicates their importance for ligand binding and activation. For GPR37L1, these residues are E3.32, Y5.43, E6.51, N6.52 and Q7.39, which create an overall negative charge in the pocket. From our homology model, the E3.32 represents an uncompensated charge, indicating preference for interaction with positively charged or amine-containing ligands. Discussion. An acidic residue in position 3.32 is observed in only a few receptor subfamilies in the entire GPCR family. Biogenic amine receptors have an aspartic acid at 3.32 that enables recognition of N+ of the endogenous amine. For GPR37L1, the glutamic acid at 3.32 makes it suitable to interact with basic residues or the N-terminus of a peptide. Furthermore, E6.51, located directly opposite E3.32, may be compensated by K7.45 but ‘switch’ interacting partners upon ligand binding. Supporting our ‘pocket’ analysis, no activation of GPR37L1 by prosaptide was observed \textit{in vitro} likely because the prosaptide peptide sequence does not contain basic residues. We are currently looking for ligands that can complement the unique binding pocket features of GPR37L1.

Characterizing Novel Aspects of Signaling Bias from GLP-1 Receptor using RNA-Seq.
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Introduction. The glucagon-like peptide 1 receptor (GLP-1R) is a key physiological regulator of β-cell function and survival and currently targeted for treatment of type II diabetes mellitus (TIIDM). TIIDM is characterized by hyperglycemia, insulin resistance and β-cell dysfunction. Long acting GLP-1R agonists, exendin-4 and liraglutide, are approved therapeutics for this disease. The molecular mechanisms underlying GLP-1R physiology are highly complex and poorly understood, but Gₛ-mediated cAMP production and signaling via β-arrestins is implicated in augmentation of glucose sensitive insulin secretion, enhanced β-cell proliferation and anti-apoptotic effects. Limitations of GLP-1 peptidomimetics have led to a continued interest in the search for small molecule GLP-1R agonists as therapeutics.

Aims. This study evaluates biased signalling at the GLP-1R mediated by peptide ligands with/without allosteric modulators in an endogenously expressing cell system. Subsequently, global cellular changes are assessed to identify various signaling proteins accountable for the positive profile of GLP-1R ligands in INS-1 832/3 cells.

Methods. Biased signalling by different ligands was assessed by cAMP accumulation, insulin secretion, ERK1/2 phosphorylation, iCa²⁺ mobilization, proliferation and apoptosis in both high and low glucose. In-depth RNA-Seq was also performed on RNA extracted from cells treated in presence of different orthosteric ligands at different time points.

Results. We show glucose-dependent effects of GLP-1R ligands on insulin secretion, ERK1/2 phosphorylation, iCa²⁺ mobilization, proliferation and survival that in some cases were modulated in presence of small compounds. RNA-Seq identified differentially expressed genes in response to GLP-1R peptides that were associated with insulin secretion and cell survival mechanisms, some of which were subsequently validated using an RT-qPCR array.

Conclusion. These results reveal biased-signalling profiles of multiple ligands in an endogenously expressing GLP1R system. They also elucidate modulation of GLP-1R signaling in presence of small molecule allosteric modulators. This may help in designing small molecule agonists with potential to either augment/mimic the positive actions of GLP-1, while reducing side-effect profiles thus identifying better therapeutics for the treatment of TIIDM.
Determination of signaling signature of 40 SNPs found in melatonin type 2 receptor: from ligand-biased signaling to mutation-bias signaling.

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Introduction. Melatonin binds two high affinity G protein-coupled receptors (GPCRs): MT1R and MT2R. Genome-wide association studies revealed 40 single nucleotide polymorphisms (SNPs) of MT2R. Some SNPs are found exclusively in patients suffering from type 2 diabetes (T2D) or having preclinical signs of T2D while others are found in both T2D and normal subjects or in normal subjects only. All mutant MT2R exhibit normal receptor expression and only 4 of them show loss of melatonin-binding.

Aims. Given that GPCRs can engage multiple signaling pathways, the objective of this study is to assess the effects of each SNP on the activation of different pathways to generate a «signaling signature».

Methods. Using bioluminescence resonance energy transfer (BRET)-based and protein-complementation assays, we monitored the basal and melatonin-mediated G_i1 and β-arrestin activation for all SNPs. The relative melatonin-induced responses were determined using the operational model and expressed as Δlog(μ/Ka), while the constitutive receptor activation was expressed as normalized difference between wild-type and each SNP. By plotting these data on radial graphs, we obtained each mutant receptor-signaling signature.

Results. Our results indicate that 14 mutants display drastic overall signaling impairment. Half of these impaired mutations are only found in the T2D patients or individuals with preclinical signs of T2D. Most importantly, their radial graphs are asymmetric.

Discussion. This asymmetry underscores the mutation-induced bias signaling. Indeed, some mutations preferentially reduce G_i1 activation over β-arrestin responsiveness while other mutations have the opposite effect. Asymmetry also indicates that constitutive and agonist-mediated activities are not always similarly affected by SNPs. Together, our signaling signatures reported for MT2R SNPs may help understanding the etiology of T2D but also illustrate the GPCR pluridimensionality concept and extend the concept of bias signaling from ligand-bias to SNP-bias.

Determination of receptor binding kinetics of M1 muscarinic acetylcholine receptor (M1 mAChR) antagonists using whole cell binding.

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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.

Aims. Using competition binding kinetics3 and whole cell [3H]-NMS binding we investigated the receptor binding kinetics of cold competitive antagonists acting at the M1 receptor.

Results. Test compounds displayed a range of k_off values, varying from 0.73 ± 0.54 (n=4) to 0.006 ± 0.001 min⁻¹ (n=3) resulting in mean t_1/2 values of 1.5 ± 1.1 to 129.3 ± 29.7 min. A near perfect correlation of 0.98, with a P value of <0.0001 determined using Deming linear regression, was obtained from classical competition binding studies (pKᵣ) and the kinetically derived pKD values. A good correlation was identified between the pKᵣ values and both the Log k_on and Log k_off values, with r² values of 0.81 and 0.58, respectively.

Discussion. In this study we have demonstrated that the use of competition binding kinetics enables the kinetic parameters of unlabelled compounds to be identified. We identified a wide range of t_1/2 values of the compounds tested. In addition a good correlation between the pKᵣ and k_on and k_off values were determined. This study has shown that compounds with similar pharmacological parameters and closely related chemical structures can display differential binding kinetics, highlighting the necessity for more rigorous pharmacology profiling. Ultimately, this increased pharmacology profile may help explain any unusual effects often observed in the clinic.

Use of hemi-equilibrium to determine the dissociation kinetics of histamine H₃ receptor antagonists.
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Introduction. Antagonism of the histamine H₃ receptor has been shown to be a potential benefit for patients of obesity, allergic rhinitis and cognitive disorders, including schizophrenia and Alzheimer’s¹. There are two major isoforms of the histamine H₃ receptor, hH₃-445 and hH₃-365, which have been shown to display differential pharmacology. However, no investigation into the kinetic parameters of histamine H₃ antagonists, at either isoform, has been performed.

Aims. As both radioligands available, [¹²⁵I]-iodoproxyfan, display agonist activity, we utilized an intracellular calcium release assay run under hemi-equilibrium² conditions, in combination with a novel analytical approach, to determine the dissociation kinetics of a range of histamine H₃ antagonists.

Results. Test antagonists displayed a range of kᵦᵣ values, resulting in t½ values of 1.0 ± 0.0 to >70 min (the limit of quantification for slowly dissociating antagonists). In most cases there was little difference between the t½ values obtained for each compound against both isoforms. However, both PF-03654746 and thioperamide displayed differential binding kinetics at the two isoforms; PF-03654746 rapidly dissociated from the hH₃-365 isoform with a t½ of 5.5 ± 16.0 min compared to >70 min from the hH₃-445 isoform. Conversely, thioperamide dissociated rapidly from the hH₃-445 isoform with a t½ of 3.8 ± 2.6 min compared to 67.0 ± 30.1 min from the hH₃-365 isoform.

Discussion. These data demonstrate the utility of functional assays run under hemi-equilibrium conditions to determine the binding kinetics of unlabelled histamine H₃ antagonists. The difference in binding kinetics between the major isoforms may prove a novel mechanism in developing isoform specific compounds.


GPRC6a mediates cellular responses to L-amino acids, but not osteocalcin variants
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Introduction. By analysis of KO mice, the promiscuous class C GPCR, GPRC6a, has been shown to be involved in regulation of metabolism, inflammation and endocrine function. Such effects are described as mediated by L-amino acids, the bone-derived peptide osteocalcin or the male hormone, testosterone. However, verification of activity of osteocalcin and testosterone at GPRC6a has proved somewhat elusive.

Aims. This study characterized the pharmacology and signalling of recombinantly and endogenously expressed GPRC6a in response to all putative GPRC6a ligands, including recombinant and synthetic osteocalcin.

Methods. Receptor function was assessed in assays of Ca²⁺ mobilisation, cAMP and inositol phosphate accumulation, ERK1/2 phosphorylation (HEK293-mGPRC6a cells) and GLP-1 and insulin release (GLUTag and GLUTag-GPRC6a ligand). Finally, stimulation of GPRC6a by basic L-amino acids in GLUTag and βTC-6 cells resulted in GLP-1 and glucose-dependent insulin secretion, respectively. No effect of osteocalcin variants or testosterone could be detected in either of these cellular assays.

Discussion. These data do not support a role for osteocalcin or testosterone as ligands for GPRC6a, suggesting that the reported in vivo effects requiring GPRC6a may be indirect, rather than via direct activation of the receptor.

Figure 1. GPRC6a-mediated Ca²⁺ mobilization in response to L-amino acids in HEK293-mGPRC6a cells.
Stimulation of glucose uptake by $\alpha_{1A}$-adrenoceptors involves mTORC2, AMPK, and Rac1

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

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

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

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

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

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

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

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

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

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

Stimulus bias of allosteric modulators at the metabotropic glutamate receptor subtype 5.
Kathy Sengmany, Michelle L. Halls, Gregory D. Stewart, Sanja Bosnyak, Chris J. Langmead, Arthur Christopoulos

Introduction. Allosteric receptor modulators offer the advantage of spatial and temporal fine-tuning of endogenous agonist activity, allowing for increased selectivity, reduced adverse effects and improved clinical outcomes. Some allosteric compounds can also differentially modulate distinct signalling pathways arising from the same receptor, a phenomenon referred to as stimulus bias or biased modulation. Emerging evidence for the glutamatergic theory of schizophrenia suggests the metabotropic glutamate receptor subtype 5 (mGlu5) is a promising target. Current allosteric modulators of mGlu5 have largely been classified based solely on their effects in modulating intracellular calcium responses of orthosteric agonists alone, resulting in a narrow cataloguing of compounds.

Aims. To assess mGlu5 allosteric modulators across multiple endpoints of receptor activation, in order to determine their potential for engendering biased agonism and/or modulation.

Methods. Intracellular calcium mobilisation, IP₃ accumulation and PKC phosphorylation were assessed in stably transfected mGlu5 HEK293 cells and mouse striatal and cortical neurons. Allosteric ligands were profiled alone and in the presence of orthosteric agonist.

Results. VU0403602 is a positive allosteric modulator and agonist of glutamate-mediated calcium mobilisation ($\log IC₅₀=1.02\pm0.09$, $\log I₄₅₀=-0.126\pm0.06$), an agonist of IP₃ accumulation (pEC₅₀=9.01±0.28) and a neutral allosteric ligand of glutamate-mediated PKC phosphorylation. VU0360172 is a positive allosteric modulator of glutamate-mediated calcium mobilisation ($\log IC₅₀=0.83\pm0.12$), a partial agonist of IP₃ accumulation (pEC₅₀=7.84±0.12), a negative modulator of glutamate-mediated IP₃ accumulation ($\log IC₅₀=-0.12\pm0.038$), with a trend towards negative allosteric modulation of glutamate-mediated PKC phosphorylation.

Discussion. Our results highlight that stimulus bias is operative between pathways that were traditionally considered to be linearly linked. Stimulus bias may provide a potential explanation for the diverse in vivo effects of mGlu5 allosteric modulators. Ultimately, we will lay the groundwork for rational drug design of pathway-targeted compounds in the treatment of an otherwise refractory CNS disorder.

μ-Opioid receptor signaling mechanisms: quantifying bias and kinetics
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Introduction. Attempts to develop opioid analgesics with reduced ability to produce tolerance and dependence after chronic exposure, have recently focused on ligand-direct signaling or biased agonism, whereby different agonists at a G protein-coupled receptor differentially interact with distinct signaling effectors. Understanding of unique characteristics of each agonist-receptor pair including agonist efficacy, affinity and the kinetics are extremely important for discovery of novel biased analgesics that selectively activate the signaling pathways mediating therapeutic effects but not adverse side effects.

Aims. To determine biased agonism and kinetics of a range of MOPr ligands for different signaling pathways including GIRK activation, Ser³⁷⁵ phosphorylation, β-arrest2 recruitment and internalization in AtT20-MOPr cells.

Methods. Electrophysiology for GIRK current recording, immuno-staining for Ser³⁷⁵ phosphorylation, β-arrest2 recruitment and internalization and BRET technique to determine β-arrest2 translocation.

Results. The present study illustrated that the kinetics of MOPr for these signaling pathways were agonist dependent. There was a strong positive correlation for the agonist off-rate among all pathways. Furthermore, the off-rate kinetics were correlated with the agonist ability to induce receptor internalization.

Discussion. Slowly dissociating agonists such as endomorphins produce greater endocytosis relative to their initial signaling efficacy than rapidly dissociating agonists; therefore the internalization ability of an agonist can be characterized by duration of receptor occupancy that is intrinsic for each ligand-receptor pair.

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Investigating a novel mechanism of activation of the relaxin family peptide 2 receptor (RXFP2)
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Introduction. The INSL3 (insulin-like peptide 3) receptor, RXFP2, is a unique GPCR as it contains a large extracellular domain, containing a single low density lipoprotein class a module (LDLa), and 10 leucine rich repeats (LRRs). The LDLa is critical for receptor activation, whereas the LRRs comprise the major binding site. Evidence has suggested that this linker has important functions in RXFP2. A deeper understanding as to how the linker in RXFP2 acts to couple the ligand binding LRRs to the LDLa is required to facilitate future drug discovery efforts targeting RXFP2.

Aims. To determine the role of the linker for RXFP2 function with mutagenesis, binding assays, cell signaling assays and with structural biology.

Methods. Transfected HEK293T cells expressing mutant (alanine substituted residues within the linker) receptors, generated using Quickchange site directed PCR, were assayed using a colorimetric CRE reporter assay, measuring effective receptor activation. Following this, an europium labelled INSL3 peptide was used to measure peptide binding affinities. Finally, the LDLa module attached to the linker was generated, and peptide interactions were studied using HSQC NMR.

Results. Mutations made in RXFP2 did not affect cell surface expression, nor peptide binding affinity as demonstrated through saturation binding. Functional analysis of these receptors as measured by changes in cAMP activation highlighted that EC50 values were not affected other than at residues D46 and W50, however, decreases in Emax’s were seen largely from mutations in linker residues D46, W50, and less from residues G45, G49 and F54.

Discussion. The results suggested that particular residues in the linker play an important role in the activation mechanism of RXFP2, but not in ligand binding. It is assumed that reduced activity demonstrated primarily by residues D46 and W50 are a result of altering secondary/tertiary structure in the linker, and thus preventing presentation of the module for adoption of active conformations, however this hypothesis needs further exploration.

Does RXFP1 form heteromers with GPCRs relevant in heart failure?
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Introduction. Relaxin is a 2 chain, 3 disulphide bonded member of the insulin/relaxin peptide superfamily that is the cognate ligand for the relaxin family peptide receptor 1 (RXFP1). Despite relaxin successfully completing a Phase III clinical trial for the treatment of acute heart failure, the mechanisms responsible for these effects are yet to be identified. Recent studies suggest that the anti-fibrotic actions of relaxin are dependent on the formation of heteromers of RXFP1 with the angiotensin receptor AT2.

Aim. This study investigates the formation of heteromers between RXFP1 and G protein-coupled receptors (GPCRs) that are known to be dysregulated in cardiac failure and whether heteromer formation is associated with functional consequences.

Methods. Interactions between RXFP1 and other GPCRs was examined in transiently transfected HEK-293 cells using bioluminescence resonance energy transfer (BRET). BRET Heteromer Investigation Technology (HIT) was used in transiently transfected HEK-293 cells and stably expressing HEK-RXFP1 cells to examine possible functional interactions.

Results. BRET saturation assays showed constitutive heteromerization between RXFP1 and angiotensin II receptor type 1 (AT1R), β2- and β3-adrenoreceptors and endothelin type A (ETaR) and B (ETbR) receptors. BRET HIT studies in HEK cells transiently transfected with AT1R, RXFP1 and G protein-coupled receptor kinase 2 (GRK2) showed a decreased signal between AT1R and GRK2 following activation of RXFP1 with relaxin.

Understanding RXFP1 interaction with other GPCRs and their subsequent signalling profiles may identify roles for RXFP1 that may be relevant to heart failure.

Interaction of agonists and antagonists with the transmembrane region of Protease Activated Receptor 2
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Introduction. Protease Activated Receptor 2 (PAR2) is a GPCR involved in metabolism, inflammation and cancers. It is self-activated by intramolecular binding of the new N-terminus exposed by serine proteases. Short synthetic peptides can also activate PAR2 and are routinely used as surrogates for proteases in PAR2 studies. The ECL2 of PAR2 has been reported to be important in receptor-ligand interactions for both native and synthetic agonists.

Aims. To identify the ligand binding site(s) on PAR2 in mediating activation, antagonism and intracellular signalling associated with PAR2.

Methods. 28 hPAR2 mutants were studied by site-directed mutagenesis, guided by computer-based ligand docking studies. Each cell line was treated with trypsin, synthetic agonists (2f-LIGRLO-NH₂, GB110), and antagonist (GB88) and tested for intracellular Ca²⁺ release. EC₅₀/IC₅₀ generated by each mutant was compared to WT.

Results. Mutations clustered in the TM region were found to affect PAR2 interaction with the ligands (max potency reduction >100-fold). There was a high correlation between these effects and different PAR2 ligands suggesting similar binding locations. However, there was no significant correlation between the profiles observed for synthetic compounds versus those for trypsin, indicating different binding sites for these ligands.

Discussion. Understanding ligand interaction with PAR2 is fundamental to understanding receptor activation, signal transduction and rational drug design. This study illustrated the significance of key TM residues in PAR2 and has pinpointed the binding sites of synthetic agonists and an antagonist. These three ligands clearly have a different binding site from the protease-cleaved tethered ligand and highlights potential problems in biomedical studies to date of inferring that synthetic ligands can be used as surrogate agonists for endogenous proteases.


Comprehensive analysis of factors influencing quantification of biased signalling at the mu-opioid receptor
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Introduction. The dynamic structure of GPCRs allows different ligands to stabilise the receptor into unique active conformations, which can result in differential activation cell signalling pathways and, eventually, in distinct physiological outcomes. This phenomenon, known as “signalling bias” or “functional selectivity”, can be exploited to design new opioids that selectively activate signalling pathways that lead to analgesia whilst minimising side effects that are elicited by activation of other signalling pathways. However, bias is not an absolute quality, rather it is a dynamic and multidimensional phenomenon; it is always relative to a reference ligand, and it is dependent on both cellular protein complement as well as the spatiotemporal properties of the different signalling endpoints.

Aims. This work has investigated how the cellular background influences the direction of biased agonism of μ opioid receptor (MOR) agonists and the impact of signalling kinetics on the detection of bias.

Methods. We have quantified signalling bias at the MOR across multiple different signalling pathways in CHO-MOR overexpressing different signalling effectors, and quantified biased signalling at multiple time points.

Results. This work has revealed that the relative signalling bias of set of ligands is different in CHO cells and AtT20 cells. Additionally, this work has shown that the time points selected for measuring activation of different signalling effectors can significantly alter the bias observed across different pathways.

Discussion. Altogether this demonstrates the changeable nature of ligand bias, which presents a significant challenge when attempting to predict biased signalling in vivo. However these studies have also identified ligands that maintain unique signalling bias profiles in different cell lines, which may be predictive of distinct physiological outcomes. A greater understanding about biased signalling in native tissue and in vivo, will enable links between distinct signalling bias profiles and specific physiological responses to be established, providing essential new information for the design opioids with effective analgesia and less side effects.
Small molecule inhibitors of invertebrate dopamine receptors as leads for new mode-of-action insecticides
Catherine A Hill1, Andrew B Nuss1, Karin F K Ejendal2, Jason M Meyer1, Jason M Conley2, Trevor B Doyle2, Val J Watts2. Dept Entomology, Purdue University1, West Lafayette, IN, USA; Dept Medicinal Chemistry and Molecular Pharmacology, Purdue University2, West Lafayette, IN, USA. (introduced by Arthur G Christopoulos, Monash University, Melbourne, VIC).

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

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

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

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

Discussion. These studies provide proof of concept for a target-based approach to discover novel small molecule inhibitors of invertebrate dopamine receptors as leads for development of pest-selective, safer insecticides.
Pharmacological Characterisation of Synthetic & Putative Endogenous Allosteric Modulators of the $M_2$ Muscarinic Acetylcholine Receptor (mACHr).

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Introduction. G protein–coupled receptors (GPCRs) are the major mediators of intercellular chemical communication. The recognition that virtually all GPCRs possess additional allosteric binding sites that can bind synthetic ligands, termed allosteric modulators, has revolutionised the field.

Aims. The widespread presence of allosteric sites across virtually all GPCRs suggests that there may be hitherto unappreciated roles for these sites in both health and disease, including interaction with endogenous modulators [1]. Certainly, the G protein itself is the best example of an endogenous allosteric modulator of GPCRs. Another striking example is the human eosinophil Major Basic Protein (MBP), a peptide constituting ~50% of the inflammatory peptides released following eosinophil degranulation, that binds allosterically to the $M_2$ mAChR [2]. We hypothesised that other endogenous proteins bearing similar structural features to MBP, such as polycationic, and arginine-rich peptides, may also interact allosterically with the $M_2$ mAChR [3,4].

Methods. The pharmacological properties of L-arginine (L-Arg), poly-L-arginine (PLA), dynorphin-A (Dyn-A), and myelin basic protein (MyBP), were investigated, at the $M_2$ mAChR.

Results & Discussion. In $[^3H]$NMS radioligand binding assay, both the synthetic peptide, PLA, and the endogenous proteins, Dyn-A and MyBP appeared to bind allosterically with the $M_2$ mAChR. At the level of function, both in ERK1/2 phosphorylation and calcium mobilisation assays, all three putative modulators allosterically altered ACh-mediated responses. Collectively, these data highlight the potential role of an allosteric site on a GPCR in regulating physiology and/or pathology via endogenous allosteric ligands.


New fluorescent antagonists for the histamine H$1$-receptor

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Introduction. Molecular tools to better understand G protein-coupled receptors (GPCRs) are vital for drug design and optimization. GPCR drug discovery often focuses on characterizing the affinity and efficacy of ligands but rarely considers ligand kinetics. The dissociation kinetics of a fluorescent GPCR ligand can be directly monitored using confocal microscopy coupled to a perfusion system (May et al).

Aims. To develop small molecule-based fluorescent probes for the histamine H$1$ receptor (H$1$R), and demonstrate utility of these molecules as drug discovery tools, in particular in reference to receptor-ligand kinetics.

Methods. Fluorescent probes based on the H$1$R antagonist mepyramine and the recently described antagonist VUF131816 (de Graaf et al) were synthesised. The affinities of these compounds for the H$1$R were analyzed, and the H$1$R dissociation rates of the fluorescent probes determined.

Results. All the fluorescent probes tested retained affinity at the H$1$R compared to the parent pharmacophores. Confocal imaging showed that displaceable membrane binding, with low levels of non-specific intracellular accumulation, could be detected. Kinetic experiments revealed a very slow dissociation rate at the H$1$R.

Discussion. Fluorescent probes are very powerful drug discovery and development tools, and can be used to interrogate a receptor-ligand interaction at the molecular level in a single, live cell. Fluorescent ligands with similar affinity can display different dissociation kinetics at the H$1$R.

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The Calcitonin gene-related peptide family of receptors exhibit G-protein coupling bias.
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Introduction. Calcitonin gene-related peptide (CGRP), adrenomedullin (AM) and adrenomedullin 2 (AM2) are peptide hormones and are involved in vascular angiogenesis, vasodilation, heart disease and pain response. These peptides signal through the CGRP, AM1 and AM2 class B G protein-coupled receptors with different potencies (1). These receptors couple to G0i and are obligate heterodimers of the calcitonin receptor-like receptor (CLR) and receptor activity-modifying proteins (RAMPs) 1-3 (2). The CGRP and AM1 receptors when stimulated with CGRP or AM respectively can couple to G0i (3). However, there is no complete picture of G0i coupling to these receptors.

Aims. To investigate G0i coupling to the CGRP, AM1 and AM2 receptors on binding of CGRP, AM and AM2.

Methods. We expressed the CGRP, AM1 and AM2 receptors in a yeast system with a G0i and G0s chimera adapted to couple to the yeast-mating pheromone-reporter system (4) upon treatment with CGRP, AM or AM2. We verified this coupling in HEK293S and Cos7 cells by pertussis toxin inhibition of G0i, coupling to the CGRP, AM1 or AM2 receptors and measurement of cAMP accumulation.

Results. The yeast reporter system indicated coupling of all three receptors to G0i upon CGRP, AM and AM2 binding. In HEK293 cells G0i coupled to the CGRP and AM1 receptors upon CGRP stimulation. In Cos7 cells G0i coupled to the CGRP receptor when stimulated with AM and the AM2 receptor on stimulation with AM and AM2.

Discussion. The yeast system provides essential information on the ability of the CLR based receptors to couple to different G-proteins so directing further investigation of G-protein coupling in mammalian systems. Our results suggest that the CGRP, AM1 and AM2 receptors show G0i coupling-bias dependent on the cell line.


Probing agonist and antagonist interactions at the relaxin-3 receptor RXFP3
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Introduction. Relaxin-3 is a highly conserved neuropeptide involved in the modulation of stress, feeding and cognitive processes. Its receptor is the Class A G protein coupled receptor GPCR135, now known as RXFP3. Structural knowledge of relaxin-3/RXFP3 interactions will assist structure-based drug design of smaller blood brain barrier penetrating relaxin-3 mimetics to treat neurological disorders. The residues contributing to binding (R8, R12, R16, I15, F20) are on the relaxin-3 B-chain central helix whereas the C-terminal residues R26 and W27 are important for activation. We have developed an RXFP3 specific antagonist which retains the core binding residues but with the C-terminal activation residues replaced by a non-native Arginine residue. Importantly this Arginine contributes an additional high affinity binding interaction.

Aims. To identify the sites of agonist and antagonist binding in RXFP3 using mutagenesis and homology modelling.

Methods. Conserved glutamic (E) and aspartic (D) acid residues in the N-terminus and extracellular loops of RXFP3 were mutated to Alanine to charged interactions. This approach successfully identified interaction sites for R12, R16 and R26 and was used to generate an RXFP3 relaxin-3 binding model to further predict potential ligand interactions with the transmembrane domains of RXFP3 for activation. These residues were similarly mutated to Alanine and tested for receptor binding and activation using cAMP activity assays.

Results. E141A, D145A and E244A in extracellular loops 1 (EL1) and 2 (EL2) showed significant decreased agonist and antagonist binding compared to wild type RXFP3. Seven transmembrane mutations (W138A, T162A, V241A, L246A, K271A, F364A and T346A) resulted in markedly decreased agonist binding without significant reduction in antagonist binding compared to wild type RXFP3. Interestingly, one transmembrane mutation (W339A), which showed the same level of wild type RXFP3 binding, demonstrated complete loss of activity.

Discussion. Based on the model, E141 and D145 in EL1 and E244 in EL2 are important for agonist binding to R26, R16 and R12 respectively. The significant decrease in activity despite no change in binding affinity suggests that W339 might be involved in cation-π interaction or π-π stacking with W27.
**Heterodimerization of the Calcium-Sensing Receptor with T1 Taste Receptors**
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Introduction. The calcium-sensing receptor (CaSR), and T1 taste receptors are all class C G-protein coupled receptors that are co-expressed in GI-associated endocrine tissue including insulin-secreting pancreatic islet beta cells. Although T1Rs form heterodimers of the type T1R1/2:T1R3, it is not known whether the CaSR forms heterodimers with any of the T1Rs and, if so, the consequent functional significance.

Aims. The study aimed to determine whether heterologous co-expression of the CaSR and one of the T1Rs in CHO cells results in heterodimer formation and whether any novel receptors arising have distinctive functional properties.

Methods. The methodology used was immunoprecipitation, using anti-FLAG beads (Sigma). IPone assays were conducted using CISBIO IPone kit.

Results. The results indicate that the CaSR forms heterodimers preferentially with T1R3 and to a lesser extent with T1R2. The calcium-sensing properties are comparable to that of the wild-type CaSR as reported by the IPone signalling pathway. Experiments aimed at defining the macronutrient-sensing properties of the novel receptors are in progress.

Discussion. Heterodimerization of the CaSR with T1R3 may be stabilized by a disulphide between conserved Cys residues, i.e., C129 and C129 in both receptors. The impacts of hetero-dimerization of the CaSR with the T1Rs on nutrient sensing and receptor-dependent biased signalling properties require investigation.

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**Harnessing unnatural selection: engineering thermostabilised α1-adrenoceptors for drug discovery**
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Introduction. α1A- and α1B-Adrenoceptors (α1A-AR & α1B-AR) are G protein-coupled receptors (GPCRs) that play critical roles in regulating the cardiovascular and nervous systems. Recent evidence suggests α1A-AR & α1B-AR drive opposing responses to adrenaline or noradrenaline activation in modulating cardioprotection, neuroprotection and seizure suppression. However the lack of subtype selective ligands makes it difficult to probe the individual roles of α1A-AR & α1B-AR and validate them as targets for heart and neurological diseases. To facilitate structural biology and thus structure based drug design of more subtype selective compounds, we use directed evolution techniques such as Cellular High-throughput Encapsulation Solubilisation and Screening (CHESS) to engineer thermostabilised GPCRs. Stabilised GPCRs can be purified and applied to biophysical techniques such as X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR) to aid structure-based drug design (SBDD).

Aims. To thermostabilise α1A and α1B-ARs using CHESS and enable structural investigation for drug discovery.

Methods. CHESS is a novel directed evolution technique which converts libraries of α1-AR cells into microcapsules which retain the plasmid of encoding receptors after detergent solubilisation. Microcapsules containing stabilised α1-AR were probed with fluorescent Prazosin (QAPB) and microcapsules containing stable mutants selected using Fluorescence activated Cell Sorting (FACS). 10 rounds of CHESS were conducted on both α1A-AR & α1B-AR.

Results. Several highly stable α1A-AR & α1B-AR mutants were engineered, which could be purified in detergents and retained the ability to bind to known ligands. These mutants carried only 10-14 mutations and their high expression levels and stability makes them perfect candidates for X-ray crystallography. Their stability over long time periods in purified form also enabled us to measure the binding of methoxamine and noradrenaline using Saturation Transfer Difference (STD) NMR, a method commonly used for fragment screening, but has never been applied to GPCRs.

Discussion. This project will broaden our fundamental structural understanding of α1-ARs and validate our cutting edge technology for enabling the application of X-ray crystallography and NMR to GPCRs to facilitate SBDD.
The role of phosphorylation sites in rapid desensitization of the µ-opioid receptor
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Introduction. Tolerance and addiction to opioids are serious clinical and social problems that result in part from the loss of µ-opioid receptor (MOPr) function when activated by different opioids.

Aims. The aim of this study was to determine the influence of C-terminal phosphorylation sites on rapid desensitization of MOPr.

Methods. Wild type MOPr, 3S/T-A, 6S/T-A and 11S/T-A mutants were expressed stably in AtT20 cells. Using perforated patch-clamp recording we examined the effects of MOPr activation, desensitization and re-sensitization using activation of GIRK channels by a submaximal concentration of Met5-Enkephalin (10 nM) to measure receptor activity and somatostatin (100 nM) coupling to native SSTR2 to determine heterologous desensitization.

Results. MOPr desensitization and resensitization produced by 5 min exposure to Met-Enkephalin (10 μM) at 37°C for 3S/T-A and 6S/T-A mutants did not differ from wild type but desensitization was abolished in the 11S/T-A mutant. However, upon exposure to morphine (10μM) at 37°C, all three mutants showed desensitization similar to wild type MOPr, which was found to be PKC dependent. Desensitization, when detected, was largely homologous for both met-enkephalin and morphine.

Discussion. Because 3S/T-A suppresses, and 6S/T-A abolishes MOR endocytosis, these findings suggest that homologous desensitization can occur independently of the phosphorylation and arrestin-dependent mechanisms that drive endocytosis. However, C-terminal phosphorylation sites are necessary for desensitization because mutation of all C-terminal sites (11S/T-A) abolishes desensitization with Met-Enkephalin.