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Combined administration of insulin and leptin significantly increased Fos production in the arcuate nucleus and renal sympathetic nerve activity

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Introduction. Leptin has sympatho-excitatory effects on renal sympathetic nerve activity (RSNA). Insulin also induces increases in RSNA. There is considerable interest in the central actions of leptin and insulin on sympatho-excitation. The brain regions mediating insulin's effects are unclear, compared to leptin.
Aims. This study (i) investigated whether leptin and insulin together elicits greater increases in renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP) and heart rate (HR) than when given alone, and (ii) quantified the number of activated neurons in brain regions influencing SNA, to identify potential central sites of interaction.
Methods. Anesthesia of Male Sprague-Dawley rats was induced with isoflurane (2-5%) in O_2 and maintained with intravenous urethane (1.4-1.6 g/kg). RSNA, MAP and HR were recorded prior to and for 3 hours following intracerebroventricular (ICV) saline (control; n=5), leptin (7 µg; n=5), insulin (500 mU; n=4) and the combination (leptin administered 15 minutes after insulin; n=4).
Results Following leptin or insulin alone, RSNA was significantly increased (74% and 62% respectively), but together, the RSNA increase (124%) was significantly greater than either alone. Insulin alone increased HR significantly but this was prevented by leptin. MAP was not significantly different between groups. Only in the arcuate nucleus did leptin and insulin together increase the number of Fos-positive cell nuclei significantly more than the increases following leptin or insulin alone. In the lamina terminalis and rostroventrolateral medulla, leptin and insulin together increased Fos similar to leptin alone.
Discussion. The results suggest that where leptin and insulin are elevated, there are greater effects on RSNA. This may contribute to cardiovascular complications. The arcuate nucleus may be a common site of cardiovascular integration.

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A small-molecule inhibitor of NLRP3 inflammasome activity, MCC950, reduces blood pressure and restores renal function in hypertensive mice

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Introduction: Inflammasomes are interleukin-1 processing complexes and master regulators of inflammation. We recently showed that one-kidney/deoxycorticosterone acetate/salt (1K/DOCA/salt)- and angiotensin II-dependent hypertension in mice are associated with elevated expression of the NLRP3 inflammasome in the kidneys. Moreover, genetic deficiency of a key subunit critical for NLRP3 inflammasome activity, protected mice against renal inflammation and chronic pressor responses associated with these models.
Aims: We investigated whether a highly-specific, small-molecule inhibitor of NLRP3 inflammasome activity, MCC950, similarly reduces the deleterious effects of 1K/DOCA/salt on blood pressure (BP) and renal function.
Methods: Male C57BL/6J mice were implanted with telemetry probes for recording of BP (mean arterial (MAP), systolic, diastolic) and heart rate (HR), or placed in metabolic cages for 24 h urine collections to assess renal function. Once baseline parameters were established, mice were uninephrectomized and received a DOCA pellet (2.4 mg/kg/d, *s.c.*) and 0.9% saline to drink. Following establishment of hypertension (10 d), mice were implanted with an osmotic minipump containing either MCC950 (10 mg/kg/d, *s.c.*) or vehicle (saline) and followed for 28 d.
Results: MAP increased from 102±2 to 133±3 mmHg over the 10 d following 1K/DOCA/salt surgery. In vehicle-treated mice, MAP remained at this elevated level until the end of the treatment period. By contrast, MAP of mice treated with MCC950 gradually declined such that at d 38 it was 15 mmHg lower than that of vehicle-treated mice. Systolic and diastolic BP responses were similar to MAP, whereas HR was unaffected by MCC950. Urine flow, Na^+ and albumin excretion, and osmolality were markedly increased after 10 d of 1K/DOCA/salt treatment. Consistent with its effect on BP, MCC950 reduced each of these parameters by 30-40%, whereas vehicle had no effect.
Discussion: We have shown that an inhibitor of NLRP3 inflammasome activation reduces BP and restores renal function in mice with established hypertension, highlighting MCC950 as a promising candidate for future therapies.

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Synergistic effect of the combined capsaicin and dihydrocapsaicin on in vitro platelet aggregation

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Introduction. Capsaicinoids, including capsaicin (CAP) and dihydrocapsaicin (DHC), the pungent principles of pepper fruits, individually inhibit *in vitro* platelet aggregation. However, their effects when present in the relative proportions that they are found in different fruits, i.e., ~60% CAP and ~40% DHC, are not known.
Aims. To compare the effects of CAP and DHC alone and in combination on *in vitro* platelet aggregation.
Methods. Platelet-rich and -poor plasma were obtained from venous blood of four healthy subjects. The effects of 12.5 µmol/L CAP and DHC, and their combination (CAP:DHC, 3.75:2.5 µmol/L and 7.5:5 µmol/L) on arachidonic acid (AA; 300 µg/mL), ADP (5 µmol/L), and collagen (4 µg/mL) induced platelet aggregation, were determined. Aggregation data (% AUC; mean±SEM) were compared using ANOVA/linear regression.
Results. Compared to control (64.6±2.1%), CAP (49.6±2.7%) and DHC (48.3±2.3%) individually inhibited AA-induced aggregation (both $p<0.01$). The combination of both agents produced further inhibition in AA-induced aggregation (CAP:DHC 3.75:2.5 µmol/L, 41±6.5%, $p<0.01$; 7.5:5 µmol/L, 27.5±7.7%, $p<0.001$) compared to the control. Moreover, the inhibitory effect of combined CAP:DHC (7.5:5 µmol/L) was larger than individual capsaicinoids (12.5 vs 7.5:5 µmol/L, 49.6±2.7% vs 27.5±7.7%, $p<0.05$, 12.5 vs 7.5:5 µmol/L, 48.3±2.3% vs 27.5±7.7%, $p<0.05$, CAP and DHC, respectively). In contrast to the AA-induced aggregation, neither CAP nor DHC, or their combination, had a significant inhibitory effect on ADP- or collagen-induced aggregation.
Discussion. The combination of CAP and DHC in the proportions they are present in pepper fruits, produces a significantly greater inhibitory effect against AA-induced platelet aggregation, compared to the individual capsaicinoids. These results warrant further investigation to determine whether these capsaicinoids may be exploited for therapeutic benefit.

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NTU-D Against Angiotensin-induced Heart Failure

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Introduction: Myocardial injury is associated with oxidative stress. NTU-D is a novel synthesized antioxidant.
Aims: The study is aimed to evaluate whether NTU-D elicits intrinsic cardioprotection in response to chronic stress, such as angiotensin (Ang) administration.
Methods: Effects of NTU-D on mice heart under chronic Ang treatment were assessed. The morphology and function of heart were measured.
Results: NTU-D reduced myocardial hypertrophy and fibrosis, and preserved cardiac function in chronic Ang-treated mice, $P<0.05$. In Ang-treated mice, the elevation of NOX4 was ameliorated by NTU-D, $P<0.05$, indicating NTU-D exerts cardioprotection through a reduction of oxidative stress generation.
Discussion: NTU-D diminishes myocardial remodeling caused by chronic Ang treatment through a NOX4 reduction mechanism. This makes NTU-D a potential reagent for the treatment of chronic stress-induced heart failure. To clarity its clinical application, the mechanism of NTU-D on heart during chronic stress should be further evaluated.

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The β3-adrenoceptor agonist mirabegron increases human atrial force through β1- but not β3-adrenoceptors

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Introduction. Mirabegron is a β_3-adrenoceptor agonist approved for overactive bladder syndrome. It has been associated with incidences of hypertension, cerebrovascular and cardiac events.
Aims. To determine the cardiac effects of mirabegron and β_3-adrenoceptors in human heart.
Methods. Right atrial trabeculae obtained from patients undergoing coronary artery bypass, aortic valve or myomectomy surgery, were set up on electrode blocks and electrically stimulated. Single concentrations of mirabegron (1 or 10 μM) were added in the absence or presence of the phosphodiesterase inhibitor 3-isobutyl-1 methylxanthine (IBMX 10 μM), β_3-adrenoceptor antagonist L-748,337 (100 nM), β_1-adrenoceptor antagonist CGP 20712A (300 nM), neuronal uptake inhibitors desipramine (1 μM) or phenoxybenzamine (5 μM incubation followed by washed out) and contractile force measured.
Results. Mirabegron (1 and 10 μM) increased contractile force in human right atrium (1 μM, 7.6 ± 2.6 %, n = 7; 10 μM, 10.2 ± 1.5%, n = 22 compared to (-)-isoprenaline). In the presence of IBMX, mirabegron (10 μM) caused a greater contraction (31.9 ± 2.3 %, n = 19, % (-)-isoprenaline effect). L-748,337 (100 nM) had no effect on the increase in contractile force caused by mirabegron (10 μM). In contrast mirabegron (10 μM) reduced contractile force in the presence of CGP 20712A (300 nM) which was not affected by L-748,337 (100 nM). Mirabegron (10 μM) also reduced contractile force in the presence of desipramine or trabeculae treated with phenoxybenzamine.
Discussion. Mirabegron caused cardiostimulant effects in human atrium that were prevented by β_1-adrenoceptor blockade and drugs, desipramine and phenoxybenzamine that block neuronal uptake. A non-specific cardiodepressant effect was observed when cardiostimulant mechanisms were prevented. There was no evidence that β_3- adrenoceptors modulated contractility. These studies may provide insight into clinical adverse cardiac event observations associated with mirabegron.

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Sex-specific cardiovascular effects of orphan receptor, GPR37L1

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Introduction. Expressed abundantly in the central nervous system, GPR37L1 has a role in neurodevelopment, motor learning and coordination (Marazziti et al, 2013). Concurrently, GPR37L1 expression is reportedly downregulated in human heart failure patients, and knockout mouse models exhibit severe hypertension and cardiac hypertrophy (Min et al, 2010). As such, this receptor is potentially involved in centrally mediating blood pressure homeostasis and hypertension pathogenesis.
Aim. To assess the cardioprotective capacity of GPR37L1 in response to chronic cardiac stress (hypertension).
Methods. Male and female wildtype and GPR37L1 knockout mice (C57Bl/6J background) were subjected to vehicle or angiotensin II sc infusion (2mg/kg/day) via osmotic mini pump for a 7-day period, before invasive haemodynamic measurements by catheterisation under isoflurane anaesthesia (1-2% in O_2, 0.5L/min through nose cone) and tissue harvest. Cardiac hypertrophy as a response to this cardiac stress model was assessed by left ventricular (LV) weight, cardiomyocyte density and RT-qPCR for hypertrophy markers. As a further indicator of maladaptive response, intramyocardial fibrosis was quantified by Masson's Trichrome stain and ImageJ software.
Results. We found a baseline increase in mean arterial pressure in female GPR37L1 knockout mice (9.0 mmHg; n=13-15; p=0.018), but not males. Paradoxically, LV weight/tibia length ratio revealed significantly more severe hypertrophy after cardiac stress in GPR37L1 knockout compared to wildtype, but only in the male cohort (n=8-11; p<0.0001). Preliminary results from histology and quantitative PCR appear to support this.
Discussion. Exacerbated hypertrophic response in knockout animals indicates that loss of GPR37L1 is detrimental to cardiac stress responses, and that the receptor contributes to cardiovascular compensatory mechanisms.

Marazziti D et al (2013) Proc Natl Acad Sci USA 110:16486-91
Min KD et al (2010) Biochem Biophys Res Commun 393:55-60

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α1A-Adrenoceptors promote glucose uptake and cell growth by activating mTOR

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Introduction: Cardiomyocyte viability is linked to pathways involving Akt and mTOR, as well as increased glucose uptake and utilization. The ability of G protein-coupled receptors to modulate mTOR activity is a newly emerging paradigm (Sato et al 2014) with the potential to link druggable cell surface receptors with mTOR pathways involved in cell growth and survival.
Aim: It was to determine whether the α_{1A}-adrenoceptor (AR) couples to these canonical protective pathways and glucose uptake.
Methods: Glucose uptake was measured in neonatal rat ventricular cardiomyocytes (NRVC) with ^{3}H 2-deoxy-glucose. Incell Western (Licor) were performed to measure phosphorylation of mTOR in CHO-K1 cells stably expressing the human α_{1A}-AR and GLUT4myc. Translocation of exofacially myc-tagged GLUT4 was determined by immunocytochemistry.
Results: Activation of α_{1A}-AR stimulated phosphorylation of mTOR at S2448 (Noradrenaline (NA) pEC_{50} 6.5±0.2, A61603 pEC_{50} 8.2±0.1, Oxymetazoline (Oxy) pEC_{50} 7.4±0.2, n=5-7), and at S2481 (NA pEC_{50} 8.3±0.3, A61603 pEC_{50} 9.8±0.3, Oxy pEC_{50} 8.8±0.5, n=5-6). mTOR inhibitor, KU0063794 (KU) inhibited α_1-AR mediated glucose uptake in NRVC (KU; basal 100%, +KU 106.0±6.1%, NA 222.6±15.3% + KU 146.1±5.4%, A61603 197.3±9.3% + KU 140.0±5.0%, Oxy 187.3±5.5% + KU 126.7±2.4%; n=5). Immunohistochemical studies showed that α_{1A}-AR stimulation increased GLUT4 translocation to the plasma membrane.
Discussion: Our findings provide a novel link between the α_{1A}-AR, mTORC2 and glucose uptake in a recombinant system and in cardiomyocytes. Also, activation of α_{1A}-AR may stimulate mTORC1 mediated cell growth and survival. Our studies therefore provide an improved framework for examining the utility of α_{1A}-AR selective agonists as tools in the treatment of cardiac dysfunction.

Sato, M., et al., (2014) Diabetes 63: 4115-29

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Platelet inhibition by inhaled nitrite in healthy volunteers and beta-thalassemia / haemoglobin E patients

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Introduction. Nitrite induces vasodilation and inhibits platelet activation through its conversion to NO by reductase activity of deoxygenated haemoglobin. Nitrite administered by inhalation has entered phase 2 clinical trial for pulmonary hypertension in thalassemia. Attenuation of platelet hyperactivity may provide additional benefit for thrombosis in thalassemia.
Aims. This study investigated the effect of inhaled nitrite on platelet activity in healthy volunteers and thalassemia patients.
Methods. P-selectin expression and phosphorylated vasodilator stimulated phosphoprotein (pVASP) in platelets were measured before and nitrite inhalation by flow cytometry and western blot, respectively.
Results. Inhaled nitrite decreased P-selectin expression induced by ADP immediately after inhalation in both healthy volunteers and thalassemia patients. Phosphorylated VASP levels in platelets also increased after inhalation. Phosphorylated VASP in platelets showed a negative correlation with ADP-induced P-selectin expression.
Discussion. Inhaled nitrite can inhibit platelet activation in both healthy and thalassemia, which is consistent with the previous anti-platelet effect of nitrite, reported *in vitro*. Increased phosphorylated VASP indicates platelet inhibition is mediated through NO/guanylate cyclase/cGMP pathway.

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Effects of platelet-derived growth factor-BB and its association in antiproliferative effect of sera from thalassemia patients on pulmonary artery endothelial cells

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Introduction. Endothelial dysfunction evokes early onset of atherosclerosis and thromboembolism leading to multiple vascular complications in thalassemia patient. An increase in expression of platelet-derived growth factor BB (PDGF-BB) and its receptors has been observed in the cells taken from the atherosclerotic lesion, suggesting a possibility of PDGF-BB involvement in the pathology of endothelial dysfunction.
Aims. To investigate the role of PDGF-BB on pulmonary artery endothelial cells (PAEC) and its association in vascular complications on thalassemia patients
Methods. Fourteen thalassemia patients and ten healthy volunteers were recruited into the study. Serum PDGF-BB levels were evaluated using (ELISA). The effects of PDGF-BB and sera on endothelial cell proliferation and activation were evaluated by flow cytometry using CFSE dye dilution technique and quantitative PCR, respectively. Oxidative stress in sera was evaluated using DCF method.
Results. Thalassemia sera demonstrated higher amount of PDGF-BB compared with healthy subjects. In vitro study showed that PDGF-BB stimulates endothelial cell proliferation and activation in a concentration-dependent manner. Despite of the high serum PDGF-BB levels, thalassemia sera inhibited endothelial cell growth and suppressed mRNA expression of activated endothelial genes. Evaluation of intracellular reactive oxygen species in sera-treated endothelial cells indicated inferior antioxidative capacity of the thalassemia sera ($p<0.005$).
Discussion. In this study, we found the serum PDGF-BB level in thalassemia patients is statistically higher than healthy subjects. Contrary to the in vitro study of pure PDGF-BB compound on PAEC, sera from thalassemia patients inhibited proliferation and activation of the cells suggesting other factors in the sera override the angiogenic effects of PDGF-BB. Intracellular ROS measurement indicated that impaired antioxidative defense in the thalassemia sera might, in part, contributes to the endothelial dysfunction.

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Cavin-1 Deficiency Impairs Myocardial and Coronary Responses to Stretch and Ischemia: Roles of NOS Dysregulation

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Introduction. Caveolae and associated caveolin and cavin proteins may govern myocardial responses to mechanical and ischemic stress, and abnormalities in these proteins are also implicated in different cardiac disorders. However, specific roles of cavin-1 in cardiac and coronary responses to mechanical/metabolic stress are unclear
Aim. To identify the role of cavin-1 on myocardial and coronary responses to stretch/load and ischemia-reperfusion. Methods and Results. Myocardial and coronary phenotypes were characterized in hearts from cavin-1 knockout (KO) mice. Cavin-1 KO induced a sex-dependent fall in *in vivo* cardiac function in females (~20% lower ejection fraction). While peak contractile performance appeared comparable in *ex vivo* myocardium from KO and wild-type (WT) mice, diastolic stiffness (stretch-dependent diastolic force) was markedly increased and Frank-Starling behavior (stretch-dependent inotropy) moderately enhanced with KO. These shifts in stretch-dependent function were countered by NOS inhibition with 100 µM L-NAME (and mimicked by 100 µM nitroprusside), which exposed intrinsically depressed contractility in KO hearts. Stretch-dependent efflux of intra-cellular proteins (lactate dehydrogenase, troponin I) was exaggerated and induction of brain natriuretic peptide/c-fos inhibited in KO *vs.* WT hearts, while ERK1/2 phospho-activation was unaltered. Within the coronary vasculature, cavin-1 KO increased conductance ~70% and reactive hyperemic durations ≥3-fold in an L-NAME-sensitive manner, and appeared to exaggerate the pressure-dependence of coronary flow. Cavin-1 KO also worsened myocardial dysfunction and damage following 25 min global ischemia. Discussion. Knockout of cavin-1 reveals key roles in NOS-dependent and -independent control of myocardial and coronary responses to stretch or ischemia, and regulation of sarcolemmal fragility/permeability.

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Identification of adenosine receptors in rat isolated cardiac fibroblasts and myofibroblasts

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Introduction. In a normal healthy adult heart, cardiac fibroblast cells comprise approximately 60-70% of the heart. After a stressful cardiac event such as a heart attack, fibroblast cells convert to myofibroblast cells (Santiago et al., 2010) which are not a part of normal cardiac tissue and contribute to the progression to heart failure. Adenosine is a cardioprotective mediator that is released in high concentrations during ischaemic events. Adenosine receptors have been identified in fibroblasts and are involved in stimulating cell proliferation (Epperson et al., 2009), however little in known regarding the expression of these receptors in myofibroblasts.
Aims. To isolate cardiac fibroblasts from rat hearts, transform them into myofibroblast cells and to determine the adenosine receptor populations expressed in cardiac fibroblast and myofibroblast cells.
Methods. Hearts from 8 week old Wistar rats were homogenised with 0.1% collagenase II. Following digestion, washing and centrifugation the cells were resuspended in cell culture solution (DMEM, 10% FBS). Following 2 passages the fibroblasts were starved for 24 hrs with FBS free media then exposed to 5 ng/ml TGF-β1 for 24 hrs to stimulate their conversion to myofibroblasts. Western blot analysis was used to confirm the identity of the fibroblast and myofibroblast cells and to determine adenosine receptor subtypes.
Results. Fibroblasts tested positive for the fibroblast marker vimentin and cardiac cell marker DDR2 proteins using Western blot techniques. Fibroblasts treated with TGF-β1 expressed vimentin, DDR2 and α-smooth muscle actin proteins which indicate the transformation into myofibroblast cells. Adenosine A_1, A_{2A} and A_3 receptors were found to be present on both fibroblast and myofibroblast cells.
Discussion. Our studies demonstrate that rat cardiac fibroblast and myofibroblast cells express adenosine A_1, A_{2A} and A_3 receptors. Adenosine and its receptors may moderate myofibroblast function and may provide a therapeutic target for the treatment of heart failure.

Epperson et al, (2009) Am J Physiol Cell Physiol 296(5);C1171-7.
Santiago et al, (2010) Dev Dynamics 239; 1573-1584.

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Targeting angiotensin receptors to modulate human fibrosis in vitro

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Introduction. Excessive collagen accumulation results in organ fibrosis, which can lead to end-organ failure. Angiotensin type-1 receptor (AT_1R) antagonists and angiotensin type-2 receptor (AT_2R) agonists are known to evoke anti-fibrotic effects in animal models. Anti-fibrotic effects of AT_2R stimulation in humans is not established.
Aims. To determine the anti-fibrotic effects of the AT_1R antagonist, candesartan (cand), and the AT_2R agonist, Compound 21, (C21) in human fibroblasts, in order to develop an in vitro screen of fibrosis and its modulation by angiotensin-related mechanisms.
Methods. Human cardiac fibroblast (HCF; ScienCell, USA) and human dermal fibroblast (HDF; BJ3 cells; ATCC) were treated with recombinant human transforming growth factor beta-1 (TGF-β1) (5ng/ml) ± cand or C21 (both 10-1000 nM), incubated in Medium 199 (with 10% fetal bovine serum, 5% penicillin streptomycin and 5% fibroblast growth supplement) at 37 °C with 5% CO_2 for 72 hours. Various markers of fibrosis and collagen turnover were then assessed by western blotting using extracted protein.
Results. TGF-β1 increased collagen-I in both HCF (1.5-fold) and HDF (3-fold), associated with increased alpha-smooth muscle actin (αSMA; marker of myofibroblast differentiation), collagen-degrading matrix metalloproteinase (MMP)-13 and tissue inhibitor of metalloproteinase (TIMP)-1 (all $P<0.05$ vs control group, n=4-6). Both cand and C21 caused dose-dependent reductions in TGF-β1-stimulated collagen-I and TIMP-1 in both cell types ($P<0.05$, n=4-6). These reductions correlated with dose-dependent reductions in αSMA protein expression in HCF but not in HDF. Cand or C21 treatment also appeared to increase MMP-13 levels in TGF-β1-stimulated HCF and HDF.
Discussion. Cand and C21 evoked similar 'anti-fibrotic' effects in HCF and HDF that appear to involve inhibition of collagen production yet enhanced collagen degradation, that is likely associated with reduced TIMP-1 expression. These findings suggest that both TGF-β1-stimulated HCF and HDF are useful pharmacological screening tools for the development of novel anti-fibrotic agents; and that AT_2R activation is equally as anti-fibrotic as AT_1R blockade in human fibroblasts.

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Non-adrenergic, non-cholinergic (NANC) neurogenic dilatation of rat mesenteric arteries involves opening of potassium channels

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Introduction. Transient Receptor Potential Vanilloid 4 (TRPV4) ion channels are involved in endothelium-dependent relaxation of small arteries and are also located on perivascular nerves.
Aims. To investigate the role of TRPV4 and NO in neurogenic dilatation of rat mesenteric arteries.
Methods. First and second order mesenteric arteries were mounted onto a wire myograph and subjected to electrical field stimulation (EFS; 0.5-16Hz, 0.3ms duration, 5 second train every 100 seconds). The voltage was determined by stimulating the arteries at a constant frequency of 4Hz and increasing the voltage until 50% relaxation from U46619 (U4) contraction was obtained in the presence of the adrenergic neuron blocker guanethidine (10µM) and muscarinic receptor antagonist atropine (1µM). Responses were assessed in the presence of L-NAME (100µM, NOS inhibitor), HC067047 (10µM; TRPV4 antagonist), ODQ (10µM; soluble guanylate cyclase inhibitor), capsazepine (10µM; TRPV1 antagonist), BIBN 4096 (10µM; CGRP receptor antagonist), suramin (100µM; P2 receptor antagonist), tetraethylammonium chloride (TEA 10mM; K channel blocker), glibenclamide (10µM; K_{ATP} channel blocker), iberiotoxin (1µM; large conductance K_{Ca} channel blockers) and 4-aminopyridine (4-AP, 2mM; K_V channel blocker).
Results. EFS (16Hz) caused relaxation that was not affected by endothelium removal (95±5%, n=5), L-NAME (92±7%, n=6) or ODQ (97±3%, n=6). Furthermore there was no significant effect of capsazepine (81±5%, n=5), HC067047 (80±5%, n=5), BIBN 4096 (71±7%, n=4), suramin (89±6%, n=5), glibenclamide (86±4, n=5), iberiotoxin (77±9%, n=5) or 4-AP (68±8%, n=5). By contrast, TEA significantly reduced EFS induced relaxation (43±10%, n=6, $p<0.05$).
Discussion. EFS-induced relaxation was endothelium-independent and does not involve TRPV1 or TRPV4 the neurotransmitters CGRP and ATP. TEA did significantly reduce relaxation indicating that K channels are involved, however K_V, K_{ATP} and BK_{Ca} channels were not responsible for the relaxation seen. Therefore, K channels are involved in the neurogenic dilation however the channel sub-type is still to be determined.

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Estimation of cytokine production levels in response to panel of mitogens in healthy individuals

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Introduction. Numerous cell culture studies have been performed using different mitogens as a stimulant. However, there are very few studies that have examined the most appropriate mitogen to achieve maximum response.
Aim. To determine cell viability and cytokine production in response to different mitogens by using tetrazolium salt, 3-(4,5-dimethyl thiazol-2-yl)-2,5-indiphenyl tetrazolium bromide (MTT) assay and ELISA tests, respectively.
Method. Blood samples from healthy volunteers were obtained, diluted 1:10 with RPMI 1640 culture medium and stimulated with different concentrations of the following mitogens: Phorbolmyristate acetate (PMA) + Ionomycin, Pokeweed (PW), Lipopolysaccharide (LPS), Phytohaemagglutinin (PHA) and Concanavalin A (Con A). Cells were cultured in flat-bottomed 96-well microplates and incubated at 37°C for 24, 48 and 72 hours. Culture supernatants were then measured for Interleukin 4 (IL-4), Interleukin 5 (IL-5), GMSCF (Granulocyte macrophage-stimulating colony factor) and Interleukin-9 (IL-9) by using ELISA kits. Cell viability was assessed by using MTT assay.
Results. The highest cell viability rate was observed with PMA + Ionomycin (50ng/mL+1ug/mL) at 72 hours of incubation. Maximal cytokine production was observed for the PMA + Ionomycin (50ng/mL+1ug/mL) and Con A (25ug/mL). PMA + Ionomycin induced strong proliferative response for IL-4, IL-5 and IL-9 but weak response for GMCSF. GMCSF recorded highest concentration when stimulated with LPS at 72 hours. IL-4 was not detectable with LPS and PW stimulation. The response mean values of the five mitogens can be positioned in the following order: PMA + Ionomycin > Con A > LPS > PW > PHA > cell control.
Discussion. PMA with Ionomycin is a potent stimulator as they activate T cell proliferation in whole blood cells. LPS has been shown to induce significant levels of GMCSF secretion via mitogen-activated protein kinase (MAPK) dependent mechanism. In conclusion, PMA + Ionomycin and Con A were most effective as a stimulating agent in whole blood assay compared to other mitogens.

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Generating more relevant cellular models of the airways in asthma

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Introduction: Infiltration of mast cells into airway smooth muscle (ASM) bundles is a defining feature of asthma (Brightling et al., 2002). Thus, consideration of these cell-cell interactions, the presence of extracellular matrix (ECM) and the biomechanical influences present in a 3D environment would be predicted to deliver more useful model systems for evaluating anti-asthma drugs.
Aims: This project aimed to generate and characterise 3D spheroid models of ASM bundles and in addition examine the effect of ECM on mast cell reactivity.
Methods: Human ASM and mast cell spheroid formation used two independent protocols; the low adherence centrifugation method and the 'hanging drop' method. Tracking mast cells within spheroids and monitoring spheroid size was done using confocal microscopy. Mast cells were also grown on fibrillar collagen or fibronectin and treated with a range of anti-asthma drugs, with degranulation quantified by β-hexosaminidase release.
Results: Using the centrifugal method, ASM cells formed spheroids. Formation and subsequent remodelling of these spheroids was not affected by incorporation of mast cells. Using GFP-labelled cells, mast cells were seen to be randomly distributed throughout the spheroid. Densely packed ASM spheroids were not generated using the hanging-drop method. Seeding mast cells on a thin layer of fibrillar collagen did not affect their responses to stimuli or anti-asthma drugs.
Discussion: We have successfully generated ASM and mast cell co-cultures in a spheroid model that is more reflective of smooth muscle bundles observed in asthma. Further work is necessary to refine this model to assess its utility in screening novel anti-asthma drugs with predicted actions on ASM and mast cells

Brightling CE et al (2002) N Engl J Med 22: 169-705.

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β-hexosaminidase release and regulation in lung cells

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Introduction: β-hexosaminidase (β-hex) is an enzyme involved in glycoprotein metabolism and is generally found within secretory lysosomes (Tropak et al., 2007). Whilst β-hex levels have been largely used as a measure of mast cell degranulation, the enzyme has been identified as a modulator of human airway smooth muscle (hASM) activity and is also known for its antimicrobial properties (Lew et al., 1999; Tropak et al., 2007)
Aims: We aimed to determine if other cell types in the lung had the ability to release β-hex in a regulated fashion.
Methods: Rat basophilic leukaemia (RBL) cells, hASM cells and the human bronchial epithelial cell line BEAS-2B were examined for resting and stimulus-induced β-hex release using an enzymatic assay utilising the selective substrate p-nitrophenyl N-acetyl-β-D-glucosamide. β-hex gene levels (HEXA and HEXB) were also quantified in HASM and BEAS-2B cells by qPCR.
Results: As expected, β-hex was released from mast cells activated through the IgE pathway. However the enzyme was also released basally from hASM and BEAS2B cells. Whilst a range of stimuli involved in innate immunity did not significantly modulate this release, IFN-β was able to enhance β-hex release from BEAS2B cells.
Discussion: Our results demonstrate the presence and release of β-hex from cell types other than mast cells in the lung. Released β-hex may act in an autocrine or paracrine fashion to regulate resident cells in the airway. The regulated release of β-hex by IFN-β, an antiviral protein, suggests a role for the enzyme in innate protective mechanisms in the lung.

Lew DB, et al. (1999) Am J Respir Cell Mol Biol 21: 111-118.
Tropak MB, Mahuran D (2007) FEBS J 274: 4951-4961

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Time depended effects of dexamethasone on production and degradation of extracellular matrix in cultured human trabecular meshwork cells

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Introduction. Deposition of extracellular matrix (ECM) in trabecular meshwork (TM) is one of the crucial pathological changes leading to increased intraocular pressure in steroid-induced glaucoma that is commonly associated with corticosteroid therapy. Deposition of ECM components such as collagen, fibronectin (FN), alpha smooth muscle actin (α-SMA) is consequence of increased ECM production or reduced ECM degradation or both.
Aims. To evaluate time-depended effect of dexamethasone on production of ECM components (collagen types I, III, IV; FN; α-SMA) and ECM degrading enzymes (active and total MMP-2,-9; TIMP-1,-2) by human TM (HTM) cells.
Methods. Primary HTM cells were divided into three groups: Group 1 was cultured in Dulbecco's modified Eagle's medium (DMEM) only; groups 2 and 3 were cultured in DMEM with 0.1% DMSO and DMEM with dexamethasone (DEXA) 100 nM dissolved in 0.1% DMSO, respectively. Cells were incubated for 7, 14 and 21 days. Elisa and immunocytochemistry were done to estimate the tested parameters.
Results. ELISA showed that the expression of tested ECM components was significantly ($p<0.05$) higher in DEXA-treated group in all 3 time points. The maximum levels of FN, α-SMA, collagen I, III and IV were respectively 1.38, 1.94, 6.26, 2.74 and 1.66 fold higher compared to that in DMEM-treated cells. Immunofluorescent staining results were in line with Elisa. DEXA treatment reduced concentrations of both active and total MMP-2 and MMP-9 up to 4.67, 3.54, 5.05 and 2.41 fold respectively. At the same time DEXA significantly increased TIMP-1,-2 levels up to 1.65 and 1.5 fold compared to DMEM treated group. Most of the tested parameters were significantly changed on days 14 and 21 compared to day 7 with no significant difference between days 14 and 21 of DEXA treatment.
Discussion. DEXA significantly promoted TM ECM deposition that likely contributed by reduction in secretion of MMPs and increase production of TIMPs. The results of this study may suggest that the most significant changes in ECM deposition happen in the first two weeks of steroid treatment.

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Inflammation and altered intrapulmonary airway contraction in mice following short-term cigarette smoke exposure are differentially regulated by RAGE and TLR4

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Introduction. RAGE and TLR4 are pattern-recognition receptors that sense microbial derived products, and products of tissue damage. Both receptors share common ligands and signaling pathways and are implicated in immune and inflammatory responses in COPD. Cigarette smoke (CS) alters small airway contractile responses, but whether these changes are mediated by RAGE and/or TLR4 in an additive or synergistic manner remains to be determined.
Aims. To assess changes in airway inflammation and contraction in $RAGE^{-/-}$, $TLR4^{-/-}$ and $TLR4^{-/-}/RAGE^{-/-}$ mice in comparison with wild-type (WT) mice following exposure to short-term CS.
Methods. Male C57/Bl6 mice (8-11 weeks, n=3-7/group were exposed to room air (Air) or 9 cigarettes/day (CS) for 4 days were culled (sodium pentobarbitone, 100mg/kg i.p.) for collection of bronchoalveolar lavage (BAL) for total/differential cell counts. In separate mice, precision cut lung slices (PCLS, 150 μm) were prepared for phase-contrast microscopy to analyse *in vitro* airway contraction to MCh (10nM-3μM).
Results. There was no difference in cell number in BAL or in airway contraction to MCh in PCLS between Air groups. Total BAL cells increased by ~50% in all CS groups, with only $RAGE^{-/-}$ mice showing reduced neutrophilia (BAL neutrophils/mL: WT Air $<0.1x10^4$ WT CS $11x10^4$; $RAGE^{-/-}$ CS $6x10^4$ $P<0.0001$). Contraction to MCh was attenuated by CS in WT (Air: 51±5%; CS: 26±5%, $P<0.05$), but not $RAGE^{-/-}$, $TLR4^{-/-}$ or $RAGE^{-/-}/TLR4^{-/-}$ mice.
Discussion. These preliminary results suggest that short term CS exposure reduces MCh contraction in small airways. This finding may reflect a phenotype shift of airway smooth muscle from a contractile to more proliferative phenotype that could contribute to airway remodelling in COPD. While RAGE appears to contribute to the neutrophilia caused by CS exposure, RAGE and/or TLR4 may maintain the status quo of airway contraction. Further studies examining airway dilator and constrictor responses may determine the potential implications of manipulating RAGE and TLR4 in chronic models of respiratory diseases exacerbated by cigarette smoking.

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Characterising vascular changes in a mouse model of bronchopulmonary dysplasia (BPD)

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Introduction. BPD is a chronic respiratory disease in premature infants, resulting from perinatal inflammation and postnatal oxygen treatment. Infants with BPD have impaired lung development and greater risk of developing pulmonary arterial hypertension (PAH), which reduces survival in infants by up to 75%. Validated mouse models mimicking key features of BPD-associated PAH are required to identify potential new therapies.
Aim. To characterise changes in markers of vascular remodelling and reactivity in intrapulmonary arteries in a "double-hit" mouse model known to induce alveolar and airway changes consistent with BPD.
Methods. Following *in utero* exposure to lipopolysaccharide, C57BL6 pups were born and randomized to 21% oxygen (air) or 65% oxygen (hyperoxia) exposure for 4 weeks, for comparison with age-matched naïve mice. Mice were anesthetized with inhaled isoflurane, then killed by cervical dislocation. Lung sections were prepared for immunohistochemistry for α-smooth muscle actin (α-SMA), endothelin receptor-A (ET_A) and caveolin-1 as markers of increased contraction. Precision cut lung slices (PCLS) were prepared to assess vasoconstriction to endothelin-1 and U46619.
Results. Hyperoxia did not alter vascular expression of α-SMA (pixel count x 10^8/area: air 3.5±0.5 n=15; hyperoxia 3.7±0.4 n=20, NS), ET_A (n=13,19, NS) or caveolin-1 (n=15,21, NS). Maximum artery contraction in PCLS was similar for endothelin-1 and U46619 in all groups (U46619 max % reduction in area: naïve 34±4% n=5; air 33±14% n=4; hyperoxia 42±12% n=3, NS).
Discussion. At the 4-week time point, this model of BPD does not induce vascular remodeling consistent with the development of PAH or increased contraction of intrapulmonary arteries to vasoconstrictors *ex vivo*. Alternative models or later time points may be required to establish validated conditions to assess new treatments for BPD-associated PAH.

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Hypermutable *Pseudomonas aeruginosa* related to multi-drug resistance in cystic fibrosis patients with respiratory infections

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Introduction. Hypermutable *Pseudomonas aeruginosa* (Pa) isolates have been found in chronic respiratory infections and are associated with reduced lung function in cystic fibrosis (CF) patients. These isolates have a greatly increased mutation rate leading to an enhanced ability to become resistant to antibiotics.
Aims. This study aimed to determine the prevalence of hypermutation and antibiotic susceptibility for the first time in clinical Pa isolates from Australian CF patients.
Methods. 59 clinical Pa isolates from Australian CF patients, PAO1 and PAO*ΔmutS* (hypermutable) were characterised. Mutation frequencies (MF) on 300 mg/L rifampicin-containing agar plates were assessed in triplicate. Hypermutation was defined by the rifampicin MF being ≥20-fold greater compared to PAO1. MICs were determined using Etests and susceptibility judged by EUCAST breakpoints. Multidrug-resistance (MDR; resistance to ≥1 agent from ≥3 different antimicrobial categories) was also evaluated.
Results. 22% of the clinical Pa isolates were hypermutable. MDR was significantly more frequent in hypermutable (38%) than non-hypermutable isolates (13%; Chi-square test, $p<0.05$). Among hypermutable isolates, resistance was found in 77% of isolates for ciprofloxacin, 46% for ceftazidime, and 38% for meropenem and tobramycin. In non-hypermutable isolates, resistance was common for ciprofloxacin (52%), but less frequent for tobramycin (22%), ceftazidime (20%) and meropenem (13%).
Discussion. The prevalence of hypermutable isolates in CF patients was comparable between Australia and Europe. MDR was significantly more prevalent in hypermutable isolates and these isolates were more frequently resistant to all tested antibiotics in comparison to non-hypermutable isolates. Therefore, hypermutable Pa are playing a critical role in the antibiotic resistance problem in respiratory infections within CF patients.

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High throughput assay for simultaneous analysis of plasma samples of losartan and its metabolite EXP-3174 using automated robotic solid phase extraction coupled to LC-MS/MS

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Changes in pharmacokinetics of critical medications administered during surgeries involving cardiopulmonary bypass (CPB) have been reported. The impact of CPB on the activities of cytochrome P450 enzymes metabolising >90% of administered drugs is the key factor requiring further investigation. The metabolism of losartan to EXP3174 is a specific measure of CYP2C9 metabolic activity. The aim of this study was to develop on-line solid phase extraction (SPE) method to measure plasma concentration of losartan and EXP-3174 in samples collected from patients undergoing cardiac surgeries with CPB.
Method: To 100 µL (human plasma), 100 µL (internal standard; losartan-d4, 40 ng/mL) and 100 µL (50mM ammonium acetate) (pH=2.0) were added, and loaded on SPARK Holland C18 cartridges which were pre-conditioned with methanol (1 mL) and 50mM ammonium acetate; pH=3.0 (1 mL). Extraction was carried out using 50mM ammonium acetate (pH=3.0) and washed twice using 500 µL 20% methanol and water before eluting the sample using mobile phase (0.1% formic acid in water and 0.1% formic acid in acetonitrile) at 0.4 mL/min for 8.45 min. A C18 XTerra® analytical column was used and analytes of interest were detected using QTrap 5500 mass spectrometer (AB Sciex) with negative ion spray ionisation.
Results: The mass transitions for detection were m/z 421.045→126.900 (Losartan), 435.009→156.960 (EXP-3174) and 425.166→157.100 (Losartan-d4). The Calibration curve was linear (correlation coefficient ≥0.9987) ranging 0.4-40 ng/ml. Within-run and between run accuracy and precision for QC samples were acceptable (>88%). The retention time for losartan, EXP-3174, and losartan-d4 were 4.2, 4.9 and 4.1 min, respectively.
Discussion: Previous assays for losartan & EXP-3174 were performed through manual SPE whereas this assay involves online SPE method enabling minimal solvent usage and providing better consistency. Deuterated Internal standard (Losartan-d4) compensates for variability in chemical derivatization, sample extraction & LC-MS/MS analysis due to its nearly identical chemical and physical properties to Losartan. The lower limit of quantification (LLOQs) in this assay are lower than what has been reported previously.

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Dementia and cognitive impairment risk with proton pump inhibitor therapy - a systematic review

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Introduction. Proton pump inhibitors (PPIs) are among the most widely used medications worldwide. Dementia is an increasingly common cause of disability in older populations. Recent studies have suggested an increased risk of cognitive impairment and dementia diagnosis in people who consume PPIs.
Aims. This systematic review aims to explore the use of PPIs and acute cognitive impairment and dementia.
Methods. Systematic searches were conducted in the databases of MEDLINE, EMBASE, Cochrane Central Register of Controlled Trials (CENTRAL), PSYCinfo, Scopus, Web of Science and ClinicalTrials.gov for articles published from inception to June 30, 2016. Primary outcomes of interest were the use of PPIs and diagnosis of dementia or acute cognitive impairment. Studies conducted on people aged less than 18 years old were excluded. All study designs were eligible for inclusion. Two independent reviewers assessed study quality and extracted data from included studies.
Results. The systematic search strategy and screening process yielded 11 studies for inclusion in the systematic review. Four studies explored PPI use and dementia and seven studies explored PPI use and acute cognitive impairment. Three of the four studies exploring dementia identified a positive association with PPI use. A positive association was also observed in the majority of studies exploring acute cognitive impairment.
Discussion. Current published literature indicates that there may be an association between PPI use and dementia and acute cognitive impairment. However, further longitudinal studies, with robust bias limitation, are required to explore the use of PPIs and dementia and acute cognitive impairment, and to ascertain the existence of any causal relationships.

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Comparison of enquiries for complementary and alternative medicines with enquiries for conventional medicines at the Medicines Information Service at Christchurch Hospital from 2010 to 2016

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Introduction. Use of complementary and alternative medicines (CAMs) appears to be becoming more prevalent. As with conventional medicines, CAMs have the propensity to cause significant interactions and adverse drug reactions (ADRs), and the resources for answering these enquiries are limited.
Aim. To quantify and describe CAMs enquiries received by the Christchurch Hospital Medicines Information Service (MIS) over the last six years and compare these with enquiries for conventional medicines.
Methods. Data were extracted from the Christchurch Hospital MIS database (containing all enquiries from 2010 to 2016). Microsoft Excel® was used to analyse these data. Data extracted included medicine (CAMs versus conventional), enquirer profession, category and time taken to answer.
Results. Eight hundred and seventy (6%) of a total of 15,105 enquiries involved CAMs. For CAMs, most enquiries came from hospital pharmacists (31%) or community doctors (23%). Similarly, for conventional medicines, most came from community doctors (28%) or hospital pharmacists (23%). The most common categories for CAMs were interactions (55%) followed by ADRs (16%); whereas for conventional medicines, the most common categories were administration/dosage (24%) and contraindications/precautions (21%). Most CAMs enquiries took one to four hours to answer (36%) compared with half to one hour (35%) for conventional medicines. The proportion of CAMs enquiries increased from 4% to 9% over the six-year period.
Discussion. The proportion of CAMs enquiries to the MIS has increased over the six-year period and consumes a significant amount of time. Increase in both use and prescriber awareness of CAMs may have contributed to the increase in enquiries.

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Exploring the barriers and facilitators of vancomycin guideline compliance

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Introduction. Vancomycin is a glycopeptide antibiotic, prescribed as a first-line therapy for staphylococcal infections resistant to penicillin such as those caused by methicillin-resistant *Staphylococcus aureus*. Therapeutic Drug Monitoring (TDM) is a recommended practice for vancomycin. A set of vancomycin dosing and monitoring guidelines exist at St Vincent's Hospital (SVH).
Aims. To identify the perceived barriers and facilitators to appropriate dosing and TDM of vancomycin.
Methods. Doctors from a variety of wards were approached directly and invited to participate in a short, semi-structured interview. 16 doctors were interviewed to gain an understanding of how decisions are made with respect to vancomycin initiation, dosing and monitoring. Interviews were transcribed verbatim and analysed independently by two researchers for emerging themes.
Results. Most doctors reported utilising guidelines and had a positive perception of the SVH guidelines and antimicrobial specialists. Doctors appeared to possess good knowledge of most aspects of the guidelines and were aware of poor guideline compliance. Doctors reported the main barriers to guideline compliance to be difficulty in coordinating dosing regimens with scheduled blood collection rounds, and poor coordination of dosing and TDM among several different staff caring for each patient. Interviews with doctors also revealed a poor understanding of an aspect of the guidelines related to pending trough results.
Discussion. Doctors believed that barriers to guideline compliance were largely related to characteristics of the prescribing environment. Suggestions to circumvent these barriers included the addition of a designated TDM blood collector, improved communication between staff, and implementation of alerts to prompt TDM on the hospital's electronic prescribing system. The guidelines were well-perceived but specific aspects were poorly understood. This suggests that guideline-specific education is required.

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Drug-drug interaction and brand-prescribing alerts in an electronic prescribing and administration (ePA) system

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Introduction. Localised clinical decision support alerts can be developed to fire within ePA systems. These alerts should be aimed at minimising patient harm. An ePA system, MedChart™, has been rolled out to all Canterbury District Health Board (CDHB) public hospitals except the major acute hospitals. It has been configured to include alerts for pharmacokinetic and pharmacodynamics drug-drug interactions (DDIs) and prescribing by brand. Pharmacokinetic DDI alerts included: single drug alerts for major perpetrators. Pharmacodynamic alerts included: antiplatelet/anticoagulant alerts based on bleeding risk from the combination, and duplicate prescriptions for beta-blockers and ACE inhibitors. CDHB MedChart 2015 data showed that 582/4257 prescriptions were by brand, before the introduction of the brand alert.
Aims. 1) to examine the number of alerts firing, including prescriber alerts for DDI and brand-prescribing, 2) to compare proportion of current brand-prescribing with 2015 data.
Methods. The CDHB MedChart database was queried using a locally developed extracting tool into Excel to look for DDI and brand-prescribing alerts to establish a baseline for comparison with rollout to our main acute hospital. Analysis was performed using Tableau™ and Microsoft Excel™ Power Query.
Results. During 1 July to 31 August 2016, 561 patients were prescribed 61,771 prescriptions. These generated 6,842 alerts (11 alerts per 100 prescriptions), including: pharmacokinetic = 599 (9% of all alerts), pharmacodynamic = 157 (2%) and brand-prescribing = 20 (0.3%). Brand-prescribing was significantly lower than in 2015 ($\chi^2(1) = 8200$, $P < 0.0001$). These data will be compared with data from rollout to the major CDHB acute hospitals.
Discussion. The CDHB MedChart alert burden (11% of prescriptions) is comparable to that reported elsewhere (7 to 36%). The proportion of brand-prescribing in CDHB MedChart has decreased significantly since the introduction of the brand alert.
Polasek, T.M., Lin, F.P.Y., Miners, J.O. et al (2011). Br J Clin Pharmacol. 71: 727–36

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An investigation of the drug-drug interaction between fusidic acid and statins, leading to rhabdomyolysis

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Introduction. Statins are lipid-lowering medications which are commonly prescribed. Although normally safe, muscular-related toxicity may occur. Rhabdomyolysis is a rare side-effect of statin use which is characterised by the breakdown of muscle cells and the release of toxic intracellular contents, and may be fatal in some cases. Fusidic acid (FA) is a fungally-derived antibiotic used to treat Methicillin Resistant *Staphylococcus aureus* infections. Concomitant administration of FA and statins has been reported to result in severe statin toxicity. Although the specific mechanism has not been ascertained, it has been speculated that this interaction is mediated by the inhibition of Cytochrome P450 (CYP) 3A4 as a result if its importance in the metabolism of a number of statins.
Aims. To characterise the potential *in vitro* inhibition of the CYP (CYP 1A2, 2C8, 2C9, 2C19, 2D6 and 3A4) and UDP-glucuronosyltransferase (UGT) enzymes by FA.
Methods. The incubation samples contained: buffer, probe substrate, recombinant enzymes and FA (1 µM, 10 µM, 100 µM and 250 µM) and were pre-incubated in a 37°C shaking water bath for 5min. The reaction was initiated by the addition of a generating system or cofactor and allowed to proceed before termination with 70% perchloric acid and placement on ice. Following centrifugation, the supernatant fraction was removed and metabolite formation was quantified by HPLC. The concentration at which 50% enzyme inhibition occurs (IC_{50}) was then calculated
Results. FA demonstrated weak inhibition of CYP3A4 (IC_{50}=251±17 µM) and minor inhibition of CYP2C9 (IC_{50}=108±17 µM). FA demonstrated the greatest inhibition potential of UGT 1A1 (IC_{50} = 23±1.4 µM).
Discussion. On the basis of these results, it is unlikely that systemic inhibition of CYP 3A4 will occur at unbound plasma concentrations of FA (1.2-18 µM). It is therefore suggested that alternative mechanisms may be involved in this DDI, with the Organic Anion Transporting Polypeptides (OATP) being of interest. A recent publication (Eng et al, 2016) is consistent with our findings and implicates OATP1B1 and OATP1B3 in this DDI.

Eng, H et al. (2016) Drug Metab Dispos 44: 692-9.

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Ginsenosides: Positive modulators of P2X7 in macrophages

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Introduction: P2X7 is an adenosine 5' triphosphate (ATP) gated ion channel known to play a key role in inflammation. *Panax ginseng* is a Chinese herb well known for its anti- as well as pro-inflammatory effects. The main bioactive components in ginseng are saponins known as ginsenosides. Previous work has demonstrated that four glycoyslated protopanaxadiols, Rh2, Rd, Rb1 and Compound K, have a potent potentiating action on P2X7 channels using HEK-293 cells expressing the receptor. It is possible that the immuno-modulatory properties of ginseng *in vivo* may be attributed to the ability of ginsenosides to potentiate P2X7-mediated responses (1). In the present study, we further investigate the effect of these four ginsenosides for P2X7 mediated in macrophages.
Aims: To investigate the effect of selected ginsenosides on physiological responses mediated by P2X7 in macrophages.
Methods: Cell cultures, YOPRO uptake, Fura-2 influx, caspase 3/7 activity), cell viability using MTS assay.
Results: Ginsenoside mediated potentiation of P2X7 dependent dye uptake and calcium responses was observed in J774 macrophage cell line in the rank order of CK=Rd>Rb1>Rh2, with CK potentiating the resposes 4-folds. CK was the only ginsenoside among these four which had caused a significant increase in P2X7 mediated increase in caspase 3/7 activity and increased cell death.CK also increased the IL-1 beta release in macrophages.
Discussion: These results suggest that out of the four ginsenosides tested, CK potentiated the P2X7 responses the most. The potentiating of P2X7 by CK appears to activate the signalling pathway leading to enhanced caspase 3/7 activity very rapidly pushing the cells towards apoptosis specifically via the P2X7. IL-1β release by macrophages is observed to increase in presence of CK. Taken together, these results provide strong evidence that ginsenosides enhance the pro-inflammatory properties of immune cells and can thus, help boost immune system.
References:
1. Helliwell et al, 2015, Br J. Pharm. 172: 3326-40.

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Does an intervention by a pharmacist improve long-term adherence to cardiovascular medicines?

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Introduction. Nonadherence to cardiovascular medicines occurs in 60% of subjects with chronic cardiovascular disease and leads to poor outcomes. In an attempt to improve adherence and cardiovascular outcomes, interventions are often used. Interventions may involve a pharmacist, but it is not always clear whether these are effective.
Aims. The aim was to determine whether interventions by pharmacists improve adherence to cardiovascular medicines.
Methods. Literature search of Medline and CINAHL for 'pharmacist', 'adherence' and 'medicine'.
Results. In hypertension, there were four relatively minor interventions by pharmacists that did not improve adherence to medicines in the treatment of hypertension. Three studies have suggested that more extensive interventions by a pharmacist may improve adherence in hypertension, but the first study is complicated by the provision of blister packs to the subjects in the intervention group, which may have improved adherence. In the other two studies the improvement was small and did not translate into a reduction in blood pressure. In coronary artery disease, six studies have shown that when baseline adherence to medicines is high in subjects, a pharmacist intervention does not improve this. In studies where baseline adherence is not high, there is one study showing the intervention by a pharmacist has little effect, and four studies showing that an intervention by a pharmacist may improve adherence in subjects with coronary artery disease. Only two of the four studies reporting improved adherence also reported lowered LDL cholesterol, one reported no lowering, and one did not measure LDL cholesterol. In heart failure, six studies have reported improved adherence with a pharmacist intervention, and four of these showed a positive clinical outcome.
Discussion. Minor interventions by pharmacists, or interventions in subjects who are already highly adherent, do not improve adherence to cardiovascular medicines. In subjects with hypertension or coronary heart disease, there are mixed results as to whether interventions by pharmacists improve adherence to medicines, and whether any improvement is sufficient to improve outcomes. In heart failure, there is strong evidence that major interventions by pharmacists, improve adherence and outcomes.

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Development of a LCMSMS assay for N-methyl 2 pyrrolidone in plasma

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Introduction. N-methyl 2 pyrrolidone (NMP) is a common solvent and drug vehicle. Recently, Shortt et al (2014) showed that NMP had antineoplastic and immunomodulatory activity. In order to develop NMP as a therapeutic agent a Phase I study to examine the pharmacokinetics of NMP was undertaken.
Aims. To develop a LC-MSMS assay for NMP in plasma.
Methods. Plasma samples (20µl) were prepared by adding five volumes of methanol containing deuterated internal standard. The samples were vortexed then centrifuged and the supernatant was transferred to a vial and injected onto the LC-MSMS. The LC-MSMS system consisted of a Shimadzu UHPLC with a SCIEX 6500QTrap, a Kinetex C18 column and using a gradient of 0.1% formic acid and acetonitrile. Two patients have currently had samples analysed.
Results. NMP fitted a quadratic equation over the range of 0.01 – 20 mg/L. Intra assay precision was between 2 - 6% and intra assay bias was between -0.2 – -1.1%. Inter assay precision was between 3 - 8% and inter assay bias was between -4 – 3%. The limit of quantitation for was 0.01 mg/L. Patient plasma samples were between 0.01 and 3 mg/L.
Discussion. A validated LCMSMS method for NMP was developed using a small volume of plasma and a simple methanol extraction with a limit of quantitation of 0.01 mg/L. This method is currently being used for the analysis of NMP in a phase I clinical trial.

Shortt et al (2014) Cell Reports 7:1009-1019

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Cannabis and exercise performance

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Introduction. Cannabis has been used by participants in sport for millennia notwithstanding lack of objective evidence of any benefit.
Aims. To review the evidence relating to the effect of cannabis on exercise performance.
Methods. Systematic review of published literature
Results. Only 13 studies have investigated the effects of cannabis in association with exercise protocols. Of these studies, none showed any improvement in aerobic performance, two found that marihuana precipitated angina at a lower work-load, strength is probably reduced, exercise induced asthma is inhibited and aerobic exercises causes very small rises in THC concentrations.
Discussion. From the small number of published studies, cannabis does not improve aerobic performance or strength. Cannabis should be avoided in sportspersons with a history of cardiovascular disease.

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The effect of anticholinergic load over time in community-dwelling elderly Australians

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Introduction. A higher anticholinergic load (ACL) (cumulative effect of using multiple medications with anticholinergic effects) increases the risk of depression and cognitive impairment (Lampela P et al, 2013).
Aims. To establish whether changes in anticholinergic load over time in elderly community-dwelling persons are associated with changes in cognitive performance and depression.
Methods. Participants at baseline (n=1768), one year (n=1373) and a cohort (with possible/definite cognitive impairment) at two years (n=370) were assessed for depression using the Geriatric Depression Scale (GDS; Sheikh et al, 1986), and cognitive function using a subsection of the revised Cambridge Examination for Mental Disorders of the Elderly (CAMCOG-R; Roth et al, 1986). Participants' ACLs were determined using the Anticholinergic Drug Scale (Carnahan et al, 2006).
Results. The mean ± standard deviation (SD) ACL change from baseline to 12 months was 0.012 ± 0.99, and the mean ± SD ACL change from baseline to 24 months, and 12 months to 24 months was -0.04 ± 1.3 and -0.06 ± 1.14, respectively. The majority of participants had no change in anticholinergic load. Baseline ACL was associated with changes in GDS (but not CAMCOG-R) scores. Higher baseline ACL scores were associated with increased depression scores over time, significantly so at 24 months in those with cognitive impairment.
Discussion. The ACL of elderly participants was stable over time. This may represent lost opportunities for de-prescribing. A high ACL may be a predictor for increased levels of depression over time, particularly in the elderly with impaired cognition. This supports recommendations to limit anticholinergic medications in these patients.

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Roth M et al (1986) Br J Psychiatry 149:698-709
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Development and validation of the dementia-specific medication review electronic decision support system (D-MEDSS©)

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Introduction. People with dementia are prescribed more medications compared to people without dementia, and are particularly vulnerable to the adverse effects of high-risk medications (i.e. anticholinergics, antipsychotics and benzodiazepines). Management of high-risk medications for people with dementia can be challenging for healthcare practitioners, patients and their carers.
Aim. To develop and validate a computerised clinical decision support system (CCDSS) that incorporates pharmacological and clinical tools to aid person-centred medication management in dementia.
Methods. We are developing the Dementia-specific Medication review Electronic Decision Support System (D-MEDSS©). This study consists of two phases. A) Development: The D-MEDSS will be designed to produce information reports for healthcare practitioners, patients and their carers, and will incorporate three tools: 1) The Drug Burden Index (DBI), a measure of cumulative exposure to anticholinergic and sedative medications that is associated with functional impairment in older adults; 2) The Patients' Attitudes Towards Deprescribing (PATD) questionnaire that explores patients attitudes to deprescribing medications; and 3) a management tool for goals of care for dementia. B) Validation: Focus groups and one-on-one interviews with general practitioners, pharmacists and people living with dementia and their carers will test the D-MEDSS and information reports for usability and provide perspectives on implementation of the D-MEDSS reports in practice. The System Usability Scale and descriptive statistics will be used to summarise the validation phase. The focus groups and one-on-one interviews will be audio recorded, transcribed verbatim and qualitatively analysed to derive conceptual domains.
Discussion. The validated D-MEDSS will reliably identify anticholinergic and sedative medications, incorporate patient's attitudes to deprescribing, and list the patient's goals of care to aid management of high-risk medications for people with dementia and their carers.

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Audit of Melatonin utilisation in aged care in tertiary and secondary hospitals

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Introduction. Hypnotics lead to falls and may be a risk factor for delirium in older people. Melatonin maybe a safer alternative. Falls are harmful events and half of serious adverse events reported in Quality and Safety Commission.
Aims. Utilisation of melatonin in aged care wards in a tertiary (RPH) and secondary hospital (BH).
Methods. Retrospective electronic review of pharmacy database and chart audit of melatonin prescriptions 2013 to 2015 in RPH. Retrospective review of 2014 and 2015 pharmacy database in BH.
Results. There has been doubling in cost of melatonin from $2000 in 2013 to $4000 in 2015 for older RPH patients. A similar doubling of cost from $414 in 2014 to $928 in 2015 was seen in the BH aged care rehab ward. Two main reasons for prescriptions are for sleep and delirium on chart review at RPH. The cost increase is small relative to the potential savings from harms due to falls or delirium. Older patients at risk of falls or delirium from hypnotics are being considered for melatonin if appropriate and guidelines developed based on literature review.
Discussion. Melatonin modifies risk factors for delirium due to disruption of sleep-wake cycle and circadian rhythm. Supplementation stabilises the levels of melatonin in the older person. Serum melatonin levels decline post-surgery and with opioid use often associated with delirium. There is an increasing trend in the cost utilisation of melatonin which may reduce sleep disorders or delirium thereby avoiding the risk of harms from sedatives/hypnotics. To close the quality cycle for this project a prospective chart audit for 3 months in 2016 found 6 of 11 patients prescribed melatonin had an improvement in sleep. Avoiding use of hypnotics that lead to falls or delirium in older people.

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Al-Aama T et al (2011) Melatonin decreases delirium in elderly patients: randomised, placebo controlled trial, Int J Geriatr Psychiatry.
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Simultaneous quantification of 8 drugs (11 metabolites) commonly taken by older people with polypharmacy

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Introduction. Polypharmacy (use of ≥ 5 drugs) is common in older people but has minimal pre-clinical or clinical evidence of safety or efficacy and is associated with adverse outcomes in older people. An efficient and sensitive method to measure multiple serum drugs and metabolite levels could inform drug dosing in the setting of polypharmacy.
Aims. Establish a method that can measure drugs (and their metabolites) in polypharmacy regimens commonly used by older adults using a mouse model.
Methods. Drug levels were determined using the Agilent 6400 series Triple Quadrupole LC/MS (Agilent Technologies, Santa Clara, U.S.). Mix of drugs in mouse serum were extracted via solid phase extraction (SPE) using Oasis HLB 3cc (60mg) 3mL cartridges (Waters, NSW), followed by liquid chromatography separation using a C18 Poroshell column. The jet stream electrospray was used on the triple quadrupole mass spectrometer and both molecular and product ions were scanned and optimised before proceeding to multiple reaction monitoring (MRM) and Dynamic MRM mode. Both qualitative and quantitative analyses were performed using MassHunter B.07.
Results. In spiked mili-Q water, the lowest limit of detection (LOD) tested for the compounds ranged from 0.049 to 0.78pg on the column. On positive ionization mode, the lowest LOD for majority of the compounds (simvastatin, metoprolol, α-hydroxymetoprolol, O-desmethylmetoprolol, omeprazole, 5-hydroxyomeprazole, omeprazole sulfone, paracetamol, Irbesartan, citalopram, N-desmethylcitalopram, oxybutynin, N-desethyl oxybutynin, oxycodone, noroxycodone and oxymorphone) was ≥ 0.78pg on the column. On negative ionization mode, the LOD tested for paracetamol-sulfate, tenivastatin and paracetamol-glucuronide were 0.098, 0.049 and 0.78pg on the column, respectively. When compounds were spiked in serum, all compounds were detectable at 5ng on the column.
Discussion. We have developed a method that should be sensitive enough to measure 8 drugs (and 11 metabolites) in serum from mice. The limit of detection is sufficient to detect these compounds at therapeutic concentrations in blood and is therefore suitable for testing in mice that have been chronically administered polypharmacy. The assay may ultimately be applicable to routine assessment to optimise drug dosing in the clinic.

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Impact of polypharmacy and Drug Burden Index (DBI) on physical function in middle aged mice

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Introduction. Approximately half of middle-aged and two thirds of older Australians polypharmacy (≥ 5 drugs). Increasing DBI (measure of patient's total exposure to anticholinergics and sedatives) is associated with impaired physical function in observational studies of older people. The effects of polypharmacy and DBI on functional outcomes in middle age are unclear and cannot ethically be evaluated in clinical interventional studies.
Aims. Determine the effect of polypharmacy and increasing DBI on functional outcomes in middle aged mice.
Methods. Male C57BL/6 mice were treated with standard diet until aged ~12 months. This was followed by ~3 months of control or treated feed/water containing therapeutic doses of five drugs with Zero DBI (simvastatin, metoprolol, omeprazole, paracetamol, irbesartan), Low DBI (simvastatin, metoprolol, omeprazole, paracetamol, citalopram), or High DBI (simvastatin, metoprolol, oxybutynin, oxycodone, citalopram), or single drug (simvastatin, metoprolol, oxybutynin, oxycodone or citalopram) (n=5-6/ group). A panel of functional tests was conducted at ages 12 months (baseline) and 15 months (after treatment).
Results. Animals were administered therapeutic doses of drugs for 3 months, which were well tolerated, with non-significant reductions in mean systolic blood pressure by 10 mmHg in the zero DBI group and 5 mmHg in the low DBI group ($p>0.05$). After 3 months of drug regimens, there was a non-significant trend towards decreasing locomotor activity (open field distance, time mobile, rearing and centre exploration entries) in those administered polypharmacy with low and high DBI and in those administered citalopram alone. Drug regimens did not affect grip strength (wire hang), muscle endurance (rotor rod latency) or frailty (mouse clinical frailty index). Compared to control, chronic oxybutynin treatment increased rotor rod endurance ($p<0.05$).
Discussion. Our preliminary data suggest polypharmacy with increasing DBI administered to mice at middle age may impair locomotor activity, which may be driven by citalopram intake. Oxybutynin improved muscle endurance. These findings differ to those seen with shorter exposures in young adult mice, highlighting the importance of pre-clinical evaluation relevant to the intended clinical exposure period and population.

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HPLCMS/MS method for simultaneous determination of ten antibiotics (cefazolin, cefepime, cefotaxime, ceftazidime, ciprofloxacin, flucloxacillin, linezolid, meropenem, piperacillin and tazobactam)

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Introduction: Antibiotics have been widely used in the treatment of infection but under-dosing can lead to lack of efficacy and/or antimicrobial resistance. Monitoring their concentration is thus important in preventing resistance but also in maximizing the effectiveness of the treatment while minimizing toxicity hence, the need to develop a sensitive, fast and reliable method for their measurement.
Aims: To develop and validate a method for simultaneous determination of ten antibiotics some of which, in particular meropenem and ceftazidime, exhibit profound ionisation suppression under certain conditions.
Methods: Sample preparation consisted of protein precipitation with acetonitrile containing corresponding SIL-IS (stable isotope labeled internal standard) for each analyte. Analysis was performed on LCMS/MS operated in positive electrospray ionisation mode with chromatographic separation achieved on a C_{18} column with a water, acetonitrile and formic acid as the mobile phase. Calibration curves were constructed and QC data generated daily (n=30). Accuracy, precision and recovery were estimated for each analyte. Ionisation suppression/enhancement from both endogenous and exogenous sources was evaluated by comparing calibrators prepared identically in both pure solution and plasma. Ionisation suppression was also assessed by post extraction addition. Interference from the analyte to the IS and vice versa (*cross talk*) was assessed by injecting the highest concentration of the calibrator and of the blank plasma spiked only with the IS. Responses were monitored at the IS and analyte transitions respectively. Stability of the samples was evaluated in plasma and whole blood by storing the samples at various temperature conditions (room temperature, 4, -20 and -70°C) for up to 8 months.
Results: Coefficients of variation for all analytes were <10% at each of three concentrations except ceftazidime (14% at the lower limit of quantitation). No ionisation suppression/enhancement was observed for any of the analytes. No matrix effect was observed suggesting that the use of a SIL-IS had corrected for any changes affecting the analytes. Cross talk from analyte to IS and vice versa was not observed. Tazobactam, meropenem, piperacillin and cefepime were each more stable in whole blood than plasma. Cefotaxime, cefazolin and linezolid were stable in both matrices whereas ciprofloxacin was more stable in plasma. Long term storage in plasma matrix at -70°C was acceptable for all analytes for at least eight months.
Discussion: This assay provides sensitive, specific, reproducible, accurate and precise measurement of ten antibiotics over a wide analytical range. Ion suppression/enhancement from either endogenous or exogenous sources did not impact the performance. There was no 'cross talk' observed either from analyte to the IS or vice versa. The assay is therefore suitable for both routine use and pharmacokinetic studies.

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Impact of Educational Intervention on Surgical Prophylaxis Practices to Contain Antimicrobial Resistance

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Introduction: Irrational use of antimicrobials is common. It imposes financial burden on institutions and causes emergence of antimicrobial resistance. In the present study, surgical antimicrobial prophylaxis for elective clean uncomplicated surgeries was assessed to develop an educational learning module to orient surgeons. The module was based on American Society of Health-System Pharmacists -Clinical Practice Guidelines for Antimicrobial Prophylaxis in Surgery. The module focused on common principles, timing and duration of prophylaxis. Post-education adherence to guidelines was also assessed.

Aims:

1. To assess the surgical prophylaxis practices in a specific group of surgeries (elective clean uncomplicated surgeries).
2. To orient the surgical team regarding surgical prophylaxis and its implication by developing an educational learning module as part of antimicrobial stewardship program.
3. To assess post educational compliance by surgeons.

Methods: This was an interventional, before and after study wherein educational intervention was carried out at the local level in the Department of Surgery, Government Medical College Amritsar a 1000 bedded tertiary care health institution Consecutive patients which fitted the inclusion and exclusion criteria. Pre-existing data from case files of 75 adult patients before and 75 after intervention admitted for surgery as per inclusion and exclusion criteria was collected. Data was tabulated using Microsoft Office Excel 2010 and were later compiled to evaluate the results. Type of intervention was Educational intervention of surgeons and ancillary staff by conducting a workshop using MS-PowerPoint slides. Institutional Ethics Committee (IEC)'s approval was obtained prior to commencement of the study.

Result: Pre-interventional data revealed that antimicrobials were continued beyond 24 hours in 61% patients, though justified in only 8% patients due to prolonged surgery and post-operative pyrexia, signifying irrational use. Post-interventional data showed adherence to educational module resulting in 75% improvement in post-operative antimicrobial utilization in patients without altered incidence of surgical site infections.

Discussion: Educational intervention and training of surgeons and ancillary staff has a vital role in promoting rational use of antimicrobials and keeping the emergence of antimicrobial resistance in check. Antimicrobials are unique drugs as they lose efficacy over time and they must be used sparingly. An institutional multidisciplinary committee for guidelines development & implementation was proposed as a result of the study.

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Simultaneous determination of dabigatran, rivaroxaban and apixaban in human plasma by liquid chromatography/tandem mass spectrometry

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Introduction. Dabigatran, rivaroxaban and apixaban are new oral anticoagulant alternatives to warfarin, for indications such as atrial fibrillation and venous thromboembolism. We are investigating these new anticoagulants in relation to plasma concentrations, anticoagulation intensity and clinical outcomes. These studies require an analytical method to accurately measure the plasma concentrations of dabigatran, rivaroxaban and apixaban.

Aims. The objective of this work was to develop and validate a rapid LC-MS/MS method for the simultaneous determination of dabigatran, rivaroxaban and apixaban in human plasma using a modification of *our previously published* LC-MS/MS method [Saffian et al, 2015].

Methods. Plasma samples were pretreated with acetonitrile (sample:acetonitrile = 1:4) to precipitate the proteins. Dabigatran, rivaroxaban, apixaban and their corresponding isotopically labelled internal standards were then resolved on a C18(2) column using gradient elution of 0.05% formic acid and methanol. The six compounds were detected using electrospray ionisation in the positive mode.

Results and Discussion. For these three anticoagulants, standard curves were linear over the concentration range 1.0 to 1000 µg/L (r > 0.99) in plasma, biases were <±10%, and intra- and inter-day coefficients of variation (imprecision) were <10%. The limit of quantification was 1.0 µg/L in plasma for the three anticoagulants. The assay has been successfully applied to patient samples.

Saffian et al (2015) Bioanalysis 7:957-966

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The Pharmacokinetics of Fentanyl and its Derivatives in Children – a Comprehensive Review

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Introduction. Fentanyl and its newer derivatives sufentanil, alfentanil and remifentanil are strong opioid analgesics frequently used in pediatric patients. Despite this extensive use, there is insufficient information on PK of these drugs in neonates, infants, children and adolescents.
Aims. To perform a thorough review of the PK of fentanyl and its derivatives in children of all age groups.
Methods. PubMed was searched using specific terms relating to the pharmacology of fentanyl and its derivatives in children of all age groups. Original articles regarding the PK, PD, efficacy and safety and reviews were included. Individual PK data was reanalysed for subgroups. Linear regressions to age and weight were performed.
Results. Of the retrieved 372 articles, clinical studies were the most common type of articles, followed by case series, case and short reports, and reviews. Forty four eligible PK studies contained data of 821 pediatric patients, including more than 46 preterm infants, 64 neonates, 115 infants and toddlers, 188 children, and 28 adolescents. Special populations comprised preterm infants, children with chronic renal or liver disease, undergoing extracorporeal circulation, or with obesity. There was only a weak correlation between body weight (BW) and both clearance (CL) and volume of distribution (V) of fentanyl (r^2=0.22 and r^2=0.43, p=0.0054 and p<0.0001) in preterm infants, neonates and young infants. Sufentanil CL correlated stronger with BW (r^2=0.67, p<0.0001) than with age (r^2=0.62, p<0.0001). Alfentanil CL showed a strong correlation with both age and BW (r^2=0.71 vs. 0.72, both p<0.0001). There was an identical correlation with both age and BW for Sufentanil V (both r^2=0.81, p<0.0001) and Alfentanil V (both r^2=0.59, both p<0.0001). While remifentanil CL correlated equally strong with age and BW (r^2=0.73 vs. 0.69, both p<0.0001), BW had a greater impact on the V than age (r^2=0.73, vs. 0.63, both p<0.0001).
Discussion. Fentanyl and its derivatives show a good safety profile in children. Sample sizes were small and there remain gaps regarding PK in special populations. Future studies should be designed in such a way that they assess the PK and PD of fentanyl and its derivatives in all pediatric subpopulations.

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London care home medication administration errors: multi-compartment compliance aids and original packaging

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Introduction. No published study has been specifically designed to compare care home (CH) medication administration errors (MAEs) between original packaging (OP) and multi-compartment compliance aids (MCAs) using direct observation.
Aims. Compare the effect of OP and MCAs on the accuracy of medication administration in London CHs.
Methods. Between October 2014 and June 2015, a pharmacist researcher observed solid, orally administered tablet/capsule medications (required sample size 2,246 doses) at ten purposively sampled CHs (eight 'nursing', two 'residential') located around Greater London. The MAE rate was calculated as the number of observed doses administered (or omitted) in error according to information on medication administration records, compared to the opportunities for error (total number of observed doses plus omitted doses).
Results. Over 108.4 hours (30 days), 42 staff (36 nurses and 6 carers) were observed to administer medications to 823 residents during 88 medication administration rounds. Overall, each round lasted a median of 65.0 minutes (range: 8-245 minutes). A total of 2,452 medication doses were observed - 1,067 from MCAs and 1,385 from OP. One hundred and seventy eight MAEs were identified from 2,493 opportunities for error (7.1% MAE rate). A statistically significant difference was identified in MAE rates between OP and MCAs (9.3% and 3.1% respectively, p<0.01) using Pearson's Chi-squared test. There was no statistically significant difference in MAE rates between medications administered from 'nursing' and 'residential' CHs (6.7% and 9.1% respectively p>0.05). Of the 178 doses observed to be administered (or omitted) in error, the top three most common MAE types included timing inaccuracy, omission and wrong dose.
Discussion. Benefits of MCAs were clear in terms of MAE rates. Further research examining factors contributing to the significant MAE rate difference between OP and MCAs would be of benefit.

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Changes in cardiovascular medication use in long-term care facilities in Finland.

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Introduction. Polypharmacy is highly prevalent and burdensome to residents and staff. Multiple medications are often recommended for the management of cardiovascular conditions. Current clinical practice guidelines provide limited guidance for older people.
Aims. The aim of this study was to investigate the change in cardiovascular medication use over an eight-year period in nursing home and assisted living facilities in Helsinki, Finland.
Methods. Data from three cross-sectional studies of residents aged ≥65 years in nursing homes in 2003 (n=1987) and 2011 (n=1576) and in assisted living facilities in 2007 (n=1377) and 2011 (n=1586) in Finland were combined. The prevalence of cardiovascular medication use across time periods were compared. Polypharmacy was defined as the use of nine or more regular medications.
Results. Polypharmacy increased in assisted living facilities (44.7% to 50.6%, p<0.001) but decreased in nursing homes (40.4% to 32.4%, p<0.001). The prevalence of cardiovascular medications decreased in nursing homes (67.0% to 52.9%, p<0.001) and assisted living facilities (72.5% to 66.8%, p<0.001). The prevalence of diuretics, nitrates and digoxin decreased, but the prevalence of statins increased in both settings. The prevalence of antithrombotics decreased in both nursing homes (55.4% to 49.0%, p<0.001) and assisted living facilities (62.2% to 55.8%, p<0.001).
Discussion. The prevalence of polypharmacy significantly decreased in nursing homes but has increased in assisted living facilities. Significant reductions in the use of cardiovascular and antithrombotics suggest improved assessment of the risk-to-benefit ratio of these medications in the long-term care setting.

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The impact of frailty on the association between polypharmacy and mortality in residential aged care facilities

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Introduction: Frailty and polypharmacy are highly prevalent in residential aged care facilities (RACFs). Both frailty and polypharmacy have been individually associated with greater susceptibility to adverse events.
Aims: To investigate the impact of frailty on the association between polypharmacy and 12-month mortality in RACFs.
Methods: We conducted secondary analyses of data for 383 residents of six RACFs in South Australia. Polypharmacy was defined as the use of nine or more regular medications. Frailty status was screened using the FRAIL-NH scale. Data on 12-month mortality were obtained from RACF records. Cox proportional hazards models were used to compute adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for the association between polypharmacy and mortality stratified by frailty status. Residents who were not-frail were not included in the multivariate analyses due to the small number of deaths.
Results: In total, 263 (63.4%) residents were exposed to polypharmacy. Overall, 101 (26.6%), 142 (37.5%) and 136 (35.9%) residents were not-frail, frail and most-frail, respectively. There were 11, 35 and 38 deaths among residents who were screened as not-frail, frail and most-frail, respectively. After adjusting for gender, age, comorbidity, dementia and activities of daily living, polypharmacy was not associated with mortality among residents who were frail (HR 1.05, 95%CI 0.50-2.20) or most-frail (HR 0.72, 95%CI 0.36-1.42).
Discussion: Polypharmacy was not associated with mortality in residents who were frail or most frail. Further studies with larger sample sizes are needed to confirm these findings.

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Socioeconomic position and nonadherence to statin therapy under universal coverage

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Introduction. Previous research suggests that low socioeconomic position (SEP), especially low income, is associated with nonadherence to statin therapy, and that there may be gender-differences in this relationship.
Aims. Our aim was to determine the relationship between SEP and statin adherence in Finland, a country with universal healthcare and drug reimbursement systems.
Methods. Using data from administrative healthcare registers, we identified 116 846 individuals, aged 45–75 years, who initiated statin therapy for primary prevention of cardiovascular disease in 2001–2004. We measured adherence as proportion of days covered (PDC) over an 18-month period since initiation and identified adherence patterns based on monthly PDC with group-based trajectory modelling (GBTM). We estimated the associations between SEP and adherence using logistic regression and adjusted the odds ratios (OR) for age, marital status, region, clinical characteristics, and copayment.
Results. Low SEP predicted statin nonadherence (PDC <80%) among men (e.g., lowest vs. highest income quintile: OR 1.41, 95% confidence interval [CI] 1.32–1.50; basic vs. high education: OR 1.18, 95% CI 1.13–1.24; unemployment vs. employment: OR 1.17, 95% CI 1.10–1.25). Among women, the corresponding associations were mainly non-significant and different from those observed among men (P <0.001 for gender*income quintile, gender*education level, and gender*labor market status interactions). Based on the results from GBTM, men with SEP were likely to belong to trajectories presenting a rapid decline in adherence.
Discussion. Low SEP predicted overall and rapidly increasing statin nonadherence among men. In women, however, the associations between SEP and nonadherence were weak and inconsistent. GBTM provided additional insight into the dynamics of adherence to statin therapy.

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Negative outcomes associated with accidental and intentional extramedical use of prescription opioids in Australia: a scoping review

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Introduction. Evidence is accumulating about the negative outcomes associated with extramedical use of prescription opioids (PO). In Australia, the type and extent of these outcomes are less clear than in United States or Canada.
Aims. To undertake a scoping review of the Australian literature on the negative outcomes associated with accidental and intentional extramedical use of PO.
Methods. We searched MEDLINE, EMBASE, PsychINFO, Cumulative Index to Nursing and Allied Health Literature and the Cochrane library for original studies published between January 2000 and June 2016. Studies were eligible for inclusion if: (1) PO use was explicitly reported, (2) extramedical use was evident (3) harm was explicitly reported, (4) data were collected during or after the year 2000, and (5) the study was conducted in Australia. Studies investigating over-the-counter opioids and opioid substitution therapy were excluded.
Results. We identified 381 original articles, 14 of which met the inclusion criteria. Study designs included cohort studies (n=2), surveys (n=5) and retrospective audits of coronial data (n=7). Extramedical use included deliberate overdose, use without a prescription, PO use from an unknown source, PO illicitly obtained, injection of PO. Seven studies reported death from PO use, with drug toxicity being the most common cause. Concomitant use of PO and other psychoactive drugs was reported in 13 studies. Eleven studies reported overdose, three studies reported injection-related injuries or diseases, one study reported engagement in property and violent crime, two studies reported health care service utilisation and one study reported loss of employment.
Discussion. Extramedical use of PO in Australia is associated with a broad range of negative outcomes, including harms such as overdose, injection related injuries or diseases and death, as well as engagement in crime, loss of employment and health care service utilisation. Research published to date does not provide direct data on the incidence of these outcomes in relation to prescribing of PO for legitimate clinical indications.

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Mitigating risk and improving processes of care related to polypharmacy in residential aged care services

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Introduction. The process of prescribing and administering medicines is becoming increasingly complex. The quality use of medicines (QUM) is one of four central objectives of Australia's National Medicines Policy. Our research on behalf of the Department of Health and Human Services (DHHS) has identified and prioritised 16 possible strategies to address polypharmacy. Three of those strategies involve optimising processes of care.
Aims. To trial the implementation of three strategies to address polypharmacy in 27 Public Sector Residential Aged Care Services (PSRACS) in Western Victoria in 2016-2017, with a view to informing state-wide adoption.
Methods. Strategy 1: Implementation of three new medicines-related sub-indicators (1: Proton pump inhibitor use, 2: Antipsychotic use and 3: Number of daily administration times). Sub-indicator data will be collected and reported quarterly using the electronic template already in use for the existing PSRACS quality indicator program.
Strategy 2: Development and implementation of 'deprescribing scripts' to facilitate deprescribing. A series of sample dialogs will be developed. These will be pilot tested for face validity among residents and clinicians in up to 10 PSRACS, through semi-structured interviews and focus groups. Feedback will be incorporated into the final scripts, which will then be implemented across the 27 PSRACS with a multi-faceted education strategy using the RACS online learning platform and face-to-face approach.
Strategy 3: Optimising the role of local and regional medication advisory committees (MACs). Qualitative semi-structured focus groups and interviews will be held with each of the four regional MACs and key stakeholders in each health service. Based on the data, a series of expert recommendations will be developed relating to what is currently working well, what could be improved and strategies to better support MACs to address medicines appropriateness and reduce unnecessary polypharmacy.

Jokanovic N, Wang K et al (in press). Prioritizing interventions to manage polypharmacy in Australian aged care facilities. Research in Social and Administrative Pharmacy.

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Are medications associated with fall-related hospital admissions from residential aged care facilities? A case-control study

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Introduction. Falls are a leading cause of preventable hospitalizations from residential aged care facilities (RACFs). Polypharmacy and falls-risk medications are potentially modifiable risk factors for falling.
Aim**.** To investigate whether polypharmacy and falls-risk medications are associated with fall-related hospital admissions from RACFs.
Methods. This was a matched hospital-based case-control study of patients aged ≥65 years hospitalized from RACFs between 2013 and 2015. Polypharmacy was defined as use of ≥nine regular pre-admission medications. Falls-risk medications included psychotropic medications and those that can cause orthostatic hypotension. Conditional logistic regression was used to calculate adjusted odds ratios (OR) and 95% confidence intervals (CI) for the associations between polypharmacy and falls-risk medications with fall-related hospital admissions.
Results. There was no association between polypharmacy and fall-related hospital admissions (adjusted OR=0.97; 96%CI=0.63-1.48). However, the adjusted odds of fall-related hospital admissions increased by 16% (95%CI=3%-30%) for each additional falls-risk medication. In sub-analyses, medications that can cause orthostatic hypotension (adjusted OR=1.25; 95%CI=1.06-1.46) but not psychotropic falls-risk medications (adjusted OR=1.02; 95%CI=0.88-1.18) were associated with fall-related hospital admissions. The association between medications that can cause orthostatic hypotension and fall-related hospital admissions was strongest among residents with polypharmacy (adjusted OR=1.44; 95%CI=1.08-1.92).
Discussion. Polypharmacy was not an independent risk factor for fall-related hospital admissions. However, medications that can cause orthostatic hypotension were associated with fall-related hospital admissions, particularly among residents with polypharmacy. The risk of falls should be considered among older residents in RACFs prior to prescription of medications that can cause orthostatic hypotension.

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Polypharmacy and medication regimen complexity as risk factors for hospitalisation among residents of aged care facilities: a prospective cohort study

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Introduction. Polypharmacy and complex medication regimens are highly prevalent in residential aged care facilities (RACFs). Understanding whether polypharmacy or medication regimen complexity are related to hospitalisations from RACFs is important because medication exposure may be potentially modifiable.
Aims. To investigate the relationship between polypharmacy and medication regimen complexity with time to first hospitalisation, number of hospitalisations and number of hospital days among residents of aged care facilities.
Methods. A 12-month prospective cohort study including residents of six South Australian RACFs was conducted. Cox regression and Poisson regression models were used to determine the association between polypharmacy (≥9 regular medications) or Medication Regimen Complexity Index (MRCI) score and each hospitalisation outcome, with adjustment for age, sex, length of stay in RACF, comorbidities, activities of daily living and dementia severity.
Results. Of the 383 residents included, 63% took ≥9 regular medications and the median MRCI score was 43.5 (IQR 33-56). There were 0.56 (95%CI 0.49-0.65) hospitalisations per person-year and 4.52 (95%CI 4.31-4.76) hospital days per person-year. In the adjusted analyses, polypharmacy was associated with time to first hospitalisation (Hazard Ratio (HR) 1.84; 95%CI 1.21-2.79) number of hospitalisations (Incident Rate Ratio (IRR) 1.51; 95%CI 1.09-2.10) and hospital days per person-year (IRR 1.39; 95%CI 1.24-1.56). Similarly, adjusted analyses showed a 10-unit increase in MRCI was associated with time to first hospitalisation (HR 1.17; 95% CI 1.06-1.29), number of hospitalisations (IRR 1.15; 95%CI 1.06-1.24) and hospital days per person-year (IRR 1.19; 95% CI 1.16-1.23).
Discussion. Polypharmacy and medication regimen complexity are associated with hospitalisations from RACFs. It is important for clinicians to be alert to the risk of hospitalisation among residents with polypharmacy or complex medication regimens, and consider whether interventions such as ongoing medication reviews may be beneficial.

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SImplification of Medications Prescribed to Long tErm care Residents (SIMPLER): protocol for a cluster randomised controlled trial (RCT)

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Introduction. Complex medication regimens are highly prevalent in residential aged care facilities (RACFs). Strategies to reduce unnecessary complexity are likely to be important for residents and aged care providers because complex medication regimens can be burdensome for residents and are costly in terms of nursing time.
Aims. To investigate application of a structured process to simplify medication administration for residents.
Methods. SIMPLER is a non-blinded, matched-pair, cluster RCT of a multidisciplinary intervention to simplify medication regimens using a structured decision aid. Trained study nurses will recruit permanent residents aged ≥65 years from up to 8 South Australian RACFs. Medications taken by residents in the intervention arm will be assessed to identify opportunities to reduce the number of administration times (e.g. by administering medications at the same time, or through the use of longer-acting or combination formulations) or routes (e.g. administering orally where possible). Residents in the control group will receive routine care. Participants will be followed for up to 36 months after study entry. The primary outcome will be the total number of charted medication administration times at 3 and 6 months post-intervention. Secondary outcomes will include time spent administering medications, medication incidents, resident satisfaction, quality of life, falls, hospitalisation and mortality. Individual-level analyses that account for clustering will be undertaken to determine the impact of the intervention on the study outcomes.
Results. Recruitment will commence in early 2017. Findings will be disseminated to consumers, carers, clinicians, researchers, aged care providers and policy makers through the NHMRC Cognitive Decline Partnership Centre. Study results will also be disseminated through conference presentations and publications in peer reviewed journals.
Discussion. SIMPLER will enable an improved understanding of the burden of medication administration in RACFs and quantify the impact of regimen simplification on a range of outcomes important to residents and care providers.

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How often are medicines charted for pro re nata (PRN) use administered in aged care facilities?

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Introduction. Previous research suggests that, on average, people living in residential aged care facilities (RACFs) are charted 2.5 pro re nata (PRN) medicines (Dörks et al, 2016; Stokes et al, 2004). The decision to administer PRN medicines is often made by nursing staff. There are few previous studies which report how often PRN medicines are actually administered.
Aims. To determine how often medicines charted for PRN use are actually administered in RACFs.
Methods. We conducted secondary analyses of data for 383 residents from six South Australian RACFs. Data from PRN medication charts were compared to medication administration records for the previous seven days. Medicines were coded using the World Health Organization Anatomical Therapeutic Chemical Classification System.
Results. Of the 383 residents included, most were female (77.5%, n=297), and the median age was 88 years (interquartile range (IQR) 84–92). Overall, 94% (n=360) were charted ≥1 PRN medicine, of whom 27% (n=96) were administered ≥1 PRN medicine in the previous seven days. The most prevalent charted PRN medicines were nervous system medicines (77%, n=294 residents), alimentary tract and metabolism medicines (69%, n=266 residents) and dermatologicals (30%, n=115 residents). Of the 96 residents who were administered ≥1 PRN medicine, the majority received alimentary tract and metabolism medicines (65%, n=62 residents) and/or nervous system medicines (63%, n=60 residents). Residents without any charted PRN medicines (n=23) had a median length of stay of 22.8 months (IQR 15.4–32.6). Residents with five or more charted PRN medicines (n=142) had a median length of stay of 28.9 months (IQR 15.7–45.1).
Discussion. Our study found that although most residents are charted PRN medicines, rates of administration of PRN medicines are relatively low. These results suggest the contribution of PRN medicines to medicine burden in RACFs may be lower than previously thought.

Dörks M et al (2016) Eur J Clin Pharmacol 72:995-1001
Stokes JA et al (2004) Pharm World Sci 26:148-54

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The anti-inflammatory activity of Australian Eucalyptus spp. (myrtaceae) with Aboriginal ethnopharmacological significance

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Introduction. Chronic inflammation is a major driving force for the progression of a number of diseases including asthma, rheumatoid arthritis, inflammatory bowel disease, cancer, atherosclerosis, diabetes, obesity and Alzheimer's disease. The existing non-steroidal anti-inflammatory drugs can cause severe side effects, most importantly stomach ulcers, and has limited effectiveness in decreasing pro-inflammatory cytokine production. Therefore, the discovery of novel and safe anti-inflammatory compounds is in urgent demand. Numerous plant species utilized in Australian Aboriginal medicine are reputed to possess anti-inflammatory activities but their pharmacological potential has not been explored.
Aims. Isolation and identification of novel anti-inflammatory molecules from Australian native plants.
Methods. In our investigation to identify novel anti-inflammatory compounds, thirty plants known to be used by Australian Aboriginal Dharawal people to treat inflammation were selected by consulting the Dharawal Botanist and Aboriginal Elder Frances Bodkin and her pharmacopeia. Fresh leaves of 17 *Eucalyptus* spp. were collected from the Australian Botanic Gardens, Mount Annan, and extracted with ethanol. Dried extracts were evaluated for anti-inflammatory activity *via* the suppression of nitric oxide (NO) and TNF-α production induced by lipopolysaccharide and interferon gamma (IFN-γ) in RAW 264.7 cells. Cytotoxicity of the crude extracts was also examined by an Alamar blue cell viability assay.
Results. We found that 7 of the investigated *Eucalyptus* spp. (*Eucalyptus benthamii, E. bosistoana, E. botryoides, E. saligna, E. smithii, E. umbra and E. viminalis*) demonstrated strong anti-inflammatory activity (IC_{50} (NO) < 20 µg/ ml). As the *E. bosistoana* ethanol extract was very potent (IC_{50} (NO) = 7.58 µg/ml), it was selected for sequential extraction and bioassay guided HPLC fractionation. Seven fractions were collected, and – as the first compound - 4-coumaroylquinic acid was identified from one of the fractions by LRMS, HRMS, 1D and 2D NMR and has showed an IC_{50} (NO) of 100.27 µg/ml.
Discussion. The present study suggests that most of the *Eucalyptus* spp. potentially possess interesting anti-inflammatory compounds with low toxicity and the *in vitro* activity appears to support the traditional use. The isolation and identification of key anti-inflammatory compounds is underway.

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Assessment of TRPV4-mediated calcium influx induced by receptor activation in MDA-MB-468 breast cancer cells

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Introduction. The calcium permeable ion channel TRPV4 is involved in breast cancer cell invasion, migration and metastasis and appears to be associated with poorer patient outcomes. TRPV4 transactivation by G-protein coupled receptors and receptor tyrosine kinases, have been identified as important mechanisms of regulation in other tissues and pathologies. Characterisation of these transactivation pathways present an opportunity to better understand the nature of TRPV4 signalling in breast cancer cells.
Aims. To assess the sensitivity of Ca^{2+} influx elucidated by ATP, trypsin and epidermal growth factor (EGF) to the pharmacological TRPV4 inhibitor HC067047 and the Gαq inhibitor UBO-QIC in MDA-MB-468 breast cancer cells.
Methods. MDA-MB-468 cells were plated at 6 x 10^3 cells per well into 384 well black plates. For the measurement of free cytosolic Ca^{2+}, cells were loaded with 2 µM PBX no-wash calcium indicator dye for 60 min at 37°C, and were subsequently treated with TRPV4 and/or Gαq inhibitors for 15 min at room temperature. Intracellular Ca^{2+} levels associated with ATP, trypsin and epidermal growth factor (EGF) were then assessed using a Fluorescence Imaging Plate Reader (FLIPR).
Results. Ca^{2+} influx induced by the purinergic receptor activator ATP was unaffected by TRPV4 pharmacological inhibition. However, TRPV4 inhibition attenuated Ca^{2+} influx associated with trypsin-mediated activation of protease activated receptor 2 (PAR2) and EGF. Calcium influx induced by trypsin was UBO-QIC sensitive; in the presence of the Gαq inhibitor, TRPV4 inhibition had no effect on Ca^{2+} influx.
Discussion. These experiments suggest that PAR2 activation promotes Ca^{2+} influx in MDA-MB-468 breast cancer cells through a Gαq-dependent mechanism. Further investigation using siRNA silencing of TRPV4 and further assessment of the mechanistic components responsible for TRPV4 activation in breast cancer cells is now required.

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Molecular mechanisms of action of M5 muscarinic acetylcholine receptor allosteric modulators

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Recently, the first putative subtype-selective allosteric modulators of the M_5 muscarinic acetylcholine receptor (mAChR) have been described, but their molecular mechanisms of action at rat and human receptors have not been fully elucidated. Using radioligand binding and IP accumulation in recombinant (CHO) cell lines stably expressing either rat or human mAChRs, we investigated the effects of the positive allosteric modulator (PAM), ML380, and negative allosteric modulator (NAM), ML375.
In functional assays of the human M_5 receptor, ML380 caused robust enhancements in the potency of the full agonists, acetylcholine (ACh; αβ=9.1), carbachol (CCh; αβ=12.3) and oxotremorine-M (Oxo-M; αβ=6.5), while significantly increasing the maximal response to the partial agonist, pilocarpine (αβ=60). ML380 also demonstrated direct allosteric agonist activity. In contrast, ML375, displayed negative cooperativity with each of the agonists (αβ=0.001), and progressively reduced the maximal pilocarpine response. Through the use of the alkylating agent, phenoxybenzamine (PBZ), to reduce receptor reserve it was possible to identify allosteric effects on both orthosteric agonist affinity and efficacy. Addition of ML375 to PBZ-treated cells inhibited the maximal ACh response. In contrast, identical experiments performed with ML380 or the M_1 mAChR prototypical PAM, BQZ12, revealed only enhancement in ACh potency with no effect on maximal response.
Interaction studies with ML375 or ML380 in [^{3}H]-NMS binding assays showed some differences between the selectivity profile of these ligands for rat and human mAChRs. At both rat and human mAChRs, ML375 was highly selective for the M_5 mAChR over other subtypes, while ML380 not only showed PAM activity at the human M_5 mAChR but also acted as a PAM at human M_1 and M_3 mAChRs. However, the PAM activity of ML380 at rat M_1 and M_3 mAChRs was reduced as compared to their respective human orthologues.
Our findings indicate that novel small molecule modulators of the M_5 mAChR display different molecular mechanisms of action compared to previously characterized modulators of other mAChRs. Moreover, these ligands demonstrate contrasting selectivity profiles at rat and human mAChRs. Moving forward, ML375 lends itself as useful tool compound to probe M_5 mAChR activity in CNS disorders.

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Apocynin Attenuates LPS-Induced Mesangial Cell Damage by Suppressing MCP-1 Expression through the Inhibition of MAPKs

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Introduction Renal failure is observed in the pathologic progression of sepsis and septic shock. Renal mesangial cells (RMC) have been reported to be implicated in the renal failure by producing mediators such as monocyte chemoattractant protein-1 (MCP-1) and TNFα in response to lipopolysaccharide (LPS). Mitogen-activated protein kinases (MAPKs) have been demonstrated to mediate the LPS-induced inflammatory response in RMC.
Aims Although previous studies suggested a promising effect of apocynin in various inflammatory conditions, its anti-septic efficacy in RMC has not been clearly determined. In the present study, the anti-inflammatory effects of apocynin and its possible mechanism were examined in LPS-challenged RMC.
Methods The production of NO was estimated by measuring the amount of nitrite, a stable metabolite of NO, using he Griess assay. The proteins were measured with the specific antibodies against iNOS, COX-2, and MAP kinases. Statistical significance was analyzed by one way ANOVA test. All data were expressed as mean± SD. P-values < 0.05 (*) and < 0.01 (**) were considered as statistically significant.
Results Apocynin significantly inhibited NO production in LPS-challenged RMC and the expression of iNOS and COX-2 in a concentration-dependent manner. LPS-induced MCP-1 expression was significantly attenuated with apocynin. Moreover, apocynin significantly suppressed the activation of MAPKs such as Erk and p38 in mesangial cells. However, there was no significant difference on phosphorylation of JNK in a concentration-dependent manner. We also observed the significant increase in HO-1 expression via increase in activity of Nrf-2.
Discussion The present data demonstrated that apocynin exerts anti-inflammatory activity through the suppression of pro-inflammatory mediators such as NO and MCP-1 presumably through the inhibition of MAPKs signaling pathway, suggesting a therapeutic potential of apocynin in inflammation-related renal disorders. Apocynin enhances the transcriptional activity of Nrf-2, resulting in the increased expression of cytoprotective enzyme including HO-1 in mesangial cells. However, further studies are needed to clarify the exact mechanism by which apocynin exerts its anti-inflammatory effects in RMC cells.

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An assessment of the effects of doxorubicin on calcium signalling in MDA-MB-231 breast cancer cells

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Introduction. Triple-negative breast cancer is often associated with distant relapses despite successful initial therapy. An understanding of key signalling pathways modified by chemotherapy may provide novel insights into combination treatment strategies to enhance treatment efficacy. Altered Ca^{2+} signalling has recently been suggested to influence treatment efficacy in hepatocellular cancer but has not been comprehensively assessed in triple-negative breast cancer.
Aims. To assess the effects of doxorubicin on Ca^{2+} signalling in the MDA-MB-231 triple-negative breast cancer cell line and mRNA expression of calcium channels, pumps and sensors associated with these Ca^{2+} signalling changes.
Methods. Live cell imaging and cell confluency measurements were used to obtain a concentration-response curve for doxorubicin. For assessment of changes in intracellular Ca^{2+} signalling, MDA-MB-231 cells expressing the GCaMP6m genetically-encoded Ca^{2+} indicator were treated with doxorubicin (0, 0.03 and 1 µM) for 24 h and imaged using an automated epifluorescence microscope (ImageXpress Micro). Real time RT-PCR was used to investigate changes in mRNA levels of selected Ca^{2+} channels, pumps and sensors as a result of doxorubicin treatment.
Results. Doxorubicin (1 µM) treatment resulted in delayed recovery of cytosolic Ca^{2+} during store-operated Ca^{2+} entry (SOCE), without major effects on cyclopiazonic acid-induced endoplasmic reticulum Ca^{2+} store release. A higher proportion of cells also produced Ca^{2+} transients in response to 1 µM ATP addition when treated with doxorubicin (1 µM) compared to control treatment.
Discussion. Doxorubicin (1 µM) appears to be associated with a change in SOCE in MDA-MB-231 cells and an enhancement of sensitivity to ATP stimulation. Further investigation is required to determine the functional significance associated with these alterations in Ca^{2+} signalling phenotypes.

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Defining the putative binding pocket of the orphan G protein-coupled receptor, GPR37L1

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Introduction. GPR37L1 is an orphan G protein-coupled receptor (GPCR), proposed to play a role in regulating blood pressure (Min et al, 2010). Previously we identified the first surrogate ligands for GPR37L1 (Ngo et al, in press) but those ligands displayed low potency, so higher affinity ligands are sought.
Aims. To investigate the role of putative GPR37L1 binding pocket residues, including E210, Y313, E375, N376, N379, Q402 and K408, in ligand interactions by site-directed mutagenesis.
Methods. GPR37L1 was C-terminally tagged with enhanced yellow fluorescent protein (eYFP). Residues were individually mutated to alanine and stably transfected into Flp-In™ HEK293 cells. Receptor expression was confirmed with western blotting, eYFP total fluorescence and confocal microscopy.
Results. Western blotting of the majority of mutants revealed two bands, similar to wild-type (WT) GPR37L1 (Coleman et al, 2015). Interestingly, the E210A mutant could not be detected and K408A revealed a single unglycosylated species. Fluorescence data showed expression of all mutants except E210A. Confocal microscopy validated expression of most receptors on the cell surface except E210A and K408A.
Discussion. Residue E210 is analogous to the aspartic acid in biogenic amine receptors which is known to be crucial for ligand binding and protein folding, thus mutation to alanine may have been too severe for GPR37L1 folding. Similarly, the K408A mutation located within the transmembrane region renders the receptor unglycosylated for surface expression, suggesting it is crucial to receptor integrity. By understanding GPR37L1's binding pocket, we may gain further insight into the design of new GPR37L1 ligands.

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Min KD et al (2010). Biochem Biophy Res Commun 393: 55-60.
Ngo T et al (2016) Nat Chem Biol (in press).

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The novel fatty acid epoxide analogue CTU activates ER-stress in MDA-MB-231 breast cancer cells

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Introduction. The cytochrome P450-derived ω-3 epoxide of eicosapentaenoic acid activates apoptosis in cells, but its potential value as an anti-cancer agent is limited by in vivo instability (Cui et al, 2012). We prepared metabolically stable epoxide bioisosteres and found that one analogue - termed CTU - rapidly killed MDA-MB-231 breast cancer cells in vitro and in mouse xenograft models in vivo (Murray et al, ASCEPT 2015).
Aims. This study was undertaken to characterise the mechanism of cancer cell killing by CTU.
Methods. In MDA-MB-231 cells the mitochondrial membrane potential was assessed by JC-1 staining, gene profiling was undertaken by RNA-seq with validation by real-time RT-PCR, and altered protein expression was assessed by immunoblotting.
Results. In triple-negative MDA-MB-231 cells, CTU disrupted the mitochondrial membrane potential within 5 min of addition, as shown by a shift in the JC-1 green:red ratio toward the green monomer. CTU decreased the expression of the anti-apoptotic Bcl-xL protein at 6 h and increased the pro-apoptotic caspase-3/7 cleavage at 24 h. RNA-seq profiling identified 382 differentially expressed genes (≥2-fold) after 6 h CTU treatment and bioinformatic pathway analysis revealed selective enrichment of genes relating to cellular stress, especially endoplasmic reticulum (ER)-stress. Real-time RT-PCR confirmed an increase in the ER-stress genes XBP-1 and CHOP at 6 and 24 h, but not at 2 h. The selective ER-stress inhibitors AEBSF and toyocamycin attenuated the activation of caspase-3/7 by CTU.
Discussion. The novel fatty acid epoxide bioisostere CTU rapidly targets the mitochondrion and activates ER-stress in MDA-MB-231 cells leading to decreased cell viability. CTU may be the prototype of a new class of agents with activity against triple-negative breast cancers, where currently the therapeutic options are few.

Cui PH et al (2012) Br J Pharmacol 162:1143-1155.
Murray et al (2015) ASCEPT, 2015.

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In vivo assessment of M1 muscarinic acetylcholine allosteric modulators

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Introduction. Positive allosteric modulators (PAMs) of M_1 muscarinic acetylcholine receptors (mAChRs) have emerged as a potential therapeutic strategy for treating cognitive symptoms of schizophrenia (Choy et al., 2016). However, some M_1 modulators exhibit mixed modes of PAM-agonist activity, whereas others exhibit minimal agonist activity in their own right.
Aims. Two assess the *in vivo* effects of the M_1 mAChR PAM, benzyl quinolone carboxylic acid (BQCA), and the PAM-agonist, benzoquinazolinone-12 (BQZ12), in animal models of predictive of antipsychotic efficacy, as well as potential peripheral cholinergic effects in mice.
Methods. The NMDA antagonist, MK-801, was used to disrupt sensorimotor gating (as determined in an assay of prepulse inhibition; PPI), or memory formation (as determined in a Y-maze test). BQCA or BQZ12 were administered with clozapine and their effects on PPI, Y-maze, salivation and defecation were determined.
Results. BQCA enhanced the efficacy of clozapine in reversing disruption of PPI and memory formation. However, this was not observed in mice treated with the combination of BQZ12 and clozapine. Furthermore, BQZ12 treatment markedly increased salivation and defecation, which was not observed in M_1 mAChR knockout mice, while BQCA showed no peripheral adverse effects.
Discussion. This study suggests that M_1 mAChR PAMs that lack direct agonist activity have a lower propensity to mediate undesirable peripheral effects while retaining potential therapeutic central activity.

Choy KHC et al. (2016) JPET, epub 14th Sep, 2016

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Targeting CTR1 to enhance the uptake and cytotoxicity of oxaliplatin in colorectal cancer cells

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Introduction. Cu transporters are essential to the maintenance of mammalian Cu homeostasis, including Cu transporter 1 (CTR1), Cu transporting P-type ATPases ATP7A and ATP7B, which have been implicated in the transport of oxaliplatin (OXL), a clinically important drug for metastatic colorectal cancer (CRC). Impaired OXL uptake is associated with low CTR1 level in small cell lung cancer cells (Song et al., 2004). Knockdown of ATP7A gene increases OXL cytotoxicity in gastric cancer cells (Chen et al., 2007). Lower tumour level of ATP7B is associated with favourable response to OXL treatment (Martiniz et al., 2009). However, the role of these transporters in transporting OXL in CRC cells is largely unknown.
Aims. To determine the role of CTR1 in OXL uptake, explore the strategy of targeting CTR1 by Cu chelators and characterise the expression of Cu transporters in CRC cells and tumour biopsy tissues.
Methods. Overexpression of CTR1 was achieved in HEK293 and DLD-1 cells via gene transfection. Expression of Cu transporters was measured by RT-PCR, Western blotting and immunohistochemistry in CRC cells and tumour biopsies. Pt contents and cell viability were determined by ICP-MS and a MTT assay, respectively.
Results. HEK/CTR1 cells showed 1.5- to 3-fold increase in Pt accumulation and ~ 3.3-fold increase in sensitivity to OXL than mock control cells. The sensitivity of DLD-1/CTR1 cells to 0.3 to 50 µM OXL was increased by ~15% compared to mock cells (P<0.05). Cu transporters were expressed in CRC cells DLD-1, SW620, HCT-15 and COLO205. Cu chelators up-regulated CTR1 expression in CRC cells by ~ 40% (P<0.05) and enhanced OXL cytotoxicity. CTR1 expression by enterocytes of tumour biopsies is relatively weaker than that of matched normal crypts. ATP7B is detected in colonic crypts of tumour tissues, whereas ATP7A is barely detectable.
Discussion. CTR1 mediates OXL uptake in CRC cells. Cu chelators could potentiate the cytotoxicity of OXL possibly via a CTR1-mediated transport mechanism. The work is supported by a Staff Fund of UNSW, Project Grants from Royal Hobart Hospital Research Foundation and Cancer Council Tasmania.

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High throughput, quantitative analysis of human osteoclast differentiation and activity

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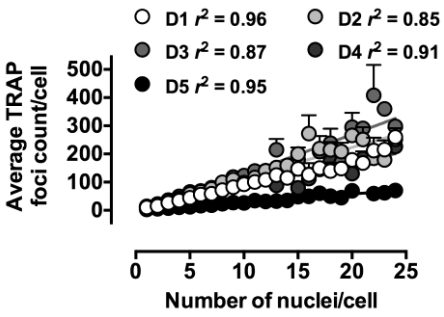
Introduction: Osteoclasts are multinuclear cells that degrade bone under both physiological and pathophysiological conditions. Consequently osteoclasts are important therapeutic targets in the treatment of osteoporosis. The nucleation state of an osteoclast is indicative of its maturation and activity; to date osteoclast activity is routinely measured at the population level with mature osteoclasts classified as having greater than 3 nuclei.

Method: CD14+ monocytes were isolated from human blood using ficoll separation and MACS CD14 selection beads. Using a fluorescent substrate for tartrate-resistant acid phosphatase (TRAP), a routinely used marker of osteoclast activity, we developed a multi-labeled imaging method for quantitative measurement of osteoclast TRAP activity at the single cell level allowing correlation with the number of nuclei/cell. This methodology also allows monitoring of osteoclast maturation by monitoring nucleation state of cells within a population. Automated image analysis enables interrogation of osteoclast populations in a high throughput manner using open source software.

Results: Human osteoclast differentiation from CD14+ monocytes in the presence of RANK-L for eight days, was monitored for five donors. RANK-L increases the number of osteoclasts (cells with >3 nuclei) to 32.6 ± 2.6 % of cells in a population. RANK-L treatment also significantly increased the number of TRAP foci in all cells an effect that was proportional with the number of nuclei demonstrating a direct correlation between TRAP activity and osteoclast maturation. Additionally, osteoclasts from differing donors were compared revealing some donor-derived cultures exhibit more TRAP activity than others (see Figure). This method could be used as a biomarker for therapeutic efficacy or applied to new therapeutic approaches targeting osteoclasts in osteoporosis.



Conclusions: A method was developed for high-throughput screening of osteoclast-targeting compounds to determine changes in maturation and activity with applications in screening therapeutics to treat osteoporosis.

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ER-LAR-GECO1 and R-CEPIA1er as tools to measure endoplasmic reticulum calcium in breast cancer cells

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Introduction. Remodelling of calcium signalling is a feature of some breast cancer subtypes. The assessment of cytosolic free Ca^{2+} is often the focus of investigations into Ca^{2+} signalling in breast cancer cells, with few studies addressing Ca^{2+} dynamics in the endoplasmic reticulum. Genetically encoded calcium sensors targeted to the endoplasmic reticulum may help assess calcium store levels during breast cancer cell division, migration and death.

Aims. To compare transfection methods for the expression of the endoplasmic reticulum Ca^{2+} sensors ER-LAR-GECO1 and R-CEPIA1er in MDA-MB-231 breast cancer cells stably expressing the cytosolic Ca^{2+} sensor GCaMP6m. To assess the ability of ER-LAR-GECO1 and R-CEPIA1er sensors to detect changes in endoplasmic reticulum calcium levels in GCaMP6m expressing MDA-MB-231 breast cancer cells.

Methods. GCaMP6m expressing MDA-MB-231 cells were transfected with either ER-LAR-GECO1 or R-CEPIA1er plasmids using Lipofectamine 3000 or Nucleofector™ Technology. Transfection efficiency and potential purinergic receptor activation-mediated changes in cytosolic free Ca^{2+} and endoplasmic reticulum free Ca^{2+} were assessed using an automated epifluorescent microscope, ImageXpress Micro (Molecular Devices).

Results. Electroporation was superior to Lipofectamine (~31% vs ~0.8% transfection). Both endoplasmic reticulum Ca^{2+} sensors appeared localised to the endoplasmic reticulum. R-CEPIA1er expression suppressed ATP-mediated increases in cytosolic free Ca^{2+}. ATP-induced increases in cytosolic free Ca^{2+} and depletion of endoplasmic reticulum Ca^{2+} stores were detected in ER-LAR-GECO1 expressing cells, with significant variability identified in the magnitude of changes in ER-LAR-GECO1 fluorescence.

Discussion. ER-LAR-GECO1 appears suitable for the detection of relative changes in endoplasmic reticulum Ca^{2+} dynamics in breast cancer cells. Future work should aim to stably express ER-LAR-GECO1 in combination with the cytosolic GCaMP6m sensor in MDA-MB-231 to help define the relationship between cytosolic and endoplasmic reticulum Ca^{2+} during the proliferation, migration and death of breast cancer cells.

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Comparison of binding and functional effects obtained with a subset of safety-related GPCRs

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Decreasing the high attrition rate in the drug discovery and development process is a main goal of the pharmaceutical industry. Extensive usage of the in vitro safety pharmacology in early development is now a major filter used during selection of lead compounds.
A set of 22 GPCRs, associated to the adverse drug reactions (ADRs) are included into a “minimum” safety panel selected by 4 big pharma companies (Bowes et al, 2012).
Different technical approaches are accessible for developing assays for these targets, particularly in the use of ligand binding and functional cell based assays.
A evaluation of these different strategies was done using a selection of specific agonists and antagonists. Results collected from different cell based assays available in “Eurofins Pharma Discovery Services” (Panlabs, Cerep and Millipore) was compared to the ligand binding assay.

Bowes J, Brown AJ, Hamon J, Jarolimek W, Sridhar A, Waldron G, Whitebread S.
Nat Rev Drug Discov. 2012 Dec;11(12):909-22

584

Novel irreversible agonists acting at the adenosine A1 receptor

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Introduction. Among an increasing number of high-resolution G protein-coupled receptor (GPCR) structures, only a few represent receptors in the active state. One of many challenges in obtaining active GPCR structures is the availability of high affinity and slowly dissociating agonists that can stabilize receptors in complex with a G protein or G protein surrogate. The adenosine A_1 GPCR (A_1AR) has been recognized as a potential therapeutic target for numerous conditions but no high-resolution structural information, that could provide insights into molecular basis for ligand binding and selectivity between subtypes of adenosine receptors, has been published.
Aims. The aim of this study was to synthesize and characterize novel, irreversible adenosine derivatives for facilitation of future A_1AR structural studies.
Methods. We tested the compounds in the AlphaScreen ERK1/2 phosphorylation assays for their ability to activate adenosine A_1ARs, as well as in a label-free xCELLigence assay to confirm real-time sensitivity to reversibility by antagonists. Selected compounds were further validated within [^{3}H]DPCPX binding, [^{35}S]GTPγS G protein activation and ThermoFluor A_1AR thermo-stability experiments.
Results. Four compounds, 8b, 15a-15b and 15d, showed similar potency and maximal response to our reference (reversible) agonist, NECA, in a pERK1/2 assay. Using a real-time, label-free xCELLigence assay we identified three compounds, 15b-15d, that were insensitive to the addition of a high concentration of antagonist, SLV320, indicating pseudo- or complete irreversibility. Compounds, 15b and 15d, were validated as irreversible agonists of the A_1AR using membrane-based [^{3}H]DPCPX and [^{35}S]GTPγS binding experiments. In addition, these compounds stabilized purified, detergent-solubilized A_1ARs in a ThermoFluor assay to a significantly higher degree than NECA, a property that could prove extremely beneficial for structural studies.
Discussion. These studies have discovered several compounds capable of prolonged A_1AR activation that may be used as pharmacological tools for future crystallization studies.

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Understanding sweet taste receptor function through heterologous expression systems

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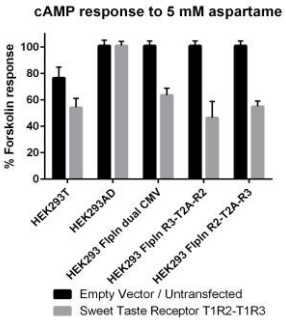

Introduction. Sweet taste receptors have recently been discovered in the pancreas and intestines, and have been implicated in obesity and diabetes. The molecular pharmacology of the sweet taste receptor is not fully understood, and suitable expression systems are needed to fully elucidate its signalling and regulation.

Aims. (i) To compare sweet taste receptor signalling and surface localisation in various heterologous expression systems. (ii) To identify the most appropriate system to investigate sweet taste receptor function.

Methods. Five heterologous expression systems were generated, using transient and stable transfections or the FlpIn system. HEK293T cells were transiently transfected with two vectors for the T1R2 and T1R3 subunits. HEK293AD stable cells were generated by sequential selection for each vector. The FlpIn system used bicistronic vectors containing both subunits with mGluR1 signal peptides, using either dual CMV promoters or the ribosomal skip sequence, T2A. Receptor expression was confirmed by western blot and localisation examined by confocal microscopy. Inhibition of forskolin-induced cAMP accumulation was measured using the BRET CAMYEL sensor.

Results. Preliminary data (above) shows that the magnitude of the functional responses to 5 mM aspartame varies between the different cell lines. The HEK293 FlpIn cell line with R3-T2A-R2 gave the strongest response. HEK293AD stable cells showed the weakest response, and transiently transfected HEK293T cells showed a substantial non-receptor-mediated response. Preliminary confocal microscopy of the HEK293AD cell line showed receptors distributed throughout the endoplasmic reticulum and cytosol, rather than localised to the cell membrane.

Discussion. The R3-T2A-R2 FlpIn cell line showed the strongest response to aspartame, suggesting that this system may have resulted in the highest concentration of functional heterodimers on the cell surface and that a 1:1 ratio of the two subunits and/or the mouse mGluR1 signal peptides may be necessary for receptor function. This result will need to be confirmed through further functional assays and confocal microscopy.

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NTU-CY Inhibits Inflammatory Response and Improves Cardiac Function during Sepsis

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Introduction. Sepsis is the invasion of microbes or their toxins into the bloodstream and characterized by a systemic inflammatory response. The excessive inflammatory response results in myocardial dysfunction leading to septic shock. The treatment of sepsis is still limited. NTU-CY is a novel synthetic alkaloid derivative.

Aims. This study is aimed to demonstrate that the effect of NTU-CY on the inflammatory disease both in cell and animal studies.

Methods. RAW 264.7, a mouse macrophage, was used. The effects of NTU-CY on lipopolysaccharide (LPS) induced inflammatory response were measured. In the animal study, rats were injected with LPS to induce septic shock. Cardiac function was measured.

Results. NTU-CY reduced LPS-induced NF-κB translocated into nucleus and cytokine secretion in macrophage, accompanied with the alleviation of iNOS and COX-2 expression. NTU-CY also alleviated LPS-induced mortality and preserved cardiac function.

Discussion. The inhibition of NF-κB activation by NTU-CY is associated with the alleviation of inflammatory gene expression, cytokine secretion, and the improvement of cardiac function. This study presents a potential compound for the therapeutic uses of sepsis.

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Measuring binding between p75NTR and BDNF peptide mimetic using the ForteBio BLItz system

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Introduction. Neurotrophins are a family of neurotrophic factors essential for normal development of both the CNS and the peripheral nervous system (PNS). They include brain-derived neurotrophic factor (BDNF), which mediates its cellular effects through two distinct classes of receptors: the TrkB receptor and the p75 neurotrophin receptor ($p75^{NTR}$). We have shown that in the PNS, BDNF promotes myelination through the $p75^{NTR}$ pathway, while inhibits myelination through TrkB activation (Xiao et al., 2009). We have previously developed a mimetic of BDNF loop-4 to selectively target $p75^{NTR}$, a peptide called cyclo-DPAKKR. We have shown that cyclo-DPAKKR exerts a pro-myelination effect in the PNS through the $p75^{NTR}$ pathway (Xiao et al., 2013). However, there is no evidence currently showing that it binds directly to $p75^{NTR}$.
Aims. To investigate if modulation of cyclo-DPAKKR via $p75^{NTR}$ can be measured using the ForteBio BLItz system, as indication of direct binding between the peptide and receptor.
Methods. The ForteBio BLItz system measures binding using an optical analysis technique, measuring wavelength changes. An analogue of cyclo-DPAKKR was biotinylated and loaded on a streptavidin sensor, $p75^{NTR}$ was then flowed across the sensor. Wavelength shifts are observed if $p75^{NTR}$ directly binds to the immoblised peptide.
Results. The BLItz system is able to detect binding between neurotrophins and their receptors. Peptide mimetics, rather than receptors, require immobilisation on the sensor to detect binding, owing to their reduced size.
Discussion. The BLItz system is an effective technique to measure binding interactions using a minimum of peptide and protein. Importantly, there are specific system features requiring development for each interaction, especially buffer controls and the linker length between the peptide and biotin, which anchors it to the sensor. This system represents a new first-round screening method for development of BDNF loop-4 peptide mimetics targeting $p75^{NTR}$.

Xiao J et al (2013) J Neurochem 125:386-398
Xiao J et al (2009) J Neurosci 29:4016-4022

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Signalling of TRV 130 in μ-opioid receptor and variants

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Introduction. TRV 130, a novel highly selective μ-opioid receptor (MOR) ligand, recently received FDA breakthrough therapy designation for the treatment of acute to moderate pain. Pre-clinical trials suggest it to be a promising opioid analgesic with reduced side effects compared with conventional opioids.
Aims. To compare the effect of TRV 130 on wild type (WT) MOR and commonly occurring single nucleotide polymorphisms of MOR (A118G and C17T). In addition, we sought to compare the efficacy of TRV 130 to morphine and the enkephalin analog DAMGO in a K channel activation assay in AtT20 cells.
Methods. Inhibition of adenylyl cyclase in CHO cells and K channel activation in AtT20 cells were studied using a fluorescence-based membrane potential assay. The efficacy of TRV130, morphine and DAMGO was determined after depleting MOR with an irreversible antagonist β-chlornaltrexamine (β-CNA, 100 nM, 20 min).
Results. The highest concentrations of TRV 130 tested reduced inhibition of the forskolin response ($P < 0.05$) in the CHO cells expressing A118G and C17T MOR polymorphisms compared to WT. TRV 130 activated K channels in AtT20 cells with a pEC_{50} of 8.1 ± 0.1, as compared to morphine (7.2 ± 0.1) and DAMGO (8.3 ± 0.1). Depleting MOR with β-CNA allowed us to calculate tau values for control and depleted conditions for DAMGO (67.6, 7.1), morphine (11.1,1.5) and TRV 130 (5.5,0.6), demonstrating that TRV 130 has a lower efficacy in this assay than morphine. Notably, in both assays, concentrations of TRV 130 ≥ 10μM produced a transient increase in fluorescence (~ 10%) suggesting a depolarisation. In WT AtT20 cells, TRV 130 (≥ 3 μM) inhibited the hyperpolarization produced by either somatostatin or the $K_{IR}3$ channel activator, ML297.
Discussion. TRV 130 appears to be a lower efficacy agonist than morphine, and its efficacy may be further reduced at common MOR polymorphisms. It remains to be established whether the ligand bias of TRV130 towards G protein signaling over β-arrestin signaling is apparent when a range of ligands are compared in conditions of similar receptor reserve, or whether the clinical effects of TRV 130 are influenced by MOR genotype.

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Comparative genotypic and phenotypic analysis of surrogate monocytic-like cell lines and human peripheral blood monocytes

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Introduction. Monocytic-like cell lines (MCLCs), including THP-1 and U-937 cells, are used routinely as surrogates for isolated human peripheral blood monocytes (PBMCs). In recent years these cell lines have been characterised based on mRNA expression levels of ion channels, G protein-coupled receptors (Groot-Kormelink et al, 2012) and inflammatory mediators (Daigneault et al, 2010).
Aims & Methods. To systematically evaluate MCLCs and PBMCs as model systems in which to study inflammation relevant to the pathogenesis of type II diabetes, we compared (i) mRNA expression of 35 inflammation-relevant genes; (ii) seven cluster of differentiation (CD) marker expression by FACS and (iii) chemotactic responses of MCLCs (with or without PMA treatment) with freshly isolated human PBMCs, differentiated GM-CSF macrophages, and GM-CSF macrophages activated with IFN/LPS.
Results. Heat map analysis of the mRNA expression data suggested that most genes were present at similar levels across all undifferentiated cells. However, hierarchical clustering and principal component analysis of the full data sets revealed clear differences between MCLCs and PBMCs (despite the former's human origin). There was little overall difference in the pattern of expression of CD markers across all cells, though absolute expression levels varied considerably, with fold changes ranging from 1.1-13.1. Functionally, THP-1 and PBMCs both migrated in response to chemoattractants in a transwell assay, with varying sensitivity, ranging from 1-100 ng/mL, to MCP-1 (CCL2), MIP-1 (CCL3) and LTB-4, in line with expression for their requisite target receptors, (CCR2 & 4, CCR1 & 5 and LTB4R1 & 2, respectively). However, despite similar gene and CD expression profiles, U-937 cells were functionally impaired as no migration was observed to any chemoattractant.
Discussion. Our analysis reveals that MCLCs only partly replicate the genotypic and phenotypic properties of human PBMCs, but also that they exhibit substantial differences to each other, highlighting the necessity for careful interpretation of data generated using these immortalized cell lines.

Daigneault M, Preston JA, Marriott HM et al (2010) PLoS ONE 5:1
Groot-Kormelink PJ, Fawcett L, Wright PD et al (2012) BMC Immunology 13:57

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Molecular Pharmacology of Human C3a Receptor Modulators

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Introduction. C3a is a product of complement activation which primes and amplifies the immune response through chemo-attraction of immune cells to sites of inflammatory SB290157 was the first compound reported by SmithKline Beecham in 2001 as a "potent" C3a antagonist. Later in 2005, it was reported to also have "potent" agonist activities in a variety of assays[1].
Aims. To explore new compounds by using Structure Activity Relationship (SAR) studies to identify more potent and selective C3aR agonists and antagonists for use in defining the physiological and pharmacological roles of C3a allowing evaluation of any therapeutic potential.
Methods. Human monocytes were isolated using Ficoll-paque density centrifugation from buffy coat of human blood. $CD14^+$ monocytes were positively selected using $CD14^+$ magnetic beads and differentiated to macrophages (HMDMs) using M-CSF. Radioligand binding assay was used for receptor affinity and selectivity study.
Results. For C3a agonists we found that N-acyl amino acid-oxazole-arginine compounds showed the optimal amino acid side chain for attachment to the oxazole scaffold was leucine or isoleucine with only slightly different binding affinity (pIC_{50}=7.71±0.08 versus 7.53±0.10) and potency (pEC_{50}=7.72±0.27 versus 7.57±0.11). SAR studies also found that the modification of SB290157 by substitution with furan (**143**) at the linker gave predominantly a C3a agonist with the binding affinity similar to SB290157 (pIC_{50}=7.43±0.10 versus 7.42±0.06) and potency (pEC_{50}=8.2±0.15). Discussion. From the study of SAR, we found that the rigid turn-like conformation has produced multiple potent C3a agonists. These stable compounds could be useful for probing the roles of C3a, which is rapidly degraded *in vivo* losing its C-terminal arginine and the resulting C3a-desArg peptide no longer binds to C3aR.

[1]Mathieu, MC, Sawyer, N et al. (2005). Immunol letters. 100(2):139-145.

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Exploring α_1 adrenoceptor ligand residence time

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Introduction. The residence time of [^{3}H]prazosin differs between the α_{1A}, α_{1B}, and α_{1D} adrenoceptors (ARs), which may account for differences in norepinephrine- and oxymetazoline-induced internalisation for these receptors. Previous docking and mutagenesis studies have identified a site at the α_{1A} AR representing a potential allosteric binding site and "extracellular vestibule". Of the implicated residues, only F86 differed between the three α_1 ARs, changing to a leucine in the α_{1B} AR and a methionine in the α_{1D} AR. Additionally, tacrine and 9-aminoacridine are stronger negative modulators of [^{3}H]prazosin dissociation at the α_{1B} AR than the α_{1A} AR.
Aims. To investigate the role of F86 in the α_{1A} AR in mediating orthosteric ligand binding and allosteric modulation.
Methods. Mutant α_{1A} ARs were generated using Dpn1-mediated site-directed mutagenesis. The effects of the mutations on orthosteric ligand binding and allosteric modulation were characterised using saturation, competition, and kinetic [^{3}H]prazosin radioligand binding assays.
Results. The dissociation rate of [^{3}H]prazosin is slower in the α_{1D} AR compared to the α_{1A} AR, and slowest in the α_{1B} AR (K_{off} (min^{-1}): α_{1A} 0.05±0.004, α_{1D} 0.03±0.002; n=3: α_{1B} ≈0.006). [^{3}H]prazosin dissociation was not significantly altered by F86L, but was significantly increased by F86M compared to wild-type (WT) (K_{off} (min^{-1}): WT 0.05±0.004, F86L 0.05±0.005, F86M 0.11±0.005; n=3, P<0.05). Additionally, the mutations did not significantly alter the affinity of NA compared to WT (NA pK_i: WT 5.45±0.06, F86L 5.42±0.12, F86M 5.48±0.14; n=3-4). The ability of 100 µM tacrine and 9-aminoacridine to negatively modulate [^{3}H]prazosin dissociation was not significantly altered by F86L and was significantly decreased by F86M (K_{obs}/K_{off}: tacrine WT 2.2±0.09, F86L 1.7±0.13, F86M 1.5±0.13; n=3, P<0.05: 9-aminoacridine WT 3.3±0.2, F86L 2.5±0.3, F86M 2.0±0.2; n=3, P<0.05).
Discussion. F86 is required for allosteric modulation at the α_{1A} AR by tacrine and 9-aminoacridine, with modulatory activity decreased by leucine or methionine substitutions. Despite α_{1B} and α_{1D} ARs displaying slower [^{3}H]prazosin dissociation rates compared to the α_{1A} AR, the F86L mutant did not significantly change [^{3}H]prazosin dissociation while the F86M mutant increased the dissociation rate, suggesting F86 does not contribute to the differences in [^{3}H]prazosin residence time between receptor subtypes.

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Therapeutic targeting of neurodegenerative disease, using transcriptomics and metabolomics in soleus muscle, compared to spinal cord, in mice overexpressing superoxide dismutase1 (SOD1) G86R

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Introduction: Drug discovery for neurodegenerative disease has not taken into account recent human evolution to run, and increase metabolic efficiency. Metabolic impairment is a major factor in Amyotrophic Lateral Sclerosis (ALS) patients and in presymptomatic (SOD1) G86R mice (Henriques et al., 2015) with changes in the ceramide/glucosylceramide ratio - inhibiting glucosylceramide synthase is deleterious to neuromuscular junctions, with associated effects on lipid metabolism. Objective: Add transcriptomic analysis of spinal cord and soleus muscle (n=4) to metabolomic analysis (n=7, 3000 lipids) to define the lesions in ALS and compare with other neurodegenerative diseases. Methods: FVB/N male mice, overexpressing the SOD1(G86R) protein ~40-fold, were used as described by Henriques et al. 2015. Extraction of total RNA was performed using RNeasy (Qiagen) and RNA quality was assessed with BioAnalyser (Agilent) with analysis by KEGG DISEASE profile.
Results. SOD1 Mice have metabolic impairment with triglycerides being almost fully depleted in plasma, muscle soleus and spinal cord at end-stage. At early symptomatic disease stage, 761 changes were detected in the spinal transcriptome of SOD1 mice when compared to wild type and 1523 in soleus muscle. Spinal cord showed immune system changes, but soleus muscle showed links to neurodegenerative disease due to downregulation of mitochondrial/Krebs cycle genes. Conclusion: Muscle pathology in early stage ALS appears more relevant than changes in spinal cord for therapeutic targeting to other neurodegenerative diseases.
Henriques et al 2015, Hum Mol Genet, 24, 7390-7405

Pathway/disease similarity to soleus changes - SOD1	Gene Set Size	Gene Candidates	p-value	q-value
Parkinson's disease	143	45 (31.5%)	5.01e-18	1.13e-15
Oxidative Phosphorylation	133	42 (31.6%)	5.72e-17	6.43e-15
Alzheimer's disease	168	44 (26.2%)	2.19e-14	1.65e-12
Huntington's disease	193	45 (23.3%)	1.01e-12	5.66e-11

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Identification of TRPC1 as a modulator of AKT phosphorylation in HCC1569 breast cancer cells

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Introduction. Active, phosphorylated AKT (pAKT) is involved in a diverse array of processes in breast cancer cells including proliferation, inhibition of apoptosis and therapeutic resistance. Ca^{2+} influx through specific channels has been linked to AKT phosphorylation in other cell types; however, this association has not been explored in breast cancer cells. HCC1569 is a HER2 positive breast cancer cell line, belonging to the basal molecular subtype. Breast cancers of the basal molecular subtype are associated with poor prognosis, hence identifying new drug targets, may lead to improved treatment of this breast cancer subtype

Aims. To develop a medium-throughput immunofluorescence assay for detection of pAKT in HCC1569 breast cancer cells and to identify Ca^{2+} channels or pumps that modulate basal pAKT levels in HCC1569 breast cancer cells.

Methods. A high content imaging screen was developed through single cell analysis of the integrated cytoplasmic fluorescence intensity of basal, epidermal growth factor (EGF) stimulated (positive control) and MK2206 (AKT inhibitor, negative control) treated HCC1569 cells. Once a robust medium-throughput (Z' score > 0) assay had been developed, an siRNA screen was performed by treating cells with siRNA targeted to different Ca^{2+} pumps, channels and channel modulators. Immunoblotting was performed as a confirmation assay.

Results. Optimisation of the developed pAKT assay led to an assay with a Z' score of 0.448, deemed suitable for a medium-throughput screen. Using this optimised assay, three proteins were identified as potentially lowering pAKT levels after silencing. Immunoblotting confirmed that TRPC1 siRNA-mediated silencing significantly reduced pAKT levels in HCC1569 breast cancer cells ($p<0.05$).

Discussion. Silencing of TRPC1 leads to a reduction of AKT phosphorylation in HCC1569 cells. Further studies are required to define the role of TRPC1 in the proliferation of HCC1569 breast cancer cells and their resistance to some inducers of apoptosis. Future studies should also assess the role of TRPC1 in other molecularly defined basal breast cancer cell lines.

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In-chain heteroatom replacements enhance breast cancer cell killing by novel omega-3 epoxyfatty acid bioisosteres

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Introduction. CTU is a metabolically stable analogue of the proapoptotic ω-3 epoxyeicosapentaenoic acid and kills MDA-MB-231 human breast cancer cells in vitro and in mouse xenografts in vivo (Murray et al., ASCEPT 2015).

Aims. We tested whether replacement of in-chain methylene groups to decrease hydrophobicity may increase the activity of CTU analogues.

Methods. Replacement of the 13-, 3- and 3,13-methylenes in CTU with sulfur atoms produced the new analogues CP1, CP3 and CP5. Cell viability was assessed by ATP formation, cell killing by annexin/7AAD staining, caspase-3/7 activity and immunoblotting for cleaved caspases, and mitochondrial membrane potential by JC-1 staining. In vivo tumour growth was evaluated in nude mice carrying MDA-MB-231 cell intramammary xenografts.

Results. CP3 and CP5 were more effective than CTU and CP1 in decreasing ATP production in MDA-MB-231 cells (26-39% versus 75-77% of control; 10 µM, 24 h). The CP analogues strongly increased annexin/7AAD staining and disrupted the mitochondrion (JC-1 staining). Caspase-3/7 activity was increased by CP3 (to ~4-fold of control; 10 µM, 24 h), and to a lesser extent CTU and CP1 (~2-fold of control), but not by CP5. After CTU treatment (40 mg/kg ip over 7 weeks) growth of MDA-MB-231 xenografts in nude mice was decreased to 42±13% of control ($p<0.05$). The CP analogues were also active, and at much lower doses than CTU.

Discussion. CTU and the CP analogues rapidly disrupt the mitochondrion, impair energy metabolism and activate apoptosis in MDA-MB-231 breast cancer cells. CP analogues were more effective than CTU in vivo. The high potency of the 3-thio-analogues CP5 and CP3 in particular is promising for the development of novel therapeutic strategies.

Murray et al (2015) ASCEPT, 2015.

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Transforming Growth Factor beta-Activated Kinase 1 (TAK1) is a novel target for retinal neovascularisation

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Introduction. The hallmark of proliferative diabetic retinopathy is the presence of retinal neovascularisation. Current treatment modalities are limited to panretinal photocoagulation, vitrectomy, and adjunctive intravitreal injection of anti-vascular endothelial growth factor (VEGF) proteins. Not all patients are responsive to anti-VEGF treatment, probably because other cytokines are involved in the complex pathogenesis of this disease. In this study, we found TAK1, may be a mediator of pro-angiogenic signalling. However, it remains unknown whether TAK1 is a suitable target for management of retinal neovascularisation.
Aims. To investigate whether TAK1 is a suitable therapeutic target in a rat model of oxygen-induced retinopathy.
Methods. MiRNA sequencing was performed on retinal RNA isolated from control (normoxic) and rats subjected to oxygen-induced retinopathy (OIR). Bioinformatic analysis was then undertaken to identify candidate pathways and genes involved in retinal neovascularisation. Identified candidate genes were then validated by RT-qPCR. TAK1 was blocked by a selective TAK1 inhibitor, 5Z-7-oxozeaenol, to assess its potential role in angiogenesis by pro-angiogenesis assays *in vitro* including tube formation, cell proliferation, cell migration and aortic ring assays. The effects of intravitreal injection of 1μM of 5Z-7-oxozeaenol were investigated further in our *in vivo* rat model of retinal neovascularisation induced by OIR.
Results. TAK1 was identified through bioinformatic analysis as a potential target gene. *In vitro* angiogenesis assays demonstrated that 5Z-7-oxozeaenol can attenuate angiogenesis in vitro and ex vivo. *In vivo,* a significant reduction of retinal neovascularisation was observed in the OIR rat model following a single intravitreal injection of 5Z-7-oxozeaenol.
Discussion. Together these data suggest that TAK1 is involved in development of retinal neovascularisation. TAK1 may represent a suitable target for the development of new therapeutics for retinal neovascularisation in diseases such as proliferative diabetic retinopathy, because it not only contributes to angiogenesis, but also activates inflammatory signals.

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Reduction of translocator protein (TSPO) A147T discrimination with carbazole acetamide analogues

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Introduction. The 18kDa translocator protein (TSPO) is a target for development of diagnostic imaging agents for glioblastoma and neuroinflammation. Clinical translation of TSPO imaging agents has been hindered by the presence of a polymorphism, rs6971, resulting in substitution of alanine for threonine at amino acid residue 147 (TSPO A147T). Most TSPO ligands lose affinity at TSPO A147T, and efforts to develop a ligand that binds with similarly high affinity to TSPO WT and A147T have been hampered by a lack of knowledge about how ligand structure differentially influences interaction with the two forms of TSPO.
Aims. This study aims to explore how modifications of a novel *N*-alkylated carbazole acetamide scaffold influences affinity at both TSPO forms.
Methods. Human embryonic kidney 293T cell lines stably over-expressing human TSPO WT and A147T were established, and the affinity of seven *N*-alkylated carbazole acetamide derivatives were determined by competition radioligand binding using [^{3}H]-PK 11195.
Results. Five of the new analogues showed high affinity to TSPO WT (K_i: 25 – 30 nM). Five also showed a 5-6-fold loss in affinity to TSPO A147T. Addition of a 3'methoxy phenyl group to the 3-position of the carbazole produced the biggest improvement in TSPO A147T affinity without imparting loss in affinity at TSPO WT (K_i TSPO WT: 29 nM; K_i TSPO A147T: 93 nM; 3.2-fold discrimination).
Discussion. Exploration of the impact of electron-donating groups to *N*-alkylated carbazole acetamides may assist future development of TSPO ligands with equally high affinity at TSPO WT and A147T, for ultimate use as diagnostic markers for glioblastoma and neuroinflammation.
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A nitrostyrene derivative inhibits human platelet aggregation through inhibition of cell surface protein disulfide isomerase

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Introduction. Platelets play a fundamental role in hemostasis. On the other hand, abnormal platelets activation is a major cause of cardiovascular diseases (CVDs). Several antiplatelet drugs, such as aspirin, have been used to prevent or treat CVDs. Recently, protein disulfide isomerase (PDI) has emerged as a potential target for antiplatelet agents. PDI is involved in the formation, reduction, and isomerization of disulfide bonds of proteins, and cell surface PDI is known to interact and regulate functions of integrins. PDI inhibition results in reduced platelet aggregation and protection from arterial thrombosis. Aims. To discover PDI inhibitors for developing new antiplatelet agents. Methods. The activity of PDI was determined by using a synthetic substrate dieosin glutathione disulfide, which becomes fluorescent after reduction by purified PDI or platelet surface PDI. Antiplatelet effects of tested compounds were measured by platelet aggregometer, flow cytometry, and flow chamber. Results. In a series of nitrostyrene derivatives, compound 1 was identified as a potent inhibitor of purified human recombinant PDI. In addition, the surface PDI activity of human platelets was also inhibited by compound 1. Thrombin-, collagen-, or a thromboxane A2 analog-induced platelet aggregation was prevented in parallel with PDI inhibition. Moreover, the activation of the integrin GPIIb/IIIa, which is regulated by PDI, was inhibited by compound 1. In an in vitro thrombotic models, compound 1 reduced platelet adhesion to collagen in flowing whole blood. Discussion. Our results suggest that compound 1 is a potent PDI inhibitor with antiplatelet activity and can be used as a lead for developing new antiplatelet agent.

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Antidepressant-like effects of RS-ketamine, but not R-ketamine, S-ketamine, R-norketamine or S-norketamine in the forced swim test using the BALB/c mouse strain

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Introduction. Racemic ketamine is showing promise as an antidepressant, as evidenced by a substantial improvement in mood scores 2-4 hours after a single IV dose (Xu et al, 2015). However, psychotomimetic effects may limit success. R-ketamine lacks these adverse effects in humans, and testing single-isomers of ketamine or its active metabolite norketamine, may identify the active moiety, not necessarily associated with the NMDA receptor.
Aims. To determine the relative potencies of RS-ketamine, R-ketamine, S-ketamine, R-norketamine and S-norketamine using rodent behavioural models of depression.
Methods. The murine forced swim test (FST) was used to compare the potencies of RS-ketamine, R-ketamine, S-ketamine, R-norketamine and S-norketamine at four doses (20-120 mg/kg), given s.c. 90 minutes beforehand. Desipramine was a positive control, and saline as baseline. Five male BALB/c mice (8-9 weeks) were used for each dose. Each animal was also tested for hyperlocomotion, using the open field test (OFT). For the second experiment, 4 BALB/c mice were injected s.c. with saline or RS-ketamine 80 mg/kg, and tested in the OFT and FST for 5 consecutive days, to assess inter-animal and inter-day variability.
Results. A dose-response relationship was obtained for RS-ketamine in the FST with an EC_{50} of 53 (95 % CI = 39-73) mg/kg and Hill slope of 1.7 (95 % CI = 0.7-2.7). S-ketamine reduced immobility in the FST by 38 ± 16 % at 20 mg/kg ($p < 0.05$) but no dose-response was evident, nor for R-ketamine or R- and S-norketamine. Only S-ketamine appeared to reduce locomotion (OFT). In the second experiment, inter-animal variability was greater for RS-ketamine (CV = 38%) than with saline (CV = 20%). However, mean intra-animal variabilities were equal at 17 %.
Discussion. This study was the first to test the isomers of ketamine and norketamine in BALB/c mice. We found a distinct dose-response relationship for RS-ketamine in the FST, but not its individual enantiomers. Multiple receptors may be responsible, and NMDA receptor inhibition is unlikely the sole mediator of the response.

Xu Y, Hackett M, Carter G et al (2015). Int J Neuropsychopharmacol. 19(4): pyv124

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Cannabinoid constituent interactions in an animal neuropathic pain model

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Introduction. Chronic neuropathic pain is a prevalent and costly health care problem. In addition, it is difficult to treat and current therapeutics produce problematic side-effects. While there is good evidence that the major psychoactive constituent of *Cannabis sativa*, D9-tetrahydrocannabinol (THC), is effective in animal models of neuropathic pain, it also produces a range of side-effects. It has been suggested that the utility of THC might be improved by administering it in combination with the non-psychoactive cannabis constituent Cannabidiol (CBD). The basis for this interaction is unknown.
Aims. We examined the pain and behavioural interactions of combination THC/CBD treatment in a nerve injury induced animal model of neuropathic pain.
Methods. Ethics approval was from Royal North Shore Hospital Animal Ethics Committee. Adult male C57BL6 mice underwent unilateral chronic constriction of the sciatic nerve (CCI) under isoflurane anaesthesia (2% in saturated oxygen). The effect of acute drug administration was tested at 8 days post-CCI (volume 0.01ml per g, in saline with 10% dimethylsulfoxide, 5% Tween80). Mechanical and cold allodynia were measured as the mechanical paw withdrawal threshold (PWT) and the number of pain-like responses to acetone application on the operated hind paw. Side-effects including motor incoordination, catalepsy and sedation were measured using the rotarod, bar test, and dark open field, respectively.
Results. Individually, THC and CBD produced a dose dependent reduction in the CCI induced mechanical and cold allodynia. THC, but not CBD produced dose dependent motor incoordination, catalepsy and sedation. Combined THC and CBD treatment reduced mechanical and cold allodynia with ED50s less than that predicted for an additive effect. Combined THC and CBD treatment produced side-effects with a profile similar to that of THC alone.
Discussion. This data indicates that THC and CBD act synergistically to reduce the allodynia associated with an animal model of neuropathic pain. By contrast, the side-effects of combination treatment are similar to those predicted for THC alone. Thus, THC/CBD combination treatment represents an alternative treatment for neuropathic pain.

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Action of centrally administered glucagon-like peptide-1 (7-36) amide to modulate blood glucose, feeding and induce emesis in *Suncus murinus*

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Introduction. Glucagon-like peptide-1 (7-36) amide (GLP-1) is released from the gut as an incretin hormone to potentiate glucose-stimulated insulin secretion. GLP-1 is also produced in the central nervous system (CNS) to regulate feeding.
Aims. To examine the potential of central administration of GLP-1 to modulate blood glucose levels, emesis and feeding in *Suncus murinus*.
Methods. To elevate blood glucose levels, anaesthetised fasted animals were administered glucose (5.55 mmol/kg, i.p.) 10 min prior to an infusion of GLP-1 (0.3-10 nmol, i.c.v), or saline (5 µl). Blood glucose levels were measured at 5-20 min intervals for 1 h. In other studies, GLP-1 (0.03-10 nmol, i.c.v.), or saline (5 µl), was administered to conscious fasted animals. Food and water consumption and spontaneous activities were measured for 1 h.
Results. The administration of glucose caused a progressive elevation of blood glucose in the saline-treated animals that peaked at 5 min and then decreased gradually afterwards. GLP-1 (10 nmol, i.c.v.) produced a 32.5% reduction in the $AUC_{0\text{-}50}$ values (GLP-1 178.1 ± 47.4 vs. saline 120.1 ± 8.1, $P<0.05$). In the conscious animals, GLP-1 (0.3 – 10 nmol, i.c.v.) reduced the distance travelled by ~60% ($P<0.05$), but had no effect on velocity ($P>0.05$). GLP-1 (0.03–10 nmol, i.c.v.) inhibited food ($P<0.05$) and water intake ($P<0.05$) significantly. GLP-1 at 3 and 10 nmol, i.c.v., induced emesis in 1 and 2 animals, respectively.
Discussion. GLP-1 (7-36) amide is involved in the modulation of blood glucose levels, locomotor activity, feeding, and emesis in *Suncus murinus*. The mechanism of GLP-1 to induce emesis and inhibit feeding requires further investigation. These studies were fully supported by a grant from the Research Grants Council of the HKSAR, China (Project no. UGC/FDS11/M02/15).

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Monoterpenes from *Cannabis sativa* do not affect human cannabinoid receptor activation of K channels in AtT-20 cells

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Introduction. *Cannabis sativa* contains a rich diversity of biologically active molecules including the cannabinoid receptor agonist Δ9-tetrahydrocannabinol (THC). It has been suggested that the behavioural effects of THC are modulated by these other molecules, including monoterpenes and related monoterpenoids such as myrcene, pinene and linalool. However, the effects of these molecules on cannabinoid receptor function remain unknown.

Aims. To determine whether monoterpenes/monoterpenoids modulate the activation of CB1 and CB2 by THC.

Methods. Cannabinoid CB1 or CB2 mediated K channel activation was measured in AtT20 cells with a membrane potential sensitive dye. Endogenous SRIF receptors served as controls for direct effects of drug on K channels.

Results. β-carophyllene, α-pinene, β-pinene, linalool, limonene (100μM) or β-myrcene (30μM) alone did not affect the membrane potential of AtT-20FlpIn-WT, CB1 or CB2 cells. Only α-pinene (100μM) produced a small inhibition of the hyperpolarization produced by subsequent application of SRIF (100nM) in AtT20FlpIn-WT cells (34±3% decrease in fluorescence in control, 30±2% in drug, $P<0.05$). THC produced a concentration-dependent hyperpolarization in AtT-20FlpIn CB1 and CB2 cells, this was unaffected by the terpenes alone (10μM), or in combination (n=6/7). To assess if terpenes had a delayed/allosteric effect on CB1 signalling, the desensitization of the THC (10μM)-mediated signal was measured in the presence of all 6 terpenes (10μM). In control, the peak decrease in fluorescence recovered by 63±6 % over 30 min, with the terpenes the recovery was 61±5 % (n=6).

Discussion. None of 6 common terpenes/terpenoids found in *Cannabis* modulated CB1 or CB2 mediated opening of K channels by THC, suggesting that they do not significantly enhance or inhibit the interaction of this agonist with these receptors. It is possible that the terpenes/terpenoids may affect activation of other signalling pathways of CB1 or CB2 receptors, but perhaps more likely is that they act on other molecular targets in neuronal circuits important for the behavioural effects of *Cannabis*.

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Antidepressants and antipsychotics appear to be overprescribed

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Introduction: PBS data show that one in six Australians take at least one psychotropic medication, mostly long term. These medications may reduce re-hospitalisation but no published data have confirmed improved clinical outcomes.

Aim: To evaluate community psychotropic prescribing.

Methods: We undertook a one week 'snapshot' audit of psychotropic prescribing at one regional and 3 metropolitan community pharmacies in Western Australia.

Results: Psychotropic medications made up 12% of the 6,228 prescriptions dispensed. This concurs with national statistics. Sixty-five percent (65%) of the prescriptions were for antidepressants, 19% for benzodiazepines, 12% for antipsychotics and 4% for stimulants. 52% of the benzodiazepines were short acting agents, presumably for insomnia. Selective serotonin reuptake inhibitors (SSRIs) accounted for 49% of the antidepressants, with mean doses of 5-15-fold their ED50 (mean population half-maximal dose).

Discussion: Given the high prevalence of mental illness and the large investment by the pharmaceutical industry in marketing new products, it is not surprising that psychotropic medications account for such a large number of community prescriptions. The known much higher prevalence of anxiety and insomnia compared to depression contrasts with the preponderance of antidepressant prescriptions and raises questions about off-label prescription since the PBS subsidy for most SSRIs requires a diagnosis of major depression. The community prevalence of ADHD is over 5-fold that of psychosis but antipsychotics are prescribed much more than stimulants, which merits closer study. SSRI doses appear excessive, compared for example to cardiovascular drugs, which are usually prescribed around 0.5-4-fold their ED50. Psychotropic medications cause a wide range of dose-related adverse effects, such as weight gain, hypotension, hyponatraemia, cardiac arrhythmia, apathy, abnormal sleep, sexual dysfunction, tremor, dependency and inappropriate mood, many potentially serious. These adverse effects may diminish potential benefits and outcomes. Meta-analyses suggest that lower doses of antidepressants can be equally effective, with the advantage of improved tolerability and safety.

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α3 Nicotinic acetylcholine receptor subunit expression is altered in the hypoglossal nucleus in Sudden Infant Death Syndrome (SIDS)

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Introduction. Pre- and/ or post-natal (maternal/paternal) cigarette smoke exposure in infants increases risk of cardio-respiratory abnormalities and Sudden Infant Death Syndrome (SIDS). Nicotine, the major psychoactive component of cigarette smoke, is a nicotinic acetylcholine receptor (nAChR) agonist. Recent work from our laboratory using animal models of cigarette smoke[1] and nicotine[2] exposure, found changes in expression of some nAChR subunits within the brainstem, the most notable being with the α3 subunit.
Aims. This study aimed to determine if α3 nAChR subunit expression is altered in the SIDS brainstem and whether cigarette smoke exposure contributes to this.
Methods. Immunohistochemical expression of the α3 subunit was studied in 8 nuclei of the brainstem rostral medulla in infants diagnosed with SIDS (n = 21) and compared to non-SIDS infants (n = 9). Two way ANOVA was performed for each nucleus, analysing main effects of SIDS status, smoke exposure status, and interaction effects.
Results. The hypoglossal nucleus of SIDS infants was found to have significantly higher expression of the α3 subunit ($F_{(1, 19)}$ = 6.238, p = 0.02) compared to non-SIDS infants, with a trend towards a significant ($F_{(1, 19)}$ = 3.430, p = 0.08) increase in the α3 subunit due to smoke exposure in SIDS infants. No significant main effects or interaction effects were found for α3 subunit expression in any other nuclei studied.
Discussion. The hypoglossal nucleus controls the tongue, thus maintaining airway patency. Changes to α3 subunit expression in this nucleus indicates changes to nAChRs that may contribute to mechanisms leading to SIDS such as difficulties is respiration during sleep, which may be compounded by pre-into post-natal cigarette smoke exposure.

[1]Vivekanandarajah A et al. (2016) *Neurotoxicology*, 53, 53-63
[2]Vivekanandarajah A et al. (2015) *Int. J. Dev. Neurosci.*, 47, 183-191

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ASIC1a inhibition preserves tissue and improves function after spinal cord injury (SCI)

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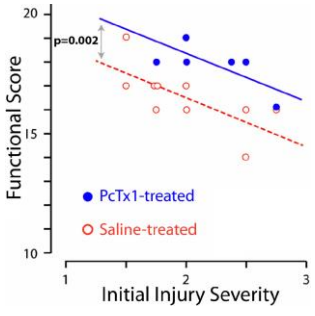
Introduction. Spinal cord injuries (SCIs) damage blood vessels resulting in ischaemia around the injury site. The consequent hypoxia and acidosis contribute to ongoing secondary tissue damage over a period of days post-injury. We surmised that preventing activation of acid-sensing ion channel 1a (ASIC1a), present on most spinal neurons, would reduce acidosis-induced excitotoxic secondary tissue loss and preserve greater residual spinal cord function.
Aims. (i) To investigate the effects of the selective ASIC1a inhibitor PcTx1 on tissue loss and spinal cord function after SCI. (ii) To investigate the molecular mechanisms of PcTx1's neuroprotective effects.
Methods. Adult, female Sprague Dawley rats were administered controlled contusion injuries to the thoracic spinal cord under deep anaesthesia (inhaled isoflurane 3% in O_2). Rats were randomly assigned to a PcTx1-treated group (12.5mg/kg s.c. then 1.08mg/h for 48h) or a saline-treated group. Immunohistochemical analysis (luxol fast blue, CNPase, Fox3), RNAseq (Illumina) and behavioural analyses (BBB score, horizontal ladder, tapered beam task) were conducted up to 6 weeks post-SCI.



Results. PcTx1 treatment significantly improved locomotor performance after SCI and this directly correlated with greater preservation of dorsolateral white matter tracts involved in locomotor function. Transcriptomic analysis did not reflect the common belief that PcTx1's neuroprotective effects were due to apoptosis reduction, instead highlighting smooth muscle response & chemokine signalling as potential contributors.
Discussion. Our data provides evidence to support the use of ASIC1a inhibitors as a therapeutic treatment to limit secondary tissue loss after SCI. Further investigation is required to elucidate the precise mechanism of PcTx1s neuroprotection, with immune response and angiogenesis highlighted by RNAseq analysis as areas for future evaluation.

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The Neurodevelopmental Profile of Dimeric and Monomeric mGluR5: Implications for Novel Therapeutics

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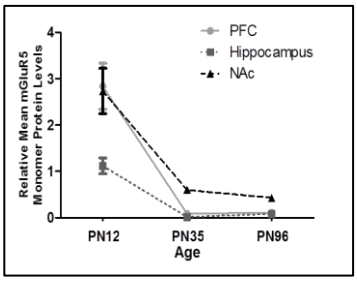

Introduction. Metabotropic glutamate receptor 5 (mGluR5) allosteric modulators are currently being investigated as novel therapeutics for the treatment of neurodevelopmental disorders such as schizophrenia, autism, depression (Cleva and Olive, 2011). Recent evidence suggests that positive allosteric modulators (PAMs) have adverse pharmacological effects when bound to the monomeric, compared to the dimeric form of mGluRs (El Moustaine et al., 2012).
Aims. To investigate the neurodevelopmental expression of dimeric and monomeric mGluR5 and identify any neurodevelopmental periods which may be vulnerable to the possible adverse effects of mGluR5 PAMs.
Methods. Rat brains were extracted on postnatal day (PN) 12, 35 and 96 (n=6/group) coinciding with juvenile, adolescent and adult time points. Immunoblots were performed on prefrontal cortex (PFC), hippocampus and nucleus accumbens (NAc) tissue under non-reducing conditions to measure dimeric and monomeric mGluR5 levels.
Results. Dimeric mGluR5 protein expression remained relatively constant throughout the time-points and brain regions investigated. In contrast, mGluR5 monomer was highly expressed at PN12, almost equal to dimeric expression and then declined to the lowest limits of detection at later time-points in all brain regions (Figure).
Discussion. Considering PAMs exert an unregulated agonist effect when bound to the monomeric form of mGluRs, administration of these compounds when mGluR5 monomer is highly abundant such as the juvenile age, may increase the risk of adverse effects such as seizures and neurotoxic effects, posing possible consequences for novel mGluR5 therapeutics.

Cleva et al., (2011), Molecules, 16, 2097-2106
El Moustaine et al., (2012), Proc Natl Acad Sci USA, 109, 16342-16347

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Portable optical fiber temperature probe to measure brain hyperthermia in deep brain structures

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Introduction. Brain hyperthermia is the major complication associated with MDMA administration and a factor that potentiates neurotoxicity (Brown and Kiyatkin, 2004). There is a requirement for a minimally invasive temperature probe, which can record region specific brain temperature changes within deep brain structures in a live ambulatory behaving rodent. Optical fibers have been used as a potential solution for this.
Aims. To develop a portable optic fiber temperature sensor capable of brain temperature measurement *in vivo*.
Methods. The optical fiber tip was dipped in molten zinc tellurite glass doped with 1 mol% erbium and 9 mol% ytterbium. Sprague-Dawley rats were implanted with telemetry devices to measure body temperature. Optical fiber temperature probes were implanted into the right striatum (A: +0.2 mm, L: +3.0 mm, V: -3.5 mm from bregma).
Results. *In vitro* results demonstrate that the measured change in fluorescence ratio can be approximated as linear with an R^2 of 0.9994 over the measured range from 22°C to 51°C, with a sensitivity of $0.00526K^{-1}$. *In vivo* results show minimal effects of ambient temperature shifts on optic fiber probe temperature recordings. *In vivo* probe results display a good correlation between brain (-0.380±0.479, n=4) and body (0.037±0.106, n=4) temperature changes from baseline across all trials.
Discussion. We have successfully demonstrated a proof-of-principle measurements for the use of an optical fiber probe to measure temperature *in vivo* based on rare-earth thermometry. Future investigations will use this method to assess brain temperature of MDMA treated rats in order to improve the understanding of the hyperthermic effects of stimulant drugs, and the pathways involved in driving the drug-induced hyperthermic response.

Brown PL and EA Kiyatkin (2004) Eur J Neurosci 20:51-58.

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GPR88 is a key regulator of striatal function

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Introduction. Dysfunction of dopaminergic and glutamatergic signalling in the striatum underpins the pathology of many neurological diseases such as schizophrenia. GPR88 is a G protein-coupled receptor, which is expressed almost exclusively in the striatum, a region of the brain that is pivotal for the integration of dopaminergic and glutamatergic inputs. Interestingly, a schizophrenia-relevant phenotype has been published from studies using GPR88 knockout mice (Quintana et al., 2012).
Aims. To determine the molecular mechanisms by which GPR88 exerts its effects on striatal neurophysiology.
Methods. CHO cells stably expressing human GPR88 (CHO hGPR88), murine embryonic striatal cultures and brain slices from adult C57/Bl6 mice were subject to a range of biochemical, second messenger and electrophysiological assays, using the synthetic GPR88 agonist, (R,R)-2-PCCA. Test assays included GTPγ^{35}S binding, cAMP accumulation, Western blotting, immunofluorescence and confocal microscopy and electrophysiological analysis.
Results. GPR88 activation with (R,R)-2-PCCA activated inhibitory $G_{i/o}$-family proteins, decreasing forskolin-stimulated cAMP accumulation in both CHO hGPR88 cells and striatal neurons (cAMP: pIC_{50} = 7.8 ± 0.1 (CHO), pIC_{50} = 6.8 ± 0.2 (striatal neurons)). Furthermore, (R,R)-2-PCCA inhibited the phosphorylation of dopamine- and cAMP-regulated neuronal phosphoprotein of 32 kDa (DARPP-32), at threonine 34, and serine 831 and 845 on GluA1-containing AMPA receptors; pGluA1 inhibtion: pIC_{50} = 5.9 ± 0.3 (pS831), pIC_{50} = 6.7 ± 0.2 (pS845). In mouse brain slice electrophysiology, bath application of (R,R)-2-PCCA (300 nM) robustly inhibited cortically-evoked and AMPA-evoked excitatory post-synaptic currents (EPSCs) in medium spiny neurons.
Discussion. These data suggest that activation of GPR88 may regulate cortico-striatal neurotransmission, a key node in disorders such as schizophrenia.

Quintana et al. (2012) Nat Neurosci. 15(11):1547-55

617

Sex differences in outcome after permanent focal cerebral ischemia

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Introduction. Stroke incidence is lower in young females than age-matched males, however females appear to experience worse long-term outcomes. Dampening of the immune system after stroke leaves the patient more susceptible to infections, but the effect of sex on this phenomenon is unknown. A key estrogen receptor, the G-protein coupled estrogen receptor (GPER), exhibits sex-dependent neuroprotective effects after stroke and could potentially influence immunosuppression.
Aims. To test whether a sex difference exists in outcomes after permanent focal cerebral ischemia, and if estrogen-mediated activation of GPER is involved.
Methods. Brain infarct volume and bacterial infection in lungs were assessed 24 h after permanent middle cerebral artery occlusion (pMCAO) in female (n=41) and male (n=20) mice. Furthermore, G-1 (GPER agonist)/vehicle was administered to ovariectomised female mice just before induction of pMCAO and infection levels were assessed 23 h later.
Results. Higher mortality was observed in females than males (27% vs. 15%). No sex difference was detected in infarct size, mortality or bacterial infection, however stroke decreased spleen weight selectively in females (n=14-28, $P<0.01$). G-1 worsened bacterial infection in females (n=4-7, $P<0.0001$) with no effect on infarct size.
Discussion. Independent of the degree of brain injury, females suffer greater mortality after permanent focal ischemia. Furthermore, a GPER agonist increases infection levels after stroke in females in the absence of endogenous estrogen. More study is needed to elucidate whether GPER modulation could be exploited for post-stroke neuroprotective therapy in older females.

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Roles of cysteinyl leukotriene receptor 1 in glioma cell migration and invasion

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Introduction. Malignant glioma is a highly invasive brain tumor with increased recurrence and treatment failure. Recent studies indicated the importance of 5-lipoxygenase (5-LOX) and cysteinyl leukotriene receptor 1 (CysLT1) in invasion of prostate and ovarian cancers. Expression of 5-LOX was found in many types of brain cancer and the inhibition of this enzyme inhibited proliferation of astrocytoma cells.
Aim. (1) To determine the expression of genes related to cyteinyl leukotriene synthesis and function in human glioma tissues. (2) To investigate the effect of leukotriene inhibition on glioma cell viability, migration and invasion.
Methods. Gene expression of 5-LOX, CysLT1 and CysLT2 in glioma and normal adjacent tissue was measured using real time PCR. Effect of montelukast, a CysLT1 antagonist, on cell proliferation, migration and invasion was tested using MTT, scratch and transwell invasion assays, respectively.
Results. We found higher expression of 5-LOX and CysLT1 in glioma tissues while CysLT2 levels were comparable between groups. At 24 hour post-exposure, the median toxic concentrations of montelukast on A172 astrocytoma cells was 7.13 µM. Montelukast greatly inhibited cell migration and cell invasion at 48 hour post-exposure.
Discussion. These results indicate the importance of leukotriene and CysLT1 in the metastasis of glioma and inhibition of CysLT1 could be a potential target for glioma treatment.

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Regulation of UGTs in Cancer

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Introduction. UDP Glucuronosyltransferases play critical roles in the conjugation and elimination of numerous therapeutic drugs as well as endogenous lipophilic molecules such as steroid hormones. UGTs are regulated by ligands that are also UGT-substrates, thus generating regulatory loops through which small molecules control their own metabolism. There is evidence that UGT activity influences the progression of steroid-dependent cancers (breast and prostate) via glucuronidation of steroids both systemically and locally. In addition, systemic and local metabolism of anti-cancer drugs may influence therapy response and the acquisition of drug resistance.
Aims. These studies aim to support the paradigm that the local regulation of UGTs in cancer cells by steroids and by various classes of anti-cancer drugs influences cancer cell growth and response to therapy.
Methods. We use a suite of gene regulation analysis tools to understand how multiple UGTs including UGT2B15, 2B17, 2B7 and 1A1, are regulated in cancer cells by steroids and by different classes of anti-cancer drugs. We also study the effects of UGT overexpression and ablation on cancer cell growth and drug resistance.
Results. We present results of studies in two areas that support our overarching paradigm. First we present new work on the regulation of UGT2B15 and UGT2B17 in breast cancer. These UGTs glucuronidate and inactivate androgens as well as some selective estrogen receptor modulators (SERMs) and aromatase inhibitors (AIs). SERMs and AIs are mainstays of hormonal breast cancer therapy that work by blocking ER function and by preventing conversion of androgens to estrogens respectively. We define the mechanism of regulation of UGT2B15 and UGT2B17 by androgens, estrogens, SERMs and AIs, and show how this could alter cancer progression and its response to hormonal therapies. Second, we present studies on the regulation of various UGTs by cytotoxic cancer drugs in various cancer cell types, including novel studies into UGT expression in cancer stem cells (CSC). Again we show how this regulation can impact on cancer cell proliferation and drug response/resistance.
Discussion. Our studies indicate that UGT regulation is an important variable in cancer progression, therapy response and drug resistance. Opportunities to modulate UGTs for therapeutic benefit in cancer will be discussed.

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Evaluation of Modafinil as a Perpetrator of Metabolic Drug-Drug Interactions (MODDI-14)

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Introduction: The capacity of the vigilance promoting agent modafinil to perpetrate metabolic drug-drug interactions (mDDIs) has been demonstrated *in vitro* for a panel of cytochrome P450 (CYP) enzymes. However, the magnitude and clinical relevance of these *in vitro* data have not yet been established. Given the expanding spectrum of indications for modafinil, the capacity for this drug to perpetrate clinically relevant mDDIs warrants consideration.
Aim: To evaluate the magnitude and clinical relevance of mDDIs affecting CYP activity perpetrated by modafinil.
Methods: MODDI-14 (ACTRN12614000451606) is a single centre, open label, cocktail interaction study. On days 0, 2 and 8, following an overnight fast, healthy participants (male and female; 21 and 35 years old) were administered an oral drug cocktail comprising caffeine (100mg; CYP1A2), dextromethorphan (30mg; CYP2D6), losartan (25mg; CYP2C9), midazolam (1mg; CYP3A4) and omeprazole (20mg; CYP2C19). Each morning from day 2 to 8 (prior to cocktail dosing on relevant days) subjects ingested modafinil (200mg PO). Timed blood samples collected prior to and at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6 and 8 hrs post cocktail dosing on days 0, 2 and 8 were analysed for probe concentrations by UPLC-MS. The area under the concentration-time curve (AUC) was determined for each probe prior to and following modafinil dosing using non-compartmental methods. The magnitude of modafinil single dose (Day 2) and steady state dosing (Day 8) mDDIs were assessed based on the probe AUC ratios in the presence and absence of modafinil.
Results: An interim analysis (n = 6) demonstrates that following a single dose of modafinil the mean AUC ratios for caffeine, dextromethorphan, losartan, midazolam and omeprazole were 0.95, 1.03, 1.05, 0.89 and 1.62, respectively. Following dosing of modafinil to steady state (200mg for 7 days), AUC ratios for caffeine, dextromethorphan, losartan, midazolam and omeprazole were 0.87, 0.76, 1.07, 0.61 and 1.89, respectively
Discussion: These data indicate that when dosed to steady state modafinil may perpetrate clinically meaningful mDDIs when co-administered with drugs metabolised by CYP2C19 (increased exposure due to inhibition of CYP2C19) and CYP3A4 (decreased exposure due to induction of CYP3A4).

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The inhibitory effect of natural alkaloid compounds on the substrate uptake mediated by human solute carrier transporters (SLCs)

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Introduction. The solute carrier transporters, particularly the organic anion transporting polypeptides and organic anion/cation transporters are the main class of solute transporters. They are important membrane proteins responsible for the cellular uptake of endogenous and exogenous substances. They are widely expressed in human key organs including the kidney, liver, intestine, placenta and brain. Inhibitors competing with drug substrates for SLCs often results in unfavourable toxicities and unsatisfactory therapeutic outcomes. Dendrobine, matrine, oxymatrine, chelerythrine and tryptanthrine are alkaloid compounds extracted from plants. These compounds are widely used clinically in Asian countries.
Aims. In this study we examined the inhibitory effect of dendrobine, matrine, oxymatrine, chelerythrine and tryptanthrine on the substrate uptake mediated by SLCs.
Methods. We investigated the inhibitory effect of these natural alkaloid compounds on substrate uptake mediated through a range of important SLC transporters in overexpressing human embryonic kidney (HEK293) cells.
Results. Our data demonstrated that chelerythrine significantly inhibits the OATP 1A2 mediated uptake (~85% of inhibition), moderately reduces the influx of substrates of OCT1 (~51% of inhibition) and OCT2 (~58% of inhibition); tryptanthine is a potent inhibitor of OAT3 (~75% of inhibition).
Discussion. Our study demonstrated that chelerythrine and tryptanthrine selectively inhibit the substrate uptakes mediated by the essential SLCs, which suggests that precautions will be needed when co-administrating drugs with these compounds so as to prevent the unfavorable drug-drug/herb interactions in human.

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Are standard dosing regimens of piperacillin/tazobactam adequate for the management of febrile neutropenia? Answers from population pharmacokinetic modelling and Monte Carlo simulations

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Introduction. Standard doses derived from trials in healthy volunteers and non-critically ill patients may become inadequate in febrile neutropenic patients with malignancies due to disease-induced PK alterations.
Aims. This study aimed to describe optimal dosing regimens of piperacillin/tazobactam for the management of febrile neutropenia in patients with haematological malignancies.
Methods. Febrile neutropenic patients with haematological malignancies were enrolled when prescribed to receive piperacillin/tazobactam. Serial blood samples were collected and piperacillin concentrations were measured. Non-parametric population PK analysis and Monte Carlo dosing simulations were performed with Pmetrics® package for R.
Results. A two compartment model with between-subject variability on clearance, adequately described the data from thirty-seven patients. Mean ± SD parameter estimates were CL, 18.0 ± 4.8 L/h and central compartment V, 14.3 ± 7.3 L. High creatinine clearance (CrCL) was associated with poor target attainment. The cumulative fraction of response was sub-optimal for conventional dosing regimens and was maximized with prolonged infusions.
Discussion. Standard doses can result in sub-optimal exposures in patients with high CrCL and against bacteria with high minimum inhibitory concentration (MIC), e.g. *Pseudomonas aeruginosa*. Extended infusion of 4.0 g piperacillin over half of the dosing interval every six or eight hourly; or a loading dose of 4.0 g plus continuous infusion with 8 to12 g maximizes the probability of achieving conventional dosing target. Guidelines should address altered dosing needs of febrile neutropenic patients exhibiting high CrCL or infected with high MIC bacteria.

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Assessing the substrate depletion of EGFR kinase inhibitor metabolism

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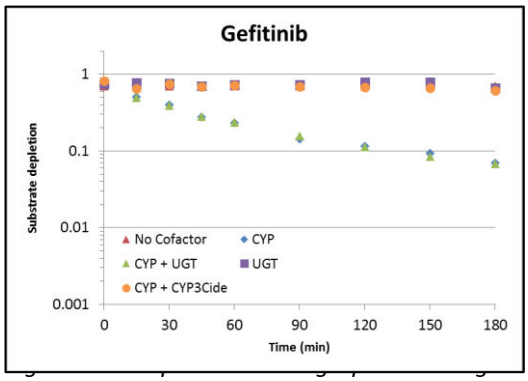

substrate depletion of gefitinib over time

Introduction. *In vitro* substrate depletion is a fast and feasible approach to assess intrinsic clearance of drugs and consequently predict intrinsic clearance *in vivo*. Epidermal growth factor receptor (EGFR) kinase inhibitors (KIs) have shown promising results in blocking cell proliferation in non-small cell lung cancer (NSCLC). However, they have a complex metabolism and therefore complicates optimal dosing. *In vitro* substrate depletion is a vital step in predicting *in vivo* clearance and hence exposure. This is particularly essential when dosing with narrow therapeutic index drugs such as KIs.
Aim. Assess substrate depletion of EGFR KIs by CYP and UGT catalysed metabolism in human liver microsomes.
Method. *In vitro* metabolism studies were performed for a panel of 5 KIs using human liver microsomes. The rate of microsomal KI metabolism was quantified in the presence and absence of CYP and UGT cofactors as well as a selective CYP3A4 inhibitor (CYP3Cide). The metabolism was assessed on the basis of the substrate depletion at an initial KI concentration of 1uM with 3 hour incubations at various time points.
Results. The substrate concentration remained essentially unchanged when incubated in the presence of UGT cofactor and CYP3Cide. Incubations assessing CYP3A4 activity (absence of CYP3Cide) with and without UGT cofactor demonstrated a similar and significant depletion in substrate for all KIs assessed (Fig.1)
Discussion. Clearances determined here by substrate depletion will be utilised in physiologically based pharmacokinetic (PBPK) modelling to predict KI exposure and clearance and assess the impact of covariates on these parameters.

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Potential role of psoralen in hepatotoxicity

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Introduction. Acceptance of complementary medicine has increased in recent years, despite the lack of evidence for safety. Herbal based Traditional Chinese Medicines (TCMs) form a significant proportion of these products, frequently containing multiple herbs. Commonly perceived as natural, therefore safe, TCMs are often taken with other pharmaceuticals or herbs. Several TCMs are linked to hepatotoxicity, both directly by herbal components or metabolites, or pharmacokinetic interactions with other drugs or herbs. Consequently, concerns of toxic adverse effects have risen. Currently, research has focussed on individual toxicity and herb-drug interactions, neglecting herb-herb interactions and polyherbacy. A recent case of fatal herbal hepatotoxicity may have involved the interaction of the herbs *Psoralea corylifolia*, *Astragalus propinquus* and *Atractylodes macrocephala*.

Aims. To determine the individual toxicity of primary components of significant herbs implicated in the fatal case of polyherbacy on two cell lines.

Methods. Primary components psoralen (from *Psoralea corylifolia*), astragaloside IV (*Astragalus propinquus*) and atractylenolide I (*Atracylodes macrocephala*) were purchased through Sigma. Products were screened at increasing concentrations in an MTT assay to establish toxicity on the hepatocyte cell line HepG2 and the intestinal epithelial cell line Caco2.

Results. Significant concentration-dependent toxicity was observed with psoralen in HepG2 cells at all concentrations ($n = 8$; $P<0.0001$) and Caco2 cells at the two highest concentrations ($n = 5$; $P<0.0001$). No significant effect was observed with astragaloside IV or atractylenolide I on either cell line ($n = 5$; $P>0.05$).

Discussion. Herb-herb interactions are poorly understood, with suitable models urgently needed. These studies show for the first time, that some major herbal medicine components can be studied in relevant tissue culture models. Interaction experiments in these models can potentially help better define the safety of herb-herb interactions.

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Investigation into the toxicity of a multi-ingredient herbal medicine in vitro

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Introduction Despite the popular belief that herbal medicines are safer compared to conventional pharmaceuticals, there continues to be reports of serious adverse reactions, including hepatotoxicity, following the use of herbal medicines. In Australia approximately 60% of adults are classified as overweight or obese. Herbal are fast becoming the most sought after weight loss treatments and thus are potentially hepatotoxic.

Aims. Survey weight loss supplements available on the Australian market for potential hepatotoxicity using toxicological substance screening an *in vitro* toxicity model.

Methods. Herbal medicines were purchased from commercial suppliers and extracts made by standard techniques. A combination of mass spectroscopy and chromatography was used to identify ingredients within the supplements as well as any contaminants or adulterants. Herbal medicine extracts and pure ingredients were tested on human hepatic carcinoma cells (HepG2) and neuronal model cells (PC12) to determine potential hepatotoxicity and neurotoxicity.

Results. Exposure of a multi-ingredient supplement containing green tea and garcinia cambogia on HepG2 and PC12 cells showed concentration-dependent hepatotoxicity and neurotoxicity with a biphasic pattern. Forensic toxicology screening of this product showed no known adulterants and contaminants.

Discussion. Drug-herb interactions and herb-herb reactions can lead to serious health effects. While there is reasonable information of drug-herb interactions, herb-herb interactions are less well understood and there is limited information regarding the exact components within herbal preparations causing toxicity and the mechanism behind this toxicity. With no known contaminants found within this supplement it leads to the question of why is this toxic to cells. There may be an interaction between hydroxycitrate acid found in garcinia cambogia and epigallocatechin-3-gallate or other catechins found in green tea. This will be further investigated in future experiments. As the popularity of these products continues to grow, further investigation is needed to understand why such serious adverse effects are occurring, the mechanism behind them to produce more effective safety advice.

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Mapping the percutaneous absorption of zinc after topical application of commercial products to human skin using synchrotron X-ray fluorescence microscopy

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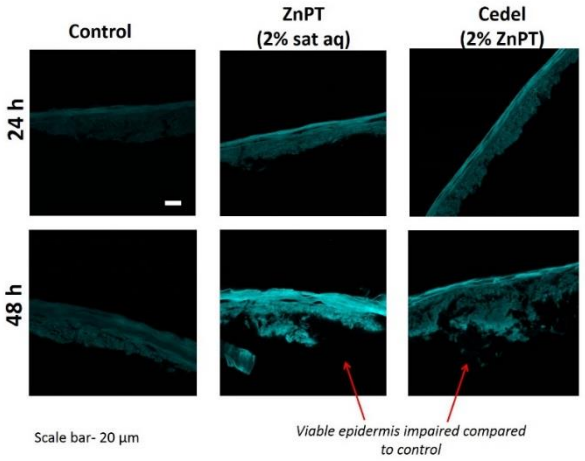
Introduction. Many topically applied consumer products contain zinc pyrithione (ZnPT). There is a gap in knowledge as to whether the release of zinc from these complexes can penetrate the skin and cause toxicity.
Aims. To determine whether there is an increase in zinc concentration within the human skin strata after topical application of ZnPT containing products. To also determine the *in vitro* cell toxicity, concentration, speciation and deposition of the permeating zinc within skin.
Methods. ZnPT containing formulations were topically applied at 10 mg/cm^2. Synchrotron light source X-ray fluorescence (XFM) was used to map and quantify the zinc within the skin strata.
Results. The areal density of zinc (mean ± SD, n=3) in untreated (control) skin was determined using XFM within the viable epidermis (VE) and was found to be 0.20 ± 0.04 µg/cm^2. After 48 h topical application of ZnPT (2% aq) the concentration increased >300 fold to 64.38 ± 8.67 within the VE. When a commercial product (Cedel) was applied for 24 h to the skin a ~ two fold increase in zinc concentration was observed within the VE 0.42 ± 0.14 cm^2. The Zinpyr-1 images (shown in Figure) support the XFM findings and various species of zinc can be found within the tissue including labile zinc (Zn^{2+}) after the application of ZnPT to the skin surface.



Discussion. We have shown ~300 fold increase in zinc concentration within the viable epidermis ZnPT (aqueous suspension) was applied topically that also coincided with a decrease in tissue integrity. The concentrations observed within the VE within this study are at concentrations that would be considered toxic.

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The effect of different concentrations of oxygen and heart drug (dofetilide) on the heart rate and lactate levels in cultured gestational day (GD 13) rat embryos.

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Introduction. Dofetilide is a potent Class III antiarrhythmic (tradename: Tykosyn) which selectively inhibits the rapid component of the time dependent outward potassium current (IKr). *In vitro* and *in vivo* it causes a significant decrease in rat embryonic heart rates (reversible) and *in vivo* is associated with hypoxia of rat embryos and birth defects (gestational days: GD11 and GD14) (Spencer et al, 1995 Ritchie et al, 2013; Ritchie et al, 2015).
Aim. Using the whole embryo culture model, this study examined the impact of hypoxia- induced bradycardia and dofetilide-induced bradycardia on the generation of a key energy metabolite of anaerobic glycolysis (lactate). Methods. Exp.1: Embryos were cultured in gas mixtures (Nil O_2 up to 80% O_2). Heart rates (HR) were recorded pre- and post- altering of the oxygen levels. Embryos were initially established in culture with a gas mixture of 95% O_2 (optimal gas condition for whole rat embryo culture). Exp. 2: Lactate content was determined in embryos cultured in 95% oxygen (normoxic), 20% oxygen (hypoxic) or dofetilide-treated embryos (cultured under normoxia).
Results. There was no significant difference between the HR of embryos cultured in 80% O_2 cf 95% O_2. However, the HRs of embryos cultured in 60%, 40%, 20% or nil O_2 rapidly decreased. Dofetilide-exposed embryos were bradycardic compared to control embryos, with a HR similar to embryos cultured in hypoxic conditions. However, lactate levels were similar in the dofetilide- and control-embryos and higher in hypoxia embryos.
Discussion. The increased lactate content under hypoxic conditions was likely a consequence of the changing ATP levels and shift from aerobic to anaerobic glycolysis driven by reduced oxygen tension. *In vivo*, it is known dofetilide-induced bradycardia in the embryonic heart is associated with embryonic hypoxia. However, *in vitro* lactate levels of dofetilide exposed embryos remained unchanged compared to hypoxic embryos (increased) suggesting the dofetilide effect on heart rate is not associated with a shift to anaerobic metabolism as a primary cause of bradycardia but related to the direct effect of dofetilide on the IKr channel.
Spencer SG et al (1994) Teratology 49:22-292; Ritchie, HE et al (2013) Birth Defects Research (Part B), 98(2), 144-153; Ritchie HE et al (2015) Birth Defects Research (Part B), 104(5), 196-203.

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Cardiovascular collapse induced by *Echis ocellatus* venom: an *in vivo* and *in vitro* examination

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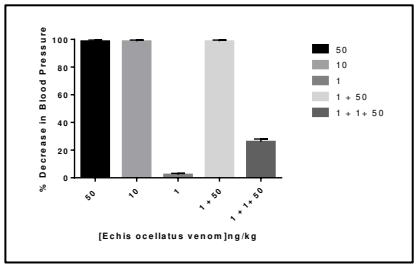
Introduction: Carpet vipers (*Echis ocellatus*) are highly venomous African snakes which cause thousands of deaths and permanent disability annually (Abubakar et al., 2010).
Aim: To investigate sudden cardiovascular collapse due to *E. ocellatus* in anesthetised rats.
Methods: Rats were anaesthetized (100 μg/kg ketamine/xylazine 10:1, i.p.) and the effects of *E. ocellatus* venom determined. *E. ocellatus* venom were also examined in rat small mesenteric arteries.
Results: Venom at 10 μg/kg (i.v.) and 50 μg/kg (i.v.), but not 1 μg/kg (i.v.), caused sudden cardiovascular collapse in anaesthetised rats as indicated by a rapid fall in blood pressure without recovery within 1-2 min. Prior administration of a single dose of venom (1 μg/kg, i.v.) or heparin (300 UI/kg, i.v.) did not protect against the subsequent addition of 50 μg/kg (i.v.) venom. However, prior addition of two doses of *E. ocellatus* venom (1+1 μg/kg, i.v.) or Brown snake (*Pseudonaja textillis*) venom (2+2 μg/kg, i.v.) at 5 min intervals prevented collapse by the subsequent addition of 50 μg/kg of *E. ocellatus* venom. Venom (0.001-1 μg/ml) induced concentration-dependent relaxation in pre-contracted mesenteric vessels although this was relatively weak in comparison to other snake venoms previously examined.



Discussion: *E. ocellatus* venom induces a sudden cardiovascular collapse in anaesthetised rats which can be prevented by the addition of small 'priming' doses, as seen previously for Australian elapid venoms. The collapse is likely due to the release of depletable endogenous mediators The venom did not cause significant relaxation in mesenteric arteries, indicating that the collapse is unlikely to be due to peripheral vasodilation.
Reference: Abubakar IS et al. (2010) PLoS Negl Trop Dis 4(7): e767

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Should CMT patients be waltzing with Lyrica?

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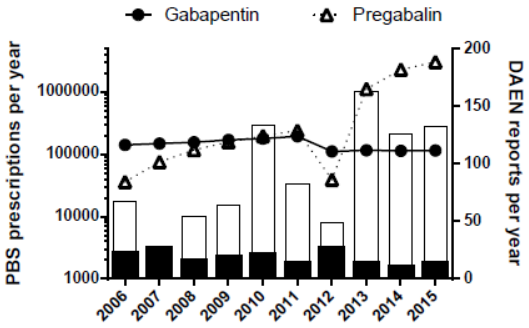
Introduction. Neuropathic pain is a common symptom of Charcot-Marie-Tooth (CMT) disease and as a consequence patients may be prescribed anticonvulsants. However these medications carry a risk of adverse effects which could be problematic for individuals with pre-existing peripheral neuropathies.
Aims. To capture the experiences of CMT patients using pregabalin and gabapentin.
Methods. The Database of Adverse Effect Notifications (DAEN) was used to capture adverse effects for pregabalin and gabapentin as experienced by a general population; prescription numbers for each were obtained from the PBS Australian Statistics on Medicines reports. Survey data from CMT patients was interrogated for self-reported side effects; follow-up in focus groups allowed us to expand on patient experiences when taking these medications.



Results. Since 2012 the prevalence of pregabalin use has steeply risen and so have the number of adverse effect notifications (see figure). For our population of 161 CMT patients 9 reported using pregabalin and 7 had experienced side effects; 2 patients reported side effects for gabapentin. Pregabalin use was independently mentioned as problematic in five out of our eight focus groups. Mixed results were obtained for whether participants believed that using these medications had permanently worsened their CMT neuropathy.
Discussion. As individuals living with an incurable chronic disease, CMT patients strive to maintain the best possible quality of life during an uncertain and unique disease progression. While chronic pain is undesirable, CMT patients may be fearful of taking drugs that could potentially worsen their peripheral neuropathy. Further research is required to understand whether adverse effects from anticonvulsants are increased in this patient population.

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Deaths from drugs and poisons in Australia, 2013

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Introduction. Mortality caused by licit and illicit drugs as well as poisons are a leading cause of injury mortality in Australia. Many of these deaths are preventable however; more annual reporting is needed to understand the problems Australia faces. Currently there are no all-encompassing studies looking at deaths from drugs, alcohol and poisons in Australia over a year.
Aim. To analyse which substances were most commonly implicated in poisoning deaths in Australia in the year 2013 and which factors were common among these deaths.
Methods. All closed medico-legal cases reported to Australian coroners in the year 2013 where drugs or poisons were involved in death were extracted from the National Coronial Information System. This data was combined with data from the Australian Bureau of Statistics to calculate specific death rates to analyse differences in factors such as age, sex, substance type, and location.
Results. Pharmaceuticals were the most common substance type found in poisoning deaths in Australia in 2013 involved in 63.2% of cases. Opioids analgesics were the most common substance with the highest nationwide death rate (2.75 per 100,000). Male death rates were higher for all substance types. The largest disparities by sex were seen amongst illicit drugs and other chemical substances. Inner regional areas had higher death rates from poisoning (8.82 per 100,000) compared to major cities (6.6 per 100,000).
Discussion. To greater prevent poisoning deaths, focus should extend beyond major cities and should focus largely on pharmaceuticals drugs, especially those with high potential for misuse and abuse.

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Pharmacology teaching and learning in the Doctor of Medicine course at the University of Western Australia

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Introduction. The 4 year postgraduate Doctor of Medicine (MD) course at the University of Western Australia (UWA) commenced in 2014, integrating the preparatory science disciplines with clinical training. Pharmacology is taught across three units during the first 18 months of the MD.
Aims. This study examined whether students engaged with and found value in a new teaching modality, namely the Treatment and Prescribing Seminar and obtained student feedback on learning experiences across the pharmacology component of the MD course.
Methods. The student learning experience was examined in surveys using a 5-point Likert scale and interviews (UWA Ethics Ref No. RA/4/1/6593 and RA/4/1/7533) in 2014 to 2016. Survey items were analysed using Kruskal-Wallis non-parametric tests. Interview transcripts were subjected to thematic analysis.
Results. More than 80% of students reported good understanding of the concept of safe and effective prescribing after the Treatment and Prescribing Seminar (Agreed or Strongly Agreed). While 92% of students in 2014 indicated that they were aware of the components required for a legal prescription (Agreed or Strongly Agreed), this decreased to 72% in 2016. Since students perceived that the use of breakout venues for discussions in 2014 was disruptive, the format was optimised in 2015 to divide students into groups within a single venue. Additionally, in 2015 and 2016, 91% of students felt that pharmacology integrated well with other subjects in the MD course (Agreed or Strongly Agreed), but only half thought pharmacology was well integrated in case-enhanced learning sessions. Students attested to the quality of pharmacology teaching in free entry comments, including "*Pharmacology lectures were stand out sessions across the pre-clinical stage of our course*" and "*I thought all/most of the lectures integrated well into the learning topics and were almost always very well prepared and considered*".
Discussion. The Treatment and Prescribing Seminar was found to engage students and provide a new and effective approach to this topic. While students recognised the value and quality of pharmacology teaching within the MD course, better integration of pharmacology into case-enhanced learning sessions needs further attention.

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Development of an international geriatric pharmacology curriculum for medical schools: systematic review and curriculum mapping

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Introduction: Understanding and application of pharmacology and therapeutics in old age and multi-morbidity is critical to ensure quality use of medicines in our ageing population. Education in geriatric pharmacology in medical schools is starting to emerge internationally.
Aims: To systematically review the published literature for evidence of existing geriatric pharmacology education in medical schools and use this to inform development of an international geriatric pharmacology curriculum.
Methods: A systematic literature review using subject terms "geriatric, pharmacology, clinical pharmacology, curriculum, medical students, medical education, skills, knowledge, and attitude in combination" was performed in MEDLINE, EMBASE, PsycINFO and Pubmed databases for studies published 2000-2016. Overall, 457 articles were screened by title and abstract. Based on inclusion criteria (available in English, focus on content or learning outcomes for pharmacology, clinical pharmacology or geriatric pharmacology, medical school education), 43 full text articles were screened further and any relevant curriculum and learning outcomes were mapped.
Results: Seventeen relevant peer-reviewed articles were identified. Of these, 12 articles described general pharmacology, 5 described clinical pharmacology and 5 included some geriatric pharmacology. Geriatric pharmacology topics included changes in pharmacokinetics and pharmacodynamics, dose adjustment to account for ageing physiology, medications that should be avoided or used with caution in older adults, consideration of the patient's goals of care, and collaboration with other health professionals to ensure safe prescribing for the elderly. No detailed, systematic or complete geriatric pharmacology curricula or learning outcomes were identified.
Discussion: This systematic review of existing literature highlighted that there is a need for a comprehensive international curriculum in geriatric pharmacology to teach medical students safe and effective prescribing for older patients.

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Evaluation of student perceptions of practical class resources

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Introduction. Practical classes are integral to science based undergraduate courses, providing students with the opportunity to obtain a range of skills, both generic and discipline specific. In addition, practicals provide an opportunity for active learning, thus enabling a deeper level of understanding of the relevant concepts. What students gain from practicals is determined not just by the in-class activity, but also by their motivation and engagement. While motivations can vary, student preparation for, and engagement with, practicals can be encouraged by providing resources including pre- and post-practical videos, as well as general "how-to" videos. The value of these resources is dependent on whether students perceive them as being helpful. Investigating student and staff perceptions of these resources, therefore, is an important component of their continued refinement.
Aims. To compare student and staff perceptions of the usefulness of supporting resources provided to students to aid preparation for the practical component of a core third year pharmacology unit at Monash University.
Methods. Student feedback was obtained via a paper-based survey (n=42), with questions relating to how they used the resources provided. In addition, students were asked to rate the usefulness of these in helping them to link the practicals to the lecture content and as preparation for the both the practicals and their related assessments. Teaching Associates (TAs) who demonstrated in the relevant practical classes were also asked for their opinions of the usefulness of these resources in helping students' understanding of the protocols and theory.
Results. The majority of students (88%) agreed that pre-practical videos helped them to link practicals to lectures and 95% agreed they helped to prepare them for each practical session. Post-practical videos helped students to understand what was happening in the practical and with the interpretation of the results (90% agreement). Although 90% of students agreed that pre-practical quizzes checked their understanding of the practical, only 45% agreed they would complete these if there was no mark associated with them.
Discussion. Overall, students do appear to find practical class resources useful and are more likely to make use of these if they are linked to marks. We need to explore alternative ways, of assessing whether the effectiveness of these resources goes beyond positive student perceptions.

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ePortfolios and reflective practice for enhancing teamwork and communication skills development in a third year Neuropharmacology course at UNSW Australia

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Introduction. Pharmacology students select the third year specialty course, *Neuropharmacology (PHAR3202)* at UNSW, as they are interested in growing their understanding of drugs and their effects on the brain. This discipline specific knowledge enables entry into pathways beyond their undergraduate degrees that include medicine, honours, pharmacy and the pharmaceutical industry. The development of co-curricular skills, such as teamwork and oral communication are critical for complimenting their knowledge and enhancing their employability upon graduation from a medical science degree program. ePortfolio pedagogy was implemented and used to build awareness of these skills and scaffold their development
Aims. To enhance the development of teamwork and communication skills that address graduate attributes at UNSW.
Methods. An innovative, authentic assessment task within PHAR3202, *'Controversies in Neuropharmacology',* applied a debate genre to allow student engagement with deep discipline-specific learning, reflective practice and research thinking. Students were placed in teams, assigned topics and provided with a series of prompt questions to start their reflective blogging journey. This allowed students to recognise their skills development as they progressed through the course, prepared for their debates and considered the effects of their experience.
Results. This approach facilitated awareness of professional skills development in teamwork and communication with a deeper understanding of research topics presented. Reflective practice increased awareness of negotiation skills, which proved useful in resolving issues that arose in teams.
Discussion. ePortfolio linked to authentic assessment in pharmacology allowed skills in teamwork, communication, research, critical evaluation which are graduate attributes important for future postgraduate studies and employability.

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Adverse Drug Reaction (ADR) Reporting and Follow Up in a Hospital Setting - A Patient's Perspective

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Introduction. Adverse drug reactions (ADRs) are a common cause of negative health outcomes for patients, often resulting in increases in hospitalisation, length of stay and the overall cost. For this reason it is important to put in place procedures that minimise risk of inadvertent re-exposure to a drug that has caused the patient an ADR.
Aims. To assess the effectiveness of the ADR reporting system and follow up process at the hospital.
Methods. Cross-sectional survey, with interviewer administered questionnaire, of people who experienced an ADR.
Results. Of the 241 eligible cases reviewed by the ADRC between 2013 and April 2016, 108 (45%) consented to the phone interview with only 82% (89) having recollection of the event. Of these 55% (49) recalled receiving a temporary ADR warning card and 73% (65) remember receiving a permanent ADR warning card post-discharge. The ADR warning card was carried by 73% (65) of participants. 85.4% (76) had told their regular GP about their ADR [41% (31) used their ADR warning card]. Only 40% (36) had told a pharmacist about their ADR [50% (18) used their ADR warning card]. 85% (76) had a regular pharmacy and of those, 53% (40) believed it necessary to tell their pharmacy and 62% (47) considered it a good idea for the hospital to automatically notify their pharmacy. Overall satisfaction was relatively high with 89% (79) agreeing that this adverse drug reaction service as a whole was valuable to them and 92% (82) agreeing that it is a good thing that a letter was sent to their regular GP. Some participants (21% (19)) also made comments with follow up issues being the most common theme.
Discussion. Overall, there was a relatively high level of satisfaction with the ADR service, provided support for this a model of care for patients who experienced an ADR. The current ADR warning cards were also found to be particularly useful for patients. There was potential for improvement, including increasing the number of patients remembering receiving a temporary ADR warning card prior to discharge. It is also evident that allowing patients to be responsible for communicating a new ADR to community pharmacist is not particularly effective so other means of notifying community pharmacists may need to be explored.

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*CYP3A5*3* genotype and *ABCB1* haplotype are associated with dose-adjusted trough blood tacrolimus concentrations in kidney transplant recipients

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Introduction. Tacrolimus (TAC) is an immunosuppressant with a narrow therapeutic window and highly variable inter-individual pharmacokinetics. It is metabolised by Cytochrome P450 3A and is a substrate of P-glycoprotein (P-gp, encoded by *ABCB1*). Pregnane X Receptor (PXR, encoded by *NR1I2*) regulates *CYP3A* and *ABCB1* expression. *CYP3A5* genotype affects TAC pharmacokinetics, however, the impact of *ABCB1* and *NR1I2* genetics remains controversial (Kravljaca et al, 2016; Li et al, 2015; Cusinato et al 2014).
Aims. To investigate the impact of *CYP3A5*, *ABCB1* and *NR1I2* genotypes on dose-adjusted trough blood TAC concentrations (C/D) in kidney transplant recipients.
Methods. Seventy-five transplant recipients were genotyped for *CYP3A5*3*, *ABCB1* 61A>G, 1199G>A, 1236C>T, 2677G>T, 3435C>T, *NR1I2* 8055C>T, -25385C>T and 63396C>T. Genotypes and *ABCB1* haplotypes were correlated with C/D at one-, three- and six-months post transplantation.
Results. Compared with *CYP3A5*1* carriers, *CYP3A5*3/*3* patients had significantly higher C/D at one-month (median [95% CI] 1.41 [1.34-1.64] *vs* 0.71 [0.53-1.32]; P=0.0001); three-months (1.50 [1.50-1.93] *vs* 0.73 [0.55-1.59]; P=0.0005) and six-months (1.68 [1.61-2.07] *vs* 0.84 [0.56-1.53]; P<0.0001) post-transplantation. *ABCB1* 61A-1199G-1236T-2677T-3435T haplotype carriers had significantly higher C/D than non-carriers (1.75 [1.64-2.33] *vs* 1.21 [1.08-1.50]; P=0.002) only at six-months post-transplantation. No other individual SNP or haplotype was associated with C/D (P>0.06).
Discussion. Our findings confirm that *CYP3A5*3* substantially affects TAC C/D. Additionally, it is the first time the *ABCB1* 61A-1199G-1236T-2677T -3435T haplotype has been reported to affect TAC C/D.

Kravljaca M. et al (2016) European Journal of Pharmaceutical Sciences, 83:109-13
Li J.L. et al (2015) Pharmacogenomics, 16(12):1355-65
Cusinato D.A.C. et al (2014) British Journal of Clinical Pharmacology, 78(2):364-72

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Beta2-adrenoceptor haplotypes associated with asthma susceptibility in a multi-ethnic population

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Introduction: Beta2-adrenoceptor (β2-AR) gene polymorphisms have been studied extensively as variations in this receptor would affect the efficacy of β2-agonists. This is a concern as β2-agonists play a major role in the management of asthma.
Aim: This study aims to determine the allelic, genotype and haplotype distribution and the linkage disequilibrium of the β2-AR gene polymorphisms and their association with asthma and ethnicity.
Methods: A total of 432 controls and 435 asthmatic patients were recruited and peripheral blood samples were taken for DNA extraction. The β2-AR gene was sequenced from the 5'-cistron leader till the end of the gene (total of 1492 bp). The allelic, genotype and haplotypes frequencies of the β2-AR gene polymorphisms were compared between controls and asthmatics. Linkage disequilibrium of the polymorphisms was determined by using Haploview.
Results: Based on literature and the GenBank, 19 polymorphic sites were identified. The allelic frequencies were not significantly different between controls and asthmatics. The genotype frequencies of A252G, A523C and C1053G were significantly associated (OR=2.25: OR=1.97: OR=1.51 respectively) with risk for asthma and ethnicity. The control group had 37 possible haplotype combinations of which 15 were not found among the asthmatics. The asthmatics had 37 possible haplotype combinations of which 17 were not found among the controls. There 20 haplotype combinations that were strongly associated with asthma. The linkage disequilibrium analysis showed that several of the polymorphisms were in strong linkage (e.g. T-47C and T-20C) and were co-inherited (r^2=0.994).
Discussion: The findings of this study identified strong association between β2-AR gene polymorphisms and susceptibility to asthma as well as ethnicity. The haplotype analysis of the 19 polymorphisms is the first in the country. These strong associations seem to indicate that the β2-AR gene polymorphisms play a major role in asthma susceptibility among the multi-ethnic population of Malaysia.

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Refereeing the Ribonucleotide Race: G-Quadruplexes, Mitochondria and Chronic Viral Infection

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Introduction. As the major biological energy currency, ribonucleotide triphosphates (NTPs) engage in myriad biological transactions besides nucleic acid synthesis. There is strong intracellular competition for NTPs, concentrations of which are stringently regulated and typically in the order ATP>>UTP>GTP>CTP. Consequently, (i) many reactions may be limited by the availability of GTP and/or CTP and (ii) overall NTP demand may be monitored by sensing the rate of flux through the CTP pool. Salvaging NMPs derived from RNA turnover - which is substantial, even in quiescent cells - saves energy, otherwise mostly derived from mitochondrial respiration. RNA turnover rates vary, depending rates of transcription, processing and nuclear export as well as the stability of processed transcripts, with secondary structure playing an important role in these processes. G-quadruplexes (GQs) are regulatory structures composed of two or more stacked groups 4 co-planar guanines which occur in both DNA and RNA. The human transcriptosome contains thousands of potential GQ-forming sequences (PGQS). Viral RNAs also contain PGGS which are presumed to have regulatory function(s). Despite its internal complexity, RNA recycling can be modelled biochemically, electronically and mathematically.

Aims and Methods. By using a variety of bioinformatics resources, (a) to identify PGQS in positive stranded RNA viruses; (b) devise mathematical and electronic models of NTP dynamics in uninfected and virus-infected cells and (c) to validate the models by applying them to published data.

Results and Discussion. Initially, genera from the Flavivirus family were chosen because of their association with widely different pathogenesis ranging from severe and acute (the Haemorrhagic Fever group), via cryptic and chronic (Hepatitis C) to symptomless and benign (Pegiviruses). Rates of development of disease symptoms were found to be inversely related to genome G+C content and PGQS (a surrogate control index). The correlation was confirmed by similar findings from representatives of other positive sense RNA viruses, implies that, in some cases, infection may confer a short-term survival on the infected cell by rapidly scavenging and conserving NMPs. Any survival advantage is lost if the infecting virus outcompetes the mitochondria in the NMP recycling race.

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CYP2C9 and *CYP2C19* genotype frequency in Aboriginal and non-Aboriginal Australians using the DMET Plus array: a pilot study.

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Introduction. A greater understanding that ethnicity can play a major role in drug efficacy and adverse effects has informed some medicines prescribing guidelines to advise that genotyping is warranted to avoid life-threatening drug toxicity, such as carbamazepine, *HLA-B*1502* and severe skin reactions. Little is known on pharmacogenomics of Aboriginal Australians, although Griese et al (2001) reported on CYPs *2D6*, *2C19* and *2E1*.

Aims. To determine the feasibility of collecting, transporting and analysing saliva samples from Aboriginal Australians and non-Aboriginal Australians for pharmacogenetic analysis of *CYP2C9* and *2C19* using the Affymetrix DMET Plus array.

Methods. Following ethics committees' approvals and informed consent, participants provided a saliva sample using Oragene DNA Saliva Kits (DNA Genotek Inc. Canada). Samples were transported unrefrigerated to Adelaide for DNA isolation and genotyping using the array that assesses, amongst others, 18 *CYP2C9* and 18 *CYP2C19* alleles.

Results. Forty self-identified Aboriginal Australians (19-50 years, 9M/31F) and 51 non-Aboriginal (24-65 years, 21M/30F) participants' samples and data were obtained. For *CYP2C9*, the frequency of the **2* and **3* (and linked 55323A>T) variants were similar (18 vs 16%; P=0.8; 5.1 vs 3.9% P=0.7, respectively). The **5*, and **12* variants were rare (0-1.25%) in both cohorts. For *CYP2C19*, the gain-of-function **17* was significantly lower in the Aboriginal Australians (8.8 vs 26%; OR 0.28 (95% CI 0.11 to 0.69), P=0.004), but **2* (19 vs 9%), **2B* (0 vs 2.0%) and 80161G>A (5.1 vs 3.9%) were not significantly different (P>0.08). The metaboliser phenotype status was UM (18 vs 37%), EM (50 vs 45%), IM (28 vs 18%), PM (5 vs 0%) and was not significantly different (P=0.08).

Discussion. The study highlights that it is feasible to conduct pharmacogenomic studies in Aboriginal Australians and suggests that drugs metabolised by CYP2C19 may have different pharmacokinetics in Aboriginal Australians in relation to the *CYP2C19* gain-of-function **17* allele. Whether this translates into altered response is unknown.

Griese UE et al (2001) Pharmacogenet 11:69-76.

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Structure based activity of small molecule pyridine $P2X_1$-purinoceptor antagonists for the development of a non-hormonal male contraceptive via the blockade of sperm transport.

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Introduction. Sperm transport through the vas deferens is regulated by sympathetically innervated smooth muscle cells that contract in response to neuronally released ATP and noradrenaline, acting at P2X1-purinoceptor ligand-gated ion channels and G protein-coupled α_{1A}-adrenoceptors, respectively. Genetic deletion of these receptors causes male infertility in mice (White et al, 2013). Pharmacological blockade at these receptors provides a therapeutic target to achieve male infertility and thus a male contraceptive. However suitable P2X1-purinoceptors are not currently available. Novel small molecules were generated for testing as $P2X_1$-purinoceptor antagonists, based on pyridine compounds that have been shown to possess antagonistic characteristics at P2X-purinoceptors.
Aims. To generate a range of favourable substituents added to the phenyl moiety of 2-phenyl-5,6,7,8-tetraydroquinoxalinen, to establish structure-activity relationships for inhibition of nerve-mediated contractions of isolated rat vas deferens.
Methods. Vasa deferentia from male Sprague-Dawley rats were mounted in isolated organ baths and isometric force was recorded in response to electrical field stimulation (60 V, 1 ms, 0.2 Hz) or exogenous application of αβ-methylene ATP (3nM-10μM), in the absence and presence of test compounds.
Results. Cumulative inhibitory concentration-response curves showed that para-substituted compounds with small non-polar aliphatic groups with methyl branching were more potent ($n = 4$, $IC_{50} = 43.7$, $P < 0.0001$). Non-polar or polar meta-substituted compounds, showed poor antagonistic activity and failed to attenuate electrical field stimulation induced contractions. Interestingly, dual substitution of small non-polar substituents in the meta and para position increased activity of the antagonist to a lesser extent than para-substituted aliphatic groups.
Discussion. Aliphatic side chains are non-polar and hydrophobic causing an increase in lipophilicity. The increased activity of small bulky aliphatic substituents in the para position suggests that the P2X1-purinoceptor binding site contains a hydrophobic pocket which may sterically hinder longer aliphatic and polar chains.
White et al (2013) PNAS 110: 20825-20830

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Visualising functional AC arrangements of the 5-HT_3 receptor

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Introduction. 5-HT_3 receptors are ligand-gated cation channels present in both central and peripheral nervous systems. 5-HT_3 receptor antagonists are used to treat various conditions such as diarrhea predominant-irritable bowel syndrome (IBS-D), chemotherapy induced nausea and vomiting (CINV) and depression. Multiple 5-HT_3 receptor subtypes exist, distinguished by the arrangement of five distinct subunits that comprise the complete receptor. Receptor subunit arrangement may contribute to differences in efficacy observed with the 5-HT_3 receptor antagonists (Yaakob et al. 2011).
Aims. To visualise functional 5-HT_3 receptors to better understand the contribution of the C subunit.
Methods. HEK293T cells were transiently transfected with constructs of 5-HT_3 receptor subunits containing fluorescent protein inserts between the 3rd and 4th transmembrane spanning region. Heteromers containing the C and A subunits were compared with homomers containing only the A subunit using whole cell patch clamp recording and super resolution microscopy.
Results. The A subunit is necessary to obtain functional AC subunits at the cell surface. Our preliminary experiments indicate that 40-50% of the receptors at the plasma membrane are AC heteromers and the remainder are A homomers ~40% with <5% C homomers. The 5-HT_3 receptor C subunits contributed subtle changes in the electrophysiological responses to 5-HT. However, ondansetron exhibited reduced efficacy on the AC heteromer relative to the A homomer.
Discussion: The C and A subunits interact to form receptors at the cell surface. Patch-clamp experiments indicate that the presence of C subunits alters the efficacy of the clinically used antagonist ondansetron. Predisposition to forming 5-HT_3 receptor heteromers could contribute to inadequate response observed in up to 30% of patients treated for CINV and IBS-D.

Yaakob N et al. (2011) Current Molecular Medicine 11:57-68

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Does the non-neuronal cholinergic system play a role in inflammatory bowel disease?

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Introduction. Cells such as epithelial and immune cells that are not innervated by cholinergic neurons secrete acetylcholine (ACh) and express nicotinic (nAChR) and muscarinic (mAChR) receptors. These receptors are involved in the paracrine and autocrine regulatory loop of ACh that is released from these non-neuronal cells (Wessler et al., 2003). The role of the non-neuronal cholinergic system (NNCS) in pathological processes is unclear.
Aims. To determine whether there are alterations in the gene and protein expression of the main components of the NNCS in the colon of patients with inflammatory bowel disease (IBD).
Methods. Quantitative real time PCR was used to determine the gene expression of the main molecular components of the NNCS (ChAT, α7 nAChR, OCT1, OCT3, M_2 and M_3 AChR) in colonic mucosa and smooth muscle of ulcerative colitis (UC) and Crohn's disease (CD) in comparison with gender and age matched control samples. Immunohistochemistry of ChAT and α7 nAChR was performed to determine their cellular distribution.
Results. In UC specimens, α7 nAChR mRNA expression was significantly increased in UC mucosa and muscle, by 5.8 fold ($P < 0.0001$) and 3.9 fold ($P = 0.0311$), respectively, compared to control. OCT3 mRNA expression was also increased in UC mucosa, but not UC muscle. Alterations for other genes in UC were not observed. Conversely, α7 nAChR mRNA expression remained unchanged in CD. However, mRNA encoding OCT1 and OCT3 in CD was increased by 3.2 fold ($P = 0.0009$) and 2.3 fold ($P = 0.0155$) respectively. ChAT and α7 nAChR immunporeactivity was localised on colonic epithelial cells, with strong staining on cells in the lamina propria area, and these cells are likely to be leukocytes. Qualitatively, there was an upregulation in ChAT and α7 nAChR protein expression.
Discussion. The positive ChAT staining in epithelial cells and leukocytes suggests the existence of the NNCS in the human colon. The altered expression of the NNCS in IBD is likely to be a factor of importance in the pathophysiology of IBD. Stimulation of α7 nAChR is known to reduce inflammatory cytokines (Kawashima et al., 2015). Thus, its upregulation in UC may indicate an adaptive response of the body to decrease inflammation.
Wessler, I. et al., (2003). *Life Sciences*, *72*(18), 2055-2061.
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Are connexins 43 and 45 ATP release channels in the porcine bladder?

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Introduction. Connexin (Cx) proteins form gap junctions between adjacent cells to regulate cell-to-cell communications and form hemichannels to release small molecules to the extracellular environment. Aims. This study was aimed to localize Cx43 and Cx45 expression in the porcine bladder, a well-recognized model to study human bladder function, and to investigate their functional role as ATP releasing channels. Methods. Immunohistochemistry (IHC) was conducted to localise Cx43 and Cx45 expression in intact porcine bladder tissues. Isolated urothelial, suburothelial and detrusor muscle cells were cultured to measure Cx43 and Cx45 mediated ATP release in response to hypotonic (~50%) induced stretch and Ca2+ depletion ([Ca2+]0, ~17nM). Results. Cx43 and Cx45 showed similar cellular distributions in the porcine bladder. In urothelium, Cx43 and Cx45 expression spans from basal layer to terminally differentiated umbrella cells. Positive signals were also seen on the surface of detrusor cell membranes as well as on some spindle shaped cells of suburothelial layer. Stretch induced a significant rise in ATP release from all three cell types ($P<0.01$). The blockage of Cx43 and Cx45 channels by their mimetic peptides significantly reduced ATP release from cultured urothelial cells in a concentration dependent manner by ~51%. For suburothelial and detrusor muscle cells, a trend of decrease of extracellular ATP release was observed in the presence of Cx43 or Cx45 mimetic peptide, but the results were not statistically significant. [Ca2+]0 also stimulated ATP release from all three cell populations which was inhibited by Cx43 peptide in all three cell types by ~50%. On the other hand, Cx45 peptide significantly reduced ATP release from urothelial cells by 52%. The effect of Cx45 peptide on other two cell types was insignificant. Discussion. Here, we report for the first time that Cx43 and Cx45 are ATP release channels in response to physiological stretch in the porcine bladder. This indicates that these channels are among several other channels, such as pannexin-1, that release ATP to initiate autocrine/paracrine signaling in response to bladder distension during the storage phase of micturition reflex. Furthermore, modulation of extracellular Ca2+ may also regulate ATP release in porcine bladder through Ca2+ sensitive Cx43 and Cx45 hemichannels.

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Biological effects on rat prostatic smooth muscle and chemical fractionation of *Costus speciosus* rhizome

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Introduction. *Costus speciosus* (Crêpe ginger or Spiral ginger) has been traditionally used by Sarawak natives to treat urological disorders.
Aims. To assess the biological effects of *C. speciosus* on prostate contractility and isolate bioactive components.
Methods. *C. speciosus* rhizome and roots were harvested from Sarawak. Extracts of dried and ground plant materials were obtained using water at different temperatures (i.e. 100°C, ~70°C, and ~21°C). Activity of these extracts was evaluated pharmacologically by assessing their effects on contractions of isolated rat prostate gland. Nerve mediated contractions were evoked electrically (0.1-20 Hz, 0.5 ms pulse duration, 60 V) while direct muscle stimulation was achieved by application of the exogenously administered agonists: noradrenaline, acetylcholine or ATP. Various pharmacological tools were used to identify mechanisms of action.
Results. *C. speciosus* rhizome and root decoction (100°C) extract (2.0 mg/mL) inhibited electrical field stimulation (EFS) induced contractions of rat prostatic smooth muscle by 64 ± 9.8% and 60 ± 11.5% at frequencies of 1.0 Hz and 2.0 Hz, respectively (p = 0.0161, n=4); whereas cold water (~21°C) extract (2.0 mg/mL) inhibited contractions by 73 ± 3.4% and 76 ± 2.2% at 1.0 Hz and 2.0 Hz, respectively (p < 0.0034, n=4). Contractions mediated by exogenous administration of noradrenaline (n=6), acetylcholine (n=6) or ATP (n=4) were not inhibited by rhizome extract (2.0 mg/mL). EFS induced contractions were still attenuated by the extract (2.0 mg/mL) in the presence of all inhibitors tested. In addition, extract (2.0 mg/mL) caused a concentration-dependent transient tonic contraction (p = 0.0092, Mean = 1.31 ± 0.32 g, n = 6) of the unstimulated prostatic tissue. The magnitude of the tonic contraction produced was also different in the presence of different pharmacology tools.
Discussion. The *C. speciosus* rhizome and root extract inhibits nerve mediated prostate smooth muscle contraction presumably by inhibition of neurotransmitter release by an unidentified pre-junctional mechanism. Tonic prostatic smooth muscle contraction induced by addition of extract indicates the existence of bioactive compounds in the rhizome extract that act via a different mechanism.

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Evaluation of Active efflux protein expression in gastrointestinal cell lines using chemically defined media

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Introduction: Previous work in our lab has suggested a link between P-gp activity and antimicrobial activity in gastrointestinal cells. Our current model is that P-gp is secreting an endogenous peptide acting as an antimicrobial agent. To isolate these proposed antimicrobial substrates it important to isolate them in an environment that is completely defined, or at least with the minimal amount of serum as possible, as the variable nature of serum can interfere with identification of such secreted product, especially if it is protein based. The level of active efflux transporters expression was examined in the different concentrations of FCS,
Aim**:** To measure the change in expression and activity of active efflux transporters, P-gp and BCRP in Caco-2, Ls174T and RKO cell lines and to detect and quantify antimicrobial activity in the conditioned medium when cultured in defined vs serum based medium.
Methods: Cell lines were cultured in chemically defined media using Insulin-transferrin-selenium-ethanolamine, with no added or 2% added FCS. In addition normal medium with 10% FCS was used as a control. Western blotting was done to compare the expression of the active efflux transporters between the cell lines and RT-PCR was done to observe mRNA changes. Trans epithelial electrical resistance (TEER) value was measured to monitor the barrier function of Caco-2 cells. Antimicrobial activity was measured and quantified from conditioned media of these cells lines.
Results: Caco 2 cells showed inconsistent active efflux protein expression in serum free media. However, 2% FCS was enough for adequate Caco-2 cell growth characteristic and P-gp expression compared to baseline levels in 10% FCS.. LS174T and RKO cell lines were able to thrive in fully defined medium devoid of FCS. Caco2 cells growing in serum free media and 2% FCS showed decreased TEER value indicating their barrier integrity diminished in comparison to cells growing in 10% FCS. Antimicrobial activity was still observed in the 2% FCS medium from Caco2 and LS174T cell lines, with P-gp induction associated with delaying bacterial growth. This indicates that a link could be established with increased P-gp and delayed growth of microorganisms suggesting the antimicrobial activity itself maybe because of the efflux mechanism of P-gp. Isolation of the causative agent is still ongoing.

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GPCRs effect on human alveolar basal epithelial carcinoma cells (A549)

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Introduction. The Transient Receptor Potential ion channel, member V3 (TRPV3) is a non selective, calcium permeable cation channel. Selective agonists and antagonists have not been identified for TRPV3, making it difficult to understand and study its physiological activation and modulation. It has been reported that TRPV3 is expressed in lung cancer cells (Li et al., 2016). The A549 lung cancer cell line is a useful to characterize TRPV3 function and to understand its role in lung and to compliment studies in TRPV3 transfected cells.
Aims. To investigate the expression of TRPV3 and the effect of a range of GPCR (Protease-activated receptors 1($PAR_{1)}$ Protease-activated receptors 2 (PAR_2) and B2 bradykinin receptor agonists on intracellular calcium levels in A549 cells. To investigate the effect of intracellular signalling on A549 cell responses to a TRPV3 agonist 2-Aminoethoxydiphenyl borate (2-APB) .
Methods: Western blotting was used to examine protein expression. Calcium imaging with Fura-2AM was used to determine receptor function in a plate-reading fluorimeter (FlexStation III and FLIPR Tetra). Thapsigargin was used to deplete intracellular calcium stores. Gq11 responses were inhibited using using UBO-QIC and TRP-channel mediated influx of calcium was inhibited using Ruthenium red (RR).
Results. We found that TRPV3 protein was expressed in A549 cells. Functional assays revealed that there was a significant increase in $[Ca^{2+}]_i$ when A549 cells were exposed to the 2-APB and to agonists of $PAR_{1,}$, $PAR_{2,}$ and to B2 bradykinin receptor. The Gq/11 inhibitor UBO-QIC blocked the response. Removal of extracellular calcium did not prevent a response to 2-APB, however depletion of intracellular stores with thapsigargin prevented it.
Discussion. TRPV3 may play a role in lung function. A better understanding of its function and responsiveness will further our knowledge of the role of this channel in the body. The finding that intracellular calcium stores are necessary for the 2-APB response of A549 cells suggests that intracellular calcium is necessary for activation of TRPV3. An alternative explanation is that 2-APB is causing increased $[Ca^{2+}]_i$ through another mechanism
Li, X., Zhang et al (2016). Int J Mol Sci, *17*(4). Switzerland

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Investigation of G-Protein-Coupled Receptor-Receptor Tyrosine Kinase heteroreceptor complexes using Bioluminescence Resonance Energy Transfer (BRET)

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Introduction. Compelling evidence exists that G-protein-coupled receptors (GPCRs) can form homodimers, heterodimers and higher-order oligomeric complexes. More recently, it has been suggested that GPCRs can physically interact with Receptor Tyrosine Kinases (RTKs) to form functional oligomeric complexes[1].

Homodimers				
Donor	**Acceptor**	**BRETmax±SEM**	**P value**	**n**
Nluc-A_1R	SNAP-A_1R	0.010 ± 0.009	< 0.05	6
Nluc-A_3R	SNAP-A_3R	0.021 ± 0.001	< 0.001	5
Nluc-A_{2A}R	SNAP-A_{2A}R	0.096 ± 0.014	< 0.001	5
Nluc-β_2AR	SNAP-β_2AR	0.088 ± 0.012	< 0.001	5
Heterodimers				
Donor	**Acceptor**	**BRETmax±SEM**	**P value**	
Nluc-VEGFR2	SNAP-A_1R	-	ns	5
	SNAP-A_3R	-	ns	5
	SNAP-A_{2A}R	0.017 ± 0.001	<0.001	6
	SNAP-β_2AR	0.015 ± 0.002	<0.01	5

Aims. In this study we have used BRET technology to investigate the potential for adenosine (AR) or β_2-adrenergic (β_2AR) receptors to form heteromeric complexes with vascular endothelial growth factor receptor-2 (VEGFR2).
Methods. HEK293 cells were transiently transfected with NanoLuc-VEGFR2 cDNA (BRET donor), together with SNAP-tagged-GPCRs (BRET acceptor). Cells were treated with cell-impermeable SNAP-Tag substrate Alexa F488 for 30min at 37°C/5%CO2 in DMEM/10%FCS. After 30min, cells were incubated with Hank's buffered salt solution (HBSS), and the NanoLuc substrate furimazine for 5min at 37°C. Emission recordings were then made with a PHERAstar FS plate reader (BMG Labtech) using 460nm (80nm bandpass; donor emission) and 535nm (60nm bandpass; acceptor emission).
Results and Discussion. BRET ratio data showed that A_{2A}R or β_2AR can associate with VEGFR2. In contrast, the adenosine receptors A_1 and A_3 subtypes did not show a significant BRET response with VEGFR-2. However, both A_1 and A_3 receptors were able to form homodimers.
[1] Ferré S, *et al.* (2014) *Pharmacological Reviews* **2**(66):413-434

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Glucagon receptor intracellular loop 1; a role Gαq/11-mediated intracellular calcium release

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	cAMP	ERK 1/2	iCa^{2+}
Ligand	**pEC_{50}**	**pEC_{50}**	**pEC_{50}**
GCG	9.5 ±0.1	6.8 ±0.2	7.0 ±0.1
Oxynt	8.0 ±0.2#	6.1 ±0.2#	6.1 ±0.2
TH-GCG	7.0 ±0.1	7.1 ±0.2	N/R
des-His	7.7 ±0.3	6.0 ±0.2#	N/R

Table 1. #, E_{max} similar to GCG, N/R, no response

Introduction: The glucagon receptor (GCGR), a family B G protein-coupled receptor (GPCR), plays an important role in regulating blood glucose levels through its ability to bind the 29 amino acid peptide hormone glucagon. Further it is one of only two family B GPCRs to have a defined crystal structure therefore making it an ideal receptor to study and to generate homology models.
Aims: To understand the contribution of ICL1 in modulating agonist bias at the GCGR.
Methods: We performed an alanine scan of the intracellular loop (ICL) 1 (ICL1) of the GCGR (residues G165-T172). The cell-surface expression of wild-type or ICL1 mutant GCGR was determined in transiently transfected HEK 293T cells using FACS analysis. Cells where stimulated with various ligands and measurement of second messengers investigated using a number assays including cAMP accumulation, ERK 1/2 phosphorylation and intracellular Ca^{2+} (Ca^{2+}i) mobilization.
Results: We found negligible difference in cell-surface expression between wild-type and alanine containing GCGR mutants with the exception of C171A, which show no detectable expression. Similarly, there was negligible difference in cAMP production or ERK 1/2 phosphorylation. In contrast, K168A, L169A, H170A and T172A showed a reduction in Ca^{2+}i (E_{max} expressed as % of wild-type GCGR: 65.2 ±5.5, 54.4 ±4.8, 50.9 ±4.9 and 70.0 ±5.3 respectively), which, through the use of a specific inhibitor; YM-254890, was confirmed to be $G\alpha_{q/11}$-mediated.
Discussion: Expanding the current knowledge available on the GCGR is of huge academic and potentially therapeutic importance. The findings presented here suggest that amino acids of ICL1 may play a role in $G_{\alpha q/11}$-mediated signaling. Due to the highly conserved nature of ICL1 within GPCRs both in terms of length and amino acid sequence, these results might also be important for other GPCRs.

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An investigation into biased signalling at the glucagon receptor

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Introduction: The glucagon receptor (GCGR), a family B G protein-coupled receptor (GPCR), plays an important role in regulating blood glucose levels through its ability to bind the 29 amino acid peptide hormone glucagon. Although the functional coupling of a single GPCR to more than one G protein has been known for years, it is a relatively new phenomenon that ligands can determine a bias towards a particular G protein [1].
Aims: To investigate alternative G proteins coupling following agonist stimulation at the GCGR.
Methods: HEK 293T cells transiently transfected with GCGR were stimulated with various ligands and secondary messengers measured using cAMP accumulation, ERK 1/2 phosphorylation (pERK 1/2) and intracellular Ca^{2+} mobilization (Ca^{2+}i) assays. Application of YM-254890 was used to assess the extent of $G\alpha_{q/11}$-mediated Ca^{2+}i.
Results: cAMP and ERK 1/2 responses were detected following stimulation with glucagon (GCG), Oxyntomodulin (Oxynt), TH-Glucagon (TH-GCG) and des-His^{1}[Glu^{9}]-Glucagon(1-29)amide (desHis) whereas Ca^{2+}i was only detected for GCG and Oxynt (Table 1). Both Ca^{2+}i responses were confirmed to be $G\alpha_{q/11}$-mediated. Further to this, mutation of TM4, previously shown in another family B GPCR to disrupt homodimerisation and Ca^{2+} signaling [2], abolished Ca^{2+}i signaling while showing small (<10-fold) affects on cAMP accumulation and pERK 1/2.
Discussion: The GCGR displays agonist bias and mobilized Ca^{2+}i in a $G_{\alpha q}$-dependent manner. Mutation of the TM4 hydrophobic interface in GCGR, also attenuates $G_{\alpha q}$-mediated Ca^{2+}i mobilization suggesting a role for dimerization in $G_{\alpha q}$-coupling.

[1] Lefkowitz RJ, Shenoy SK (2005). *Science* **308**: 512–517.

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Small molecule positive modulators of a class B GPCR, the adrenomedullin 1 receptor

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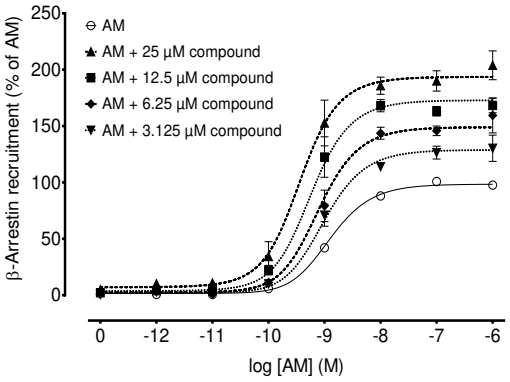
Introduction. Adrenomedullin (AM) is a vasoactive peptide hormone which mediates effects through the adrenomedullin 1 (AM_1) receptor. Targeting this receptor has therapeutic potential for cardiovascular disease, lymphoedema, and other vascular disorders. However, this is a challenging target as the AM_1 receptor shares its central G protein-coupled receptor (GPCR) component with the calcitonin gene-related peptide (CGRP) receptor. The peptide ligand specificity of each receptor is determined by the interaction of a receptor activity-modifying protein (RAMP) with the GPCR, the class B calcitonin-like receptor (CLR).



Aims. To identify specific small molecule modulators of the AM_1 receptor.

Methods. Structural information including a CGRP receptor small molecule antagonist bound to the CLR extracellular domain was used to design a series of compounds that retain the GPCR-binding motif while altering the RAMP-binding component. Compounds were screened using the DiscoveRx PathHunter β-arrestin recruitment assay at both AM_1 and CGRP receptors. Compounds were further validated by measuring receptor-mediated cAMP production in different cells to the primary screen as well as an endothelial cell line.

Results. A series of structurally-related compounds displayed positive modulation of the AM_1 receptor but not the CGRP receptor. As shown, the compounds increase the activity of AM in a concentration-dependent manner.

Discussion. These compounds are the first small molecules known to interact specifically with the AM_1 receptor. They are also one of the few examples of positive allosteric modulators of class B GPCRs.

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Dissecting the bias and binding properties of chemically distinct Glucagon-like peptide-1 receptor agonists

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Introduction: The Glucagon-like peptide-1 receptor (GLP-1R) is a class B GPCR that is a key therapeutic target for type 2 diabetes and obesity. It is pleiotropically-coupled to multiple signalling pathways and is subject to biased agonism. To date, the pathophysiological significance of biased agonism at the GLP-1R is unclear. To identify the importance of this phenomenon requires the identification of chemically distinct ligands with novel bias that can be studied in an *in vivo* setting. In addition, to be able to rationally design biased agonists for novel therapeutics requires an understanding of how biased ligands interact with the GLP-1R to promote distinct signalling profiles.

Aim: To characterise novel GLP-1R ligands for biased agonism and understand how they interact with the GLP-1R.

Methods: A series of novel peptide ligands were pharmacologically assessed in CHOFlpIn cells stably expressing the GLP-1R. Included in this study was Exendin-P5, a previously identified biased agonist (Zhang et al, 2015). Four signalling endpoints were assessed; cAMP accumulation (via a Lance HTRF assay), ERK1/2 phosphorylation (via an Alphascreen assay), iCa^{2+} mobilisation (using a Fluo4AM kinetic assay) and β-arrestin 1 recruitment (via a BRET approach). Bias was quantified using the Black-Leff operational model (Black and Leff, 1985). Mutagenesis of key residues within the GLP-1R, followed by pharmacological characterisation was used to determine differences in how a subset of identified biased peptides interact with the GLP-1R to promote their downstream signalling.

Results: The reported bias of Exendin-P5 towards G-protein mediated signalling relative to β-arrestin 1 when compared to Exendin-4 was confirmed. Two novel 11mer peptide ligands were biased agonists relative to GLP-1. Mutagenesis of a transmembrane binding cavity identified within GLP-1R molecular models revealed key residues that are important for affinity and efficacy of discrete biased ligands.

Discussion: This study has identified novel biased agonist ligands for GLP-1R and identified key residues that are critical for the affinity of different ligands and for driving biased agonism.

Zhang et al. (2015) *Nat. Comm.* 6:891
Black and Leff (1984) *BJP* 84:561-571

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Teaching old receptors new tricks: computational method to generate new GPCR/ligand complexes

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Introduction. G protein-coupled receptors (GPCRs) are a ubiquitously expressed superfamily of transmembrane proteins that play crucial roles in cell physiology. Being key drug targets in many diseases, drug discovery efforts harness the increasing structural information about these receptors to rationally design drugs in the field of structure-based drug design (SBDD). SBDD uses atomic detail GPCR X-ray binding pockets as tools to screen large libraries of potential drug lead candidates using virtual screening (VS). Retrospective VS performance of a binding pocket is assessed using the relative recovery of known ligands for that GPCR over decoy molecules. Stark differences in VS performance for some binding pockets over others are associated with small differences in structural features that include ligand/receptor interaction pattern and their predicted interaction strength. Indeed, these small changes can improve the identification of potential hit molecules, but they also determine the pharmacology of these potential hits. Currently, sparse availability of experimentally determined GPCR/ligand complex structures impedes the application of SBDD programs that aim to identify new molecules with the desired pharmacological profile.
Aims. We aim to expand on the available GPCR X-ray structures and generate accurate GPCR/ligand model complexes that have improved VS performance and are selective towards molecules of specific pharmacological profile.
Methods. We have developed a computational method that refines a GPCR pocket conformation using a single known ligand for that GPCR. The ligand directed modelling (LDM) method is a computationally efficient iterative workflow consisting of protein sampling and ligand docking.
Results. Comparison of LDM refined binding pockets to GPCR X-ray structures reveals improvement in VS performance in 21 out of 24 cases assessed. With this study focussed on seven different GPCRs bound to a range of ligands with different chemotypes and pharmacological profiles, we also identify the key receptor/ligand interaction patterns that are associated with each VS binding pocket performance.
Discussion. One key outcome is that the LDM is successful in all cases of inhibitor-bound to agonist-bound binding pocket refinement, which represents a key feature for GPCR SBDD programs. Indeed agonist ligands are often required for therapeutic intervention, however GPCR X-ray structures are mostly available in their inactive inhibitor-bound state.
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Characterisation of signalling and regulation of common calcitonin receptor splice variants and polymorphisms

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Introduction. The calcitonin receptor (CTR) is a Class B GPCR involved in bone homeostasis and remodelling and it is a clinical target for the treatment of osteoporosis. The CTR has 2 common splice variants (CTRa, CTRb) and there is also a common polymorphism (Leu/Pro substitution in the C-terminal tail), which are poorly characterised. The CTR couples to different downstream effectors such as Gαs, Gαq and Gαi proteins to trigger downstream signalling, including the formation of intracellular cAMP, mobilisation of calcium and phosphorylation of extracellular signal-regulated kinase (pERK1/2). A number of distinct calcitonin peptide agonists (including salmon CT that is used clinically) activate the receptor but it is unknown if they are biased agonists relative to each other.
Aims. To compare the signalling and regulation of splice/polymorphic CTR variants and to assess the ability of different peptides to promote biased agonism at these variants.
Methods. CTRaLeu, CTRaPro, CTRbLeu and CTRbPro were each stably expressed in COS-7 cells. Five peptide ligands were assessed in calcium mobilisation, cAMP accumulation and pERK1/2. In addition, confocal microscopy was employed to assess receptor internalisation and recruitment of β-arrestins.
Results. CTR polymorphic variants (Pro and Leu) produced similar cAMP and pERK1/2 profiles for all peptides assessed. In cAMP formation, a biphasic response was observed, however the fraction of high and low efficacy responses differed between the splice variants. Only the CTRa splice variants elicited calcium mobilisation within the concentration range of peptides assessed. In addition, no ligand was able to promote β-arrestin recruitment to any of the CTR variants. In all cases, the magnitude of cellular response was greater for the CTRa splice variants.
Discussion. The greater cellular response for CTRa splice variant relative to the CTRb may play an important physiological role when correlated with the distribution of these receptors in different tissues. In addition, biased agonism was detected at all CTR variants when activated by distinct peptides. Understanding biased agonism, how this alters physiological functions downstream of CTR activation and how signalling differs between patients with different receptor variants has therapeutic relevance and may aid in the design of novel drugs to target this receptor.

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Protease activated receptor-2 signals to and opens transient receptor potential vanilloid-4 via Gα13 and p38δ

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Introduction. We have previously shown that the pro-inflammatory G-protein coupled receptor (GPCR) Protease –activated receptor 2 (PAR_2) signals to and opens TRPV4 in HEK293 cells, sensory neurons and in an *in vivo* model of mechanical hyperalgesia (Grace et al. 2014). However, the signalling pathways responsible are not known.
Aim. To use siRNA to knockdown molecules which transduce signals from GPCRs (e.g. G-proteins, phospholipases and adenylate cyclases and protein kinases) to see if they inhibit TRPV4 opening by PAR_2.
Methods. HEK293 cells stably expressing human TRPV4 were transfected with 693 Dharmacon SMARTpool siRNAs and after 72 hrs intracellular calcium ($[Ca^{2+}]_i$) was measured using FURA-2AM dye in a plate reading fluorimeter. Targets were functionally validated and subsequently, RNA was extracted and the expression level of the target mRNA was quantified using qPCR.
Results. In hTRPV4 expressing cells, PAR_2-activating peptide (50µM) caused a biphasic $[Ca^{2+}]_i$ response mediated by (i) an initial transient release of intracellular calcium stores, followed by (ii) a more sustained increase in $[Ca^{2+}]_i$ due to an influx of extracellular calcium through hTRPV4. The sustained response is a measure of PAR_2-dependent opening of TRPV4. siRNAs that disrupted this signalling were considered “hits". Of 693 siRNAs tested, over 50 caused some inhibition. However, after stringent siRNA validation and on-target mRNA analysis by qPCR to confirm knockdown, 5 candidate molecules were chosen. Knockdown of Gα13 and Gγ8, the kinases p38δ and WNK4 and phospholipase A2 4A lead to an inhibition of the sustained $[Ca^{2+}]_i$ response by 47 ± 3%, 57 ± 5% 58 ± 5%, 42 ± 4% and 41 ± 8%, respectively, indicating that they are necessary for PAR_2-dependent opening of TRPV4.
Discussion. Heterologous expression in HEK293 cells is a powerful tool for large scale genetic screening assays like this one. Activation of most GPCRs results in multiple parallel signalling pathways being initiated. Therefore, inhibition indicates that the targeting of signalling molecules which are vital for the PAR_2-dependent opening of TRPV4 are good candidates to play a role in the physiological opening of TRPV4 by PAR_2 and by other GPCRs.
Grace MS et al., (2014) BJP 171:3881-94

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Divergent effects of strontium and calcium-sensing receptor positive modulators on osteoclast activity

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Introduction: Osteoclasts are multinucleated cells that mediate bone resorption and are a therapeutic target for osteoporosis. Strontium ranelate is an anti-resorptive therapeutic that decreases osteoclast differentiation and activity. While there are mixed reports regarding the mechanism of action of strontium (Sr^{2+}), the calcium-sensing receptor (CaSR) is thought to at least in part mediate its activity in osteoclasts. Theoretically, CaSR positive allosteric modulators (PAMs) could be used to potentiate or replicate CaSR-mediated Sr^{2+}activity in osteoclasts.
Methods: Three structurally divergent PAMs (cinacalcet, AC-265347 and BTU-compound 13) were profiled in a recombinant cell system across multiple signaling pathways ($Ca^{2+}{}_i$-mobilisation, IP_1-accumulation and ERK1/2-phosphorylation) for both Ca^{2+}- and Sr^{2+}-mediated CaSR activation. A high throughput, multi-staining imaging method was used to quantitatively measure human osteoclast tartrate-resistant acid phosphatase (TRAP) activity and nucleation state at the single cell level. Both quantitative TRAP staining and hydroxyapatite resorption assays were used to characterise the effects of PAMs on cultured human osteoclast maturation and activity.
Results & conclusions: The PAMs displayed modulatory activity for both Ca^{2+}- and Sr^{2+}-mediated CaSR signalling across three pathways with examples of probe dependence (e.g. cinacalcet modulation of ERK1/2-phosphorylation Log $\alpha\beta_{Ca2+}$ 0.56 ± 0.05 versus Log $\alpha\beta_{Sr2+}$ 0.19 ± 0.10; n = 3) and pathway bias (e.g. cinacalcet is 2.5 fold biased towards $Ca^{2+}{}_i$-mobilisation versus ERK1/2-phosphorylation) in a recombinant cell system. In human osteoclasts, Sr^{2+} (10 - 20 mM) inhibited TRAP activity (>30% reduction for cells with >11 nuclei/cell), maturation (5% reduction) and hydroxyapatite resorption (>70% reduction), but CaSR PAMs were unable to replicate or potentiate these effects. These data therefore question whether Sr^{2+} acts via CaSR to modulate these processes. Intriguingly, cinacalcet inhibited osteoclast resorption of hydroxyapatite, with no effect on TRAP activity or maturation, a profile not shared by the other PAMs suggesting divergent mechanisms regulating osteoclast function. Additionally, this is the first evidence of bone cell activity for cinacalcet, which is used clinically for secondary hyperparathyroidism.

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Dynamic ligand binding underlies the mechanism of action of a novel class of allosteric modulator of the dopamine D2 receptor

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Introduction SB269652 is the first drug-like allosteric modulator of the D2R, despite containing structural motifs consistent with an orthosteric mode of engagement. We provided a mode of action to reconcile this apparent discrepancy, where SB269652 binds in a bitopic manner to one protomer of a D2R dimer to modulate the action of dopamine at the other protomer.
Hypothesis/Aims The aims of this study were to validate the proposed mode of interaction of SB269652 at D2R, and probe both the key residues of D2R and structural motifs of SB269652 that confer its novel pharmacology.
Methodology We expressed the D2R as a monomer in a high density lipoprotein (rHDL) particles in order to explore the action of SB269652 at a purely monomeric population of D2Rs. Structural features of SB269652 and key ligand receptor interactions were identified using molecular modelling, site directed mutagenesis and the generation of structural derivatives of SB269652.
Results The pharmacology of SB269652 is maintained at monomeric D2R reconstituted in rHDL particles, consistent with a dynamic equilibrium between two binding poses, suggesting that a D2R dimer is not required for the allosteric action of SB269652. Interestingly, changes in negative cooperativity and affinity caused by either mutation of receptor residues or changes to ligand structure were positively correlated. Indeed, residues within the TM bundle responsible for orthosteric binding caused a decrease in both affinity and negative cooperativity. In contrast mutation of residues within ECL1 & 2, that might form an allosteric pocket, caused an increase in affinity and negative cooperativity. Finally, in time-resolved functional assays, SB269652 initially displays competitive pharmacology but then shifts to an apparent allosteric mode of action This is consistent with a changing distribution between orthosteric and allosteric modes of action over time in a 'flip-flop' model.
Conclusions We propose an alternative 'flip-flop' mechanism in which SB269652 can bind to either an orthosteric or an allosteric pose. In this model the apparent negative cooperativity of SB269652 will be governed by its relative affinity for these two different sites. This represent a novel mode of GPCR allosteric ligand action.

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Roles of the intraloops and C-terminus in calcium/calcimimetic selection of G-protein dependant signalling pathways from the calcium-sensing receptor

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Introduction. The calcium-sensing receptor (CaSR) is a Class C G-protein coupled receptor that contributes to the control of calcium metabolism and bone homeostasis via its expression in various tissues, including the parathyroid glands, kidney and bone. The CaSR mediates diverse effects by selecting for signalling pathways in a ligand- and cell-type-specific manner. However, the mechanisms that underlie the selection of signalling pathways are not well understood.
Aims. To investigate the mechanisms underlying the role of calcium, and calcimimetics including L-amino acids and the clinical compound, cinacalcet, in CaSR-mediated ligand dependent signalling.
Methods. We decided to perform alanine scanning site-directed mutagenesis of intraloops -1, -2 and -3, and to truncate the C-terminus. We assessed the residues critical for the coupling of the CaSR, in the presence/absence of the aforementioned calcimimetics, to distinct pathways, including downstream of PI-PLC (IP_1 accumulation), phosphorylated $ERK_{1/2}$ (pERK), and suppression of forskolin-stimulated adenylyl cyclase (intracellular cAMP levels). An enzyme-linked immunosorbance assay (ELISA) was also performed to examine the cell surface expression of these CaSR mutants.
Results. The results demonstrate that distinct residues and sub-domains mediate coupling to distinct signalling pathways downstream of the receptor. In particular, the CaSR mutant constructs A642-644 (iL-1), A701-704, F706A (iL2), A793-795, A796-798, E799A, F801A, (iL-3), none of which impaired cell surface expression, markedly attenuated PI-PLC and pERK. E803A was observed to have reduced cell surface expression. Furthermore, the C-terminal truncation mutant, F881X, markedly attenuated PI-PLC and ERKp, while R891X retained sensitivity. Most strikingly, R866X exhibited complete loss of Ca^{2+}_i mobilization but retained intact suppression of adenylyl cyclase.
Discussion. The results demonstrate that pathway selection arises from distinct domains and sub-domains of the receptor's intraloops. In addition, C-terminal residues that lie between F881 and R891 may be required for CaSR-mediated PI-PLC signalling.

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Off-target effects contribute to apparent agonist bias at human α1A-adrenoceptors

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Introduction. Agonists acting at G protein-coupled receptors (GPCRs) promote biased signalling via downstream effectors including multiple Gα subunits and β-arrestins. As biased agonism has implications for drug discovery, it is essential to consider confounding factors contributing to apparent bias. For example, agonist dissociation kinetics markedly influence bias at the dopamine D_2 receptor (Klein-Herenbrink et al, 2016). Here, we demonstrate apparent bias related to off-target effects of the α_1-adrenoceptor (AR) agonist oxymetazoline for one signalling outcome.
Aim. To determine the basis for pronounced biased agonism by oxymetazoline in CHO cells expressing the α_{1A}-AR.
Methods. Concentration-response curves for noradrenaline (NA), phenylephrine, oxymetazoline (Oxy) and A61603 were determined in CHO cells stably expressing the α_{1A}-AR (Bmax 204 fmol/mg protein). Peak intracellular Ca^{2+} release (fluoro-4) occurred within 15 sec of drug addition, cAMP accumulation was measured at 30 mins (LANCE HTRF), and Erk1/2 phosphorylation (pErk1/2) at 5 mins (AlphaScreen SureFire Kit).
Results. In CHO-α_{1A}-AR cells, Oxy stimulated Ca^{2+} release and pErk1/2, but not cAMP accumulation. Oxy displayed reduced potency for pErk1/2 (pEC_{50} 7.2 ± 0.1) compared to Ca^{2+} release (9.3 ± 0.4) but high Emax values (4.6 ± 0.13 fold over basal, NA 2.0 ± 0.5). Unlike NA and A61603, the pErk1/2 response to Oxy was blocked by Pertussis toxin but not by the Gαq/11 inhibitor UBO-QIC. We investigated endogenous Gαi/o-coupled 5-HT_{1B} receptors as a potential target. Oxy and 5-hydroxytryptamine stimulated pErk1/2 in non-transfected cells, and in CHO-α_{1A}-AR cells pErk1/2 responses to Oxy were blocked by the 5-HT_{1B} selective antagonist SB216641 (300 nM).
Discussion. Chemical similarity approaches have identified many novel drug-target associations (Keiser et al, 2009). In turn, commonly-used cell lines express a repertoire of endogenous GPCRs that may confound studies on biased agonism at recombinant receptors. It is thus critical to test all agonists against all responses in non-transfected cells.

Keiser MJ et al (2009) Nature 462:175–181.
Klein-Herenbrink C et al (2016) Nature Commun. 7:10842.

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An essential role of RGS protein in partial agonism of the m2 muscarinic receptor-mediated K^+ currents

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Introduction. GPCRs are important targets of pharmaceutical research. A subclass of GPCR ligands acts as partial agonists, meaning that they elicit a submaximal response as compared to a full agonist. Differences in the regulation of heterotrimeric G-proteins are thought to underlie the lower activation efficacy of partial agonists.
Aims. We tried to understand the molecular components and mechanism underling the partial agonism of m2 muscarinic receptor (m2R)-mediated K^+ currents.
Methods. We carried out *in vitro* electrophysiological and pharmacological analyses.
Results. The submaximal response of several m2R partial agonists relative to the full agonist, acetylcholine (ACh), was confirmed in atrial myocytes and was reconstituted in *Xenopus* oocytes with the regulator of G protein signaling (RGS) 4, a heart-abundant subtype of RGS. However, the G-protein-gated inwardly rectifying K^+ (K_G) current evoked by pilocarpine and other partial agonists in oocytes lacking RGS4 was similar to that evoked by ACh. We also found enhanced RGS-mediated inhibition of G-protein signaling during m2R stimulation by its partial agonist, under the strong influence of membrane potential. The disruption of plasma targeting of RGS4 reduced the difference in K_G current induced by pilocarpine relative to ACh. These results suggest that m2Rs are endowed with inhibition mechanism of G-protein signaling by RGS protein that determines the activation efficacy of m2R agonists. Furthermore, mutations of residues in the aspartic acid-arginine-tyrosine (DRY) motif of m2R significantly impaired RGS4-mediated inhibition of pilocarpine-evoked K_G currents. Thus our results demonstrate that voltage-dependence of RGS4 function is derived from m2R.
Discussion. The present study shows an essential role of RGS Protein in partial agonism of GPCR and provides novel insights into how membrane potential impacts G-protein signaling by modulating GPCR communication with downstream effectors.

Chen IS, Furutani K, Inanobe A, Kurachi Y. (2014) RGS4 regulates partial agonism of the M2 muscarinic receptor-activated K^+ currents. J Physiol 592(6):1237-1248.

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Targeting meta-stable binding sites with bitopic ligands – a new concept in GPCR medicinal chemistry

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Introduction. To date it has been difficult to obtain subtype selective G protein-coupled receptor (GPCR) ligands due to the fact that orthosteric binding sites are generally highly conserved across the subtypes and due to the general lack of structural information on allosteric sites.[1] A computational study on the pathway and mechanism of ligand binding to GPCRs revealed that ligands pause at transient meta-stable binding sites on their way to the orthosteric pocket.[2] Since such meta-stable binding sites are located in generally less conserved receptor areas, we hypothesize that bitopic ligands that link two identical pharmacophores to simultaneously target the orthosteric and a meta-stable binding site may provide ligands with improved efficacy and receptor subtype selectivity.

Aims. The overall aim of the present project is the development of a new class of bitopic ligands as high efficacy, subtype selective GPCR modulators by targeting meta-stable binding sites.

Methods. The bitopic ligands are designed based on docking studies on the ß$_2$-adrenergic receptor (β_2AR) using the Glide tool of the Schrödinger software suite. The synthetic strategies for such compounds start from a 2-substituted phenol or phenol derivative and can be divided into two main synthetic steps, *i.e.* the construction of the pharmacophores and the connection of the two entities. Pharmacological evaluation is achieved in β_2AR functional assays that monitor receptor deactivation by measuring the concentration of second messenger cAMP.

Results. Initial pharmacological tests indicate that the compounds antagonize the receptor as competitive antagonists, most of them with potencies and efficacies in the range of known β_2AR antagonist alprenolol.

Discussion. Based on docking studies, different symmetrical bitopic β_2AR ligands were designed and synthesized. Pharmacological tests show promising results, but testing with regard to potency, efficacy, selectivity, etc. is still on-going. In combination with the docking studies, these results will be used to identify the optimal linker composition for potential bitopic β_2AR ligands.

[1] Conn P J et al (2009) Nat Rev Drug Discov 8:41-54
[2] Dror R O et al (2011) PNAS 108:13118-13123

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Probing the functional importance of the linker region of the class B GPCR, CLR, in CGRP and adrenomedullin receptors.

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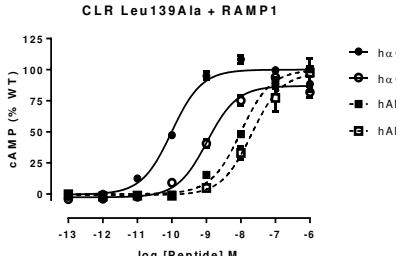
Introduction. The calcitonin-receptor-like receptor (CLR) is the backbone of three receptors, the calcitonin gene-related peptide (CGRP) receptor, and two adrenomedullin (AM) receptors; AM_1 and AM_2. CLR pharmacology is dictated by receptor activity-modifying proteins (RAMPs). The CGRP receptor (CLR+RAMP1) has been linked to migraine, and AM receptors (CLR+RAMP2/3) are involved in cardiac regulation. Therefore drugs which target these receptors could be therapeutically relevant. There is little known of the CLR linker region (an extension of transmembrane helix one which joins the extracellular domain to the transmembrane domain of class B GPCRs). We have investigated the role of individual amino acids within this region on receptor function.

Aims. Determine the functional contribution of individual amino acids within the CLR linker region.

Methods. Transfect alanine substituted CLR mutants into COS7 cells to measure cAMP responses and cell surface expression, with all three RAMPs.

Results. The majority of alanine mutations were well tolerated, causing no major change in expression or cAMP production. Three residues abolished receptor expression and cAMP signalling across all receptors. A few mutations caused up to thirty fold decreases in potency with select receptor/ligand combinations (see figure for CGRP/CGRP receptor specific interaction) but did not impact expression.



Discussion. Most alanine mutations did not significantly alter the potency of AM. Mutations which did affect AM potency caused similar reductions at all receptors, indicating similar modes of receptor interaction across tested receptors. Two CLR mutations decreased only the potency of CGRP; indicating CGRP possibly interacts with this region in a different fashion to AM. This shows that the CLR linker region has a role in receptor pharmacology.

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Insights into the allosteric binding sites of the M5 muscarinic acetylcholine receptor

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Introduction. The five muscarinic acetylcholine receptors (mAChRs M_1-M_5) are Class A G protein-coupled receptors that play a key role in regulating a wide range of physiological functions. Activation of the M_5 mAChR represents a potential path for the treatment of cerebrovascular diseases, whereas inhibition of the M_5 mAChR may provide novel therapies for the treatment of addictive behaviors. The design of subtype-selective mAChR selective ligands is challenging due to the conserved nature of the orthosteric site across mAChR subtypes. Recently, several highly selective M_5 mAChR ligands have been reported that achieve subtype-selectivity by targeting an allosteric site of the M_5 mAChR, thus enabling the closer study of the allosteric pharmacology of the receptor. Knowledge of the location, structure, and dynamics of the M_5 mAChR allosteric binding site can usher in a new era of structure-based drug design for this important protein family. Aims. The objective of this project is to obtain a comprehensive understanding of the structural properties of the allosteric site of the M_5 mAChR and its ligands in order to address the need for novel and selective M_5 mAChR-targeting therapeutics. Methods. Site-directed mutagenesis was used to generate putative allosteric site-targeted M_5 mAChR mutants. Structure-function analyses, incorporating cell-based $G_{q/11}$ protein-mediated signaling, radioligand binding, and analytical and molecular modelling, were used to study the effects of receptor mutations on novel positive and negative M_5 allosteric modulators and elucidate receptor regions contributing to their pharmacological properties. Results. We assessed multiple mutations of residues within the extracellular vestibule of the M_5 mAChR, a region implicated in allosteric drug action at other mAChRs, using functional and radioligand binding assays, and found that the activity of the M_5 mAChR allosteric ligands was surprisingly unaffected by these mutations. Equilibrium interaction binding experiments between prototypical mAChR vestibule site modulators and selective M_5 mAChR allosteric ligands also indicated that these compounds do not compete for a 'common' allosteric site. Discussion. These studies have indicated that the recently developed M_5 mAChR allosteric ligands likely bind at a distinct location from the prototypical mAChR allosteric site located in the extracellular vestibule. Efforts are underway to obtain a crystal structure of the M_5 mAChR in order to locate and fully characterize the structural properties of the M_5 mAChR allosteric site(s).

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Characterising signalling bias of Glucose-dependent Insulinotropic Polypeptide receptor ligands

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Introduction. Glucose-dependent Insulinotropic Polypeptide (GIP) receptor, is an important regulator of blood glucose levels through binding of the incretin hormone GIP (1-42). GIP (1-42), together with GLP-1 (7-36) amide, stimulates insulin secretion from pancreatic β-cells. Interestingly, these ligands have contrasting actions at pancreatic α-cells with GIP (1-42) stimulating glucagon secretion by a cAMP-dependent mechanism and GLP-1 inhibiting glucagon secretion, despite it also promoting cAMP production (Seino et al., 2010). The underlying mechanisms of GIP (1-42) and GLP-1 (7-36) amide that lead to these contrasting effects are largely unknown.
Aims. Establish signalling pathways modulated by the GIPR upon activation by endogenous and exogenous ligands.
Methods. cAMP accumulation, intracellular calcium release (iCa^{2+}) and ERK1/2 phosphorylation were assayed in response to GIPR ligands (including the DPPIV-resistant GIP analogue, GIP (D-Ala2), and the proposed GIPR antagonist, GIP (Pro3)) in HEK293 cells transiently expressing the GIPR. This pharmacological analysis was then extended to the more physiologically relevant mouse pancreatic alpha cell line, αTC1.6.
Results. Through studying the intracellular signalling pathways modulated by GIPR in HEK293 cells we have shown that GIPR activation can not only stimulate cAMP accumulation but also lead to intracellular calcium release and ERK1/2 phosphorylation. GIP (1-42) and GIP (D-Ala2) appear to be cAMP biased in their signalling, whilst GIP (Pro3) is in fact a partial agonist of the GIPR and relatively biased towards iCa^{2+} mobilisation. The use of YM254890, an inhibitor of $G\alpha_{q/11}$ signalling, and ESI-09, an EPAC1/2 non-selective inhibitor, on αTC1.6 cells has shown that iCa^{2+} mobilisation in response to GIP (1-42) and GIP (D-Ala2) is mediated via Gαs and EPAC, whilst GLP-1 is $G\alpha q_{/11/}$-dependent.
Discussion. The different pathways by which GIP (1-42) and GLP-1 (7-36) amide lead to iCa^{2+} mobilisation in pancreatic α-cells provides a mechanism to explain the contrasting actions of these incretins on glucagon secretion.

Seino, Y., Fukushima, M. & Yabe, D. 2010. GIP and GLP-1, the two incretin hormones: Similarities and differences. *J Diabetes Investig,* 1, 8-23.

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Allosteric modulators of mGlu5 desensitise multiple signalling pathways in striatal neurons

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Introduction. Although $mGlu_5$ positive, negative and neutral allosteric modulators (PAMs, NAMs and NALs) are classified based on intracellular Ca^{2+} (iCa^{2+}) mobilisation, $mGlu_5$ PAMs exhibit stimulus bias for multiple acute signalling pathways (Sengmany et al, 2016). However, little is known about the ability of these ligands to induce bias in receptor regulation pathways. Given the therapeutic potential of $mGlu_5$ allosteric modulators in a range of CNS disorders, it is important to understand the effects of prolonged exposure to such ligands on receptor function.
Aims. To determine the effects of $mGlu_5$ PAMs and NALs on the desensitisation of $mGlu_5$-mediated iCa^{2+} mobilisation and phosphoinositide (PI) hydrolysis in striatal neurons.
Methods. Primary striatal neurons were isolated from E15 mouse embryos and cultured for 6-7days before use. Acute desensitisation of $mGlu_5$ mediated-iCa^{2+} mobilisation was induced by exposing neurons to allosteric ligands +/- the orthosteric ligand DHPG 1 min prior to stimulation with a maximal concentration of DHPG. Chronic desensitisation of $mGlu_5$-mediated iCa^{2+} mobilisation and PI hydrolysis were induced by exposure to ligands +/- DHPG for 24 hours, followed by washout and DHPG stimulation.
Results. PAMs alone desensitised $mGlu_5$-iCa^{2+} mobilisation and PI hydrolysis and potentiated DHPG-induced desensitisation. VU424465 alone was the most potent (pIC_{50} 6.8±0.4 and 8.6±0.3 for iCa^{2+} and PI hydrolysis, respectively), and was biased towards both pathways relative to DHPG. All PAMs had higher affinity (K_b) but lower cooperativity (logβ) for potentiation of DHPG-induced desensitisation of PI hydrolysis compared to Ca^{2+} mobilisation. Moreover, ligands previously classified as NALs also potentiated DHPG-induced desensitisation of iCa^{2+} mobilisation, but not PI hydrolysis.
Discussion. These data suggest that PAMs and NALs of $mGlu_5$ have the ability to desensitise multiple receptor signalling pathways, both acutely and chronically. The ability of some PAMs to induce receptor desensitisation in the absence of direct receptor activation may be linked to the favourable *in vivo* safety profile of these ligands.

Sengmany K. et al (2016) Neuropharmacology. In press.

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Identifying small molecule agonists of glucagon-like peptide-1 receptor

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Introduction. Glucagon-like peptide-1 (GLP-1) is an incretin hormone released from the gut in response to meal ingestion. It plays a major role in regulating insulin secretion and thus maintaining glucose homeostasis within the body. GLP-1 acts at the glucagon-like peptide-1 receptor (GLP-1R) to elicit its physiological effects making this receptor a major therapeutic target for treatment of type 2 diabetes mellitus (DM) and associated obesity. Peptide analogues of GLP-1 are approved for the treatment of these diseases, however these are suboptimal due to their route of administration and significant side effects profiles. An orally active small molecule GLP-1R ligand would be a major advance for the treatment of DM. BETP binds to an allosteric site on the GLP-1R potentiating calcium mobilisation in response to the cognate ligand GLP-1. BETP also enhances cAMP production of the endogenous agonist oxyntomodulin and the weak partial agonist GLP-1(9-36)NH_2 that is the primary metabolite of GLP-1. Therefore, BETP provides a scaffold for SAR analysis to identify small molecules with improved clinical potential.
Aims. Screen a 415 compound SAR library based around the structure of BETP, to identify GLP-1R agonists in multiple signalling pathways. Confirm initial hits with detailed pharmacological profiling.
Methods. All cell signalling assays were carried out in a GLP-1R expressing CHO Flp-In cell line. Screening was performed at a single ligand concentration followed by full dose response analysis of hit compounds. cAMP accumulation was measured using the PerkinElmer LANCE kit, intracellular calcium mobilisation was performed using a Fluo4 based FLEX assay and pERK1/2 activation was assessed using the AlphaScreen ERK1/2 SureFire kit at both 7 min and 30 min stimulation.
Results. BETP is a very weak agonist at the GLP-1R. We have identified compounds that show higher efficacy than BETP in the pERK1/2 pathway whilst maintaining an efficacy similar to BETP in cAMP and calcium pathways.
Discussion. There has been large investment from the pharmaceutical industry to develop an orally available GLP-1R-mediated treatment for type 2 diabetes. We have shown that SAR chemistry around a known GLP-1R small molecule ligands can improve efficacy and identify SAR that induces biased agonism (relative to the parent compound) towards different cellular signaling pathways.

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Development of a Nanoluc BRET binding assay for investigating the complex relaxin-RXFP1 interaction

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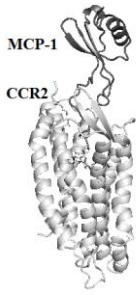
Introduction. The receptor for relaxin, RXFP1, is a G protein-coupled receptor containing a large extracellular binding domain. Relaxin binds RXFP1 via a complex mechanism with multiple interactions sites which is poorly understood, hindering efforts to develop more efficacious relaxin-like molecules. Kinetic analysis of binding can be used to elucidate binding mechanisms, thus we sought a convenient technique to investigate relaxin-RXFP1 binding kinetics. It was recently demonstrated that ligand binding can be measured by bioluminescence resonance energy transfer (BRET) signal between a fluorescent ligand and a Nanoluciferase (Nanoluc)-tagged receptor.



Aims. To develop and optimise a Nanoluc BRET based binding assay for investigating relaxin-RXFP1 interactions.

Methods. Mono-labelled Tamra-relaxin (TamRLX) was produced and purified by HPLC and Nanoluc was cloned in frame to the N-terminus of RXFP1 in pcDNA3.1/Zeo vector. For Nanoluc BRET experiments, HEK293T cells stably expressing Nanoluc-RXFP1 were seeded into 96-well white optiplates and experiments performed 24h later. Saturation binding experiments - cells were incubated with varying concentrations of TamRLX, followed by addition of furimazine and measurement of BRET. Dissociation experiments, 10 nM TamRLX was bound to equilibrium followed by addition of furimazine and 10 µM unlabelled relaxin to initiate dissociation.

Results. TamRLX showed equal potency to unlabelled relaxin, and fusion of Nanoluc to RXFP1 did not significantly perturb receptor function. Saturation binding yielded a single binding mode with a K_D of 1.28±0.04 nM, in line with previously published reports using alternative labels. Dissociation experiments also yielded a single binding site model with a k_{off} of 1.1±0.07 X 10^{-4} sec^{-1} and a calculated k_{on} of 8.6±0.3 X 10^5 $M^{-1}.sec^{-1}$.

Discussion. A convenient Nanoluc BRET binding assay for relaxin-RXFP1 has been developed. Importantly, these assays required no separation of unbound TamRLX and there was no non-specific binding signal. Future work will utilise RXFP1 receptors with mutations in known relaxin binding sites to dissect the mechanism by which relaxin binds. Nanoluc BRET represents a powerful tool for the measurement of ligand binding to cell surface receptors and we have now demonstrated that Nanoluc BRET binding can also easily be utilized for kinetic measurements for GPCR-ligand interactions with no bound from free separation.

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Structural insights into differential agonism by MCP-1 and MCP-3 at their shared receptor CCR2

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Introduction. Chemokine receptors are G-protein coupled receptors (GPCRs) that regulate the movement of leukocytes during inflammation.CCR2, a major chemokine receptor on monocytes and macrophages. The major ligands of hCCR2 include monocyte chemoattractant proteins (MCP) -1 and -3, where -1 is considered a full agonist compared to -3.

Aims. To identify the structural features of the chemokines which contribute to the differences in their potencies and efficacies at their shared receptor & to identify the receptor residues responsible for these differences.

Methods. By using a series of MCP-1 and MCP-3 chimeras, binding and activation was studied using radio-ligand binding, β-arrestin 2 and pERK-1/2 phosphorylation. Binding and activation of the CCR2 mutants were also characterized using the similar assays.

Results and Discussion. We have found that the chemokine N-terminus plays a major role towards full versus partial agonism. The affinities of the chemokine chimeras to the CCR2 also confirmed that the N-terminus makes a significant contribution to receptor binding by these two chemokines. Analysis of the CCR2 mutants have identified several residues in the trans-membrane helices of the receptor which act preferentially with either of the chemokines. Our investigation has yielded new information on chemokine receptor binding and signalling, which will guide future drug development.

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Heteromerisation of the angiotensin II type 2 receptor and the bradykinin type 2 receptor

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Introduction. Unlike the well characterised angiotensin II type 1 (AT_1) receptor, the pharmacology of the angiotensin II type 2 (AT_2) receptor remains poorly understood. Unlike most G protein-coupled receptors (GPCRs) it does not readily signal through G proteins, recruit GPCR kinases (GRKs) or arrestins, and nor does it internalise upon treatment with agonist. However, it has been shown to interact with bradykinin type 2 (B_2) receptor signalling systems, and it has been suggested that heteromerisation between the two receptors may underpin this functional crosstalk.
Aims. To investigate evidence for the existence of the AT_2-B_2 heteromer at the molecular level.
Methods. The existence of the AT_2-B_2 heteromer was investigated using the GPCR heteromer identification technology (GPCR-HIT) on the bioluminescence resonance energy transfer (BRET) platform (Johnstone et al, 2012). The GPCR-HIT assay enables identification of heteromers through interactions with tagged biomolecules. Furthermore, the assay provides functional information and can be used to investigate all aspects of GPCR pharmacology, including ligand binding, signalling and regulation, trafficking and internalisation.
Results. In contrast to the monomeric AT_2 receptor, coexpression and activation of the B_2 receptor in the GPCR-HIT assay resulted in recruitment of GRK2 and β-arrestin2 to the AT_2 receptor, following which the receptor subsequently underwent internalisation. Furthermore, although neither the AT_2 receptor nor the B_2 receptor couple to $G\alpha_z$ proteins, upon their coexpression, $G\alpha_z$ was recruited proximal to the B_2 receptor following treatment with bradykinin. In contrast, there was no evidence of alteration to B_2 receptor-mediated $G\alpha_q$ signalling, as the potency and efficacy of inositol phosphate production was unchanged upon coexpression of the AT_2 receptor. Finally, the proximity of the two receptors was confirmed using the BRET ligand binding assay in the GPCR-HIT configuration.
Discussion. This study has provided evidence for the existence of the AT_2-B_2 heteromer and the unique signalling and regulatory aspects of its pharmacological profile.

Johnstone EKM and Pfleger KDG (2012). *Front. Endocrinol.*, 3: 101.

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G protein coupled receptor mediated Smad2 linker region phosphorylation in cardiovascular disease

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Introduction. Thrombin via its G protein coupled receptor (GPCR) protease-activated receptor (PAR)-1 stimulates proteoglycan synthesis and glycosaminoglycan (GAG) chain elongation leading to increased LDL binding and retention. Thrombin mediated GAG chain elongation occurs via the transactivation of protein tyrosine kinase receptors (PTKR) and serine/threonine kinase receptors (S/TKR). We recently published that transforming growth factor (TGF)-β regulated GAG chain elongation via the phosphorylation of the Smad2 linker region. Four residues of the Smad2 linker region were phosphorylated by Erk, p38, PI3K and CDK and in turn regulated the mRNA expression of GAG synthesizing genes.
Aims. To assess the role of the Smad2 linker region in GPCR mediated GAG chain elongation in human vascular smooth muscle cells.
Methods. The mRNA expression of GAG synthesizing genes was measured and quantified by real time-PCR. Smad2 linker region phosphorylation was detected and quantified by western blotting
Results. Thrombin via the transactivation of PTKR (EGFR) and S/TKR (TGFBR1) mediate the phosphorylation of four serine/threonine residues of the Smad2 linker region. The four Smad2 linker region residues were phosphorylated by Erk, P38, PI3K or CDK. These serine/threonine kinases were involved in thrombin mediated mRNA expression of GAG synthesizing genes chondroitin 4 sulfotransferase and chondroitin synthase -1.
Discussion. All of thrombin mediated proteoglycan synthesis and GAG elongation occur via transactivation dependent pathways. We seek a common signalling intermediate which can inhibit thrombin mediated proteoglycan synthesis and GAG chain elongation. This work shows that thrombin via the transactivation of both PTKR and S/TKR leads to Smad2 linker region phosphorylation. The Smad2 linker region phosphorylation via the individual transactivation pathways was mediated by different serine/threonine kinases. Hence a common kinase mediating Smad2 linker region phosphorylation via the two transactivation pathways may serve as a therapeutic target for the prevention of atherosclerosis.

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It's only a matter of time: Investigating the temporal aspects of dopamine D_2 receptor signalling

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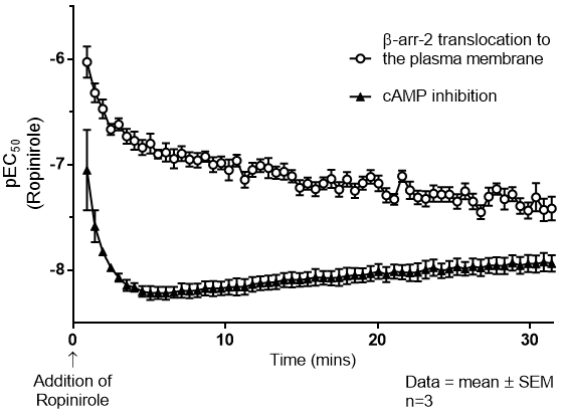

Introduction. Signalling bias is the phenomenon of one agonist that acts to preferentially activate one signalling pathway relative to another signalling pathway when compared to a different agonist at the same receptor. A number of studies have suggested that dopamine D_2 receptor (D_2R) partial agonists which preferentially activate the β-arrestin pathways may be desirable for the treatment of schizophrenia. However, pharmacologists have historically measured activation of signalling pathways at single time-points, missing out on important kinetic information.

Aims. To understand D_2R signalling over time in HEK 293 cells via multiple pathways in response to agonist.

Methods. FLPIN HEK 293 cells either stably expressing the $D_{2L}R$ or transfected with $D_{2L}R$-RLuc fusion protein were used to detect G protein activation, cAMP production inhibition, β-arrestin-2 translocation to the plasma membrane or β-arrestin-2 recruitment to the $D_{2L}R$-RLuc through use of recombinant bioluminescence resonance energy transfer (BRET) sensors.

Results. Our initial experiments revealed that the potency of the agonist ropinirole changed differently over time dependent upon the D_2R signalling pathway measured. The most striking difference was observed between β-arrestin-2 recruitment and cAMP production inhibition whereby β-arrestin-2 recruitment results in increased potency over time whereas cAMP inhibition has decreasing potency after five minutes.

Discussion. The observed increase in potency over time for β-arrestin-2 recruitment appears counterintuitive as arrestins are traditionally thought to desensitise the signalling of GPCRs. Furthermore, this study highlights the importance of measuring signalling over time to obtain a holistic picture of drug response.

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Discovery of a vasopressin V1a receptor-selective antagonist through D-amino acid substitution of the insect peptide inotocin

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Introduction. A lack of subtype-selective ligands towards the four human oxytocin (OT)/vasopressin (AVP) receptor still exists with currently available therapeutics, limiting clinical treatments. To better understand human OT-/AVP-receptor activation for the development of new subtype-selective peptides, we exploited the orthologous insect OT-/AVP-like peptide, inotocin, which bears similarities with OT and AVP. We studied the pharmacology of the native inotocin peptide and its D-amino acid substitution analogues at their insect receptors and the human receptors.

Aims. To characterise the pharmacology (and mechanism of engagement and activity) of inotocin and its analogues at insect inotocin receptors and human OT and AVP receptors.

Methods. Inotocin and its receptor from *Lasius niger* were isolated and cloned from RNA samples. Peptides were pharmacologically characterised via radioligand binding, inositol phosphate (IP_1) accumulation and cAMP accumulation in HEK293 cells transiently expressing human and insect (*L. niger* and *Tribolium castaneum*) receptor constructs. Computational modelling of inotocin (and D-amino acid analogue) binding to the V_{1a} receptor was also performed to complement these pharmacological data.

Results. Functional profiles of D-amino acid inotocin analogues revealed key roles of residues for binding and a biased agonist at the inotocin receptors. Screening these peptides at the human receptors identified an antagonist that selectively binds to the V_{1a} receptor. Computational analysis reveals a potential mechanism for this selectivity.

Discussion. This study has identified a selective antagonist for the $V_{1a}R$. The unique pharmacology of this selective antagonist provides insights into peptide and receptor structure that may be exploited to further develop selective ligands for OT and AVP receptors. Moreover, this highlights the potential for using invertebrate peptide and receptor orthologues for drug discovery of GPCRs.

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Probing the binding site(s) of calcium sensing receptor (CaSR) negative allosteric modulators (NAMs)

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Introduction. The CaSR is a G protein-coupled receptor that primarily acts to maintain extracellular calcium (Ca^{2+}_o) homeostasis, via inhibitory control of parathyroid hormone (PTH) secretion. Accordingly, CaSR NAMs have potential for treating osteoporosis, because transient increases in serum PTH induce bone formation. However, to date, CaSR NAMs have failed to improve bone density in human clinical trials, possibly due to sub-maximal and prolonged stimulation of PTH release. An understanding of the binding site(s) utilised by distinct CaSR NAMs may aid the rational design of novel drugs with greater therapeutic efficacy.
Aims. To probe the binding site(s) of the reported NAMs, NPS-2143, ronacaleret, ATF-936, BMS compound 1, pyrimidine-4-one and calhex231.
Methods. Modulation of CaSR signalling by NAMs was investigated in FlpIn HEK TRex cells expressing the wild type CaSR and alanine-substituted mutant receptors using intracellular calcium mobilisation assays. An operational model of allosterism was used to determine NAM affinity and cooperativity with Ca^{2+}_o.
Results. We have previously identified an extended cavity in the CaSR's 7 transmembrane-spanning (7TM) domain that accomodates overlapping but distinct allosteric binding sites (Leach et al, 2016). In the present study, the binding affinity of all NAMs tested was significantly altered ($P<0.05$, one-way ANOVA) following substitution of one or more amino acids in this 7TM cavity. Our data suggest that ronacaleret, NPS-2143 and pyrimidine-4-one are predicted to bind to a common allosteric site. Although calhex231 is also predicted to bind to this site, it exhibited both negative and positive allosteric modulation via subtle differences in its engagement with distinct residues. ATF-936 and and BMS compound 1 are predicted to bind in a manner distinct from the other NAMs.
Discussion. A detailed understanding of NAM structure-function may aid our understanding of CaSR negative allosteric modulation and the development of effective anti-osteoporosis therapeutics.

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Endosomal Signalling and Trafficking of the Calcitonin Gene-Related Peptide Receptor in Pain

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Introduction. G protein-coupled receptors (GPCRs) are cell-surface sensors of extracellular signals that control most pathophysiological processes. While it has been demonstrated that activated GPCRs can redistribute to endosomes, it remains unexplored whether the signals that are generated from such endosomal receptors underlie complex pathophysiological processes.
Aims. To determine whether endosomal signalling of the calcitonin gene-related peptide (CGRP) receptor is a critical contributor to pain transmission and, if so, if endosomal signalling of the CGRP receptor can be selectively targeted.
Methods. Bioluminescene and fluorescence resonance energy transfer assays were utilized to study receptor trafficking and compartmentalized signalling, respectively. The CGRP receptor antagonist, $CGRP_{8-37}$, was conjugated to cholestanol to anchor the drug to the membrane surfaces and promote endosomal delivery. The role of CGRP receptor endocytosis in pain transmission was studied by investigating the effect of $CGRP_{8-37}$, $CGRP_{8-37}$-cholestanol, and conventional inhibitors of endocytosis on capsaicin-induced mechanical hyperalgesia.
Results. We report that the CGRP receptor generates signals from endosomes that underlie pain. Endosomal receptors signalled via G proteins and β-arrestins to cause sustained stimulation of extracellular signal regulated kinases in the nucleus and protein kinase C and cAMP in the cytosol. Clathrin, dynamin and β-arrestin inhibitors blocked agonist-evoked receptor endocytosis, excitability of spinal neurons and pain transmission. Receptor antagonists, when conjugated to cholestanol to promote endosomal targeting and retention, selectively inhibited endosomal signalling and suppressed neuronal excitability and pain.
Discussion. Our results reveal a critical role for endosomal signalling of the CGRP receptor in the complex pathophysiology of pain, and demonstrate the utility of endosomally-targeted antagonists.

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ML290 is a small molecule biased agonist at relaxin family peptide receptor 1 (RXFP1)

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Introduction. The peptide hormone, relaxin, is the cognate ligand for relaxin family peptide receptor 1 (RXFP1), that is being assessed in Phase IIIb clinical trials for treating acute heart failure. ML290 was recently identified as a first small molecule agonist acting at RXFP1.
Aims. To better understand ML290-mediated signalling responses at RXFP1.
Methods. This study compared ML290 and relaxin-mediated MAPK, cAMP and cGMP signalling using Surefire or Alphascreen kits in human cells endogenously and recombinantly expressing RXFP1, and longer-term actions on markers of fibrosis (MMP2 expression and Smad2/3 phosphorylation) in human cardiac fibroblasts.
Results. In HEK-RXFP1 cells, ML290 stimulated cAMP accumulation and p38MAPK phosphorylation but had no effect on cGMP accumulation, ERK1/2 or JNK1/2/3 phosphorylation. In human primary vascular cells, ML290 increased cAMP and cGMP accumulation but not p-ERK1/2 in coronary artery (HCAEC) and umbilical vein endothelial cells (HUVEC), in umbilical artery (HUASMC) and umbilical vein smooth muscle cells (HUVSMC). In human cardiac fibroblasts (HCF), ML290 increased cGMP accumulation but had no effect on p-ERK1/2 and given chronically also activated MMP-2 expression and inhibited TGF-β1-induced Smad2 and Smad3 phosphorylation. ML290 increased p-p38MAPK only in smooth muscle but not endothelial cells. In vascular cells, ML290 was ten times more potent for cGMP accumulation and p-p38MAPK than for cAMP accumulation. ML290-mediated cAMP and cGMP accumulation was inhibited by NF449 (Gαs inhibitor) but not NF023 (Gαi inhibitor) in all vascular cells. In BRET studies ML290 caused strong coupling of RXFP1 to Gαs and GαoB but weak coupling to Gαi3.
Discussion. In summary, ML290 exhibited signalling and system bias at RXFP1 displaying a signalling profile indicative of vasodilatory and anti-fibrotic properties.

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Tendency of class B G protein-coupled secretin and GLP-1 receptors to self-associate and cross-associate when co-expressed on the same cell

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Introduction. Class B GPCRs form homo- and hetero-dimeric complexes, however the tendency to form these complexes and the functional and regulatory implications have not been established. The secretin receptor (SecR) and the glucagon-like peptide-1 receptor (GLP-1R) are both expressed on the pancreatic β-islets and activation of each of these receptors can promote glucose mediated insulin secretion.
Aims. To characterise the homo- and hetero-dimeric complexes of SecR and GLP1-R.
Methods. A CHO cell line that expresses a fixed, single concentration of fluorescently tagged SecR and inducible expression of GLP-1R with the complementary tag, was used to generate a broad variety of stoichiometric expression ratios and to examine the impact of this on formation of hetero-receptor complexes using BRET. cAMP accumulation, calcium mobilisation and internalisation of each of the receptors by GLP-1 or secretin was assessed in stable CHO cell lines expressing a fixed number of SecR and three distinct stoichiometric ratios of GLP-1R to SecR expression levels. Functional assays were also performed in isolated β-islets derived from wildtype and GLP-1R knockout mice.
Results. Homo-dimeric SecR/SecR and GLP-1R/GLP-1R complexes were more stable than the heterodimer GLP-1R/SecR complex. Cells co-expressing GLP-1R and SecR exhibited no change in the cAMP responses to each natural agonist, but displayed reduced secretin-mediated intracellular calcium mobilisation. The hetero-dimeric receptor complexes also mediated agonist-induced cross-receptor internalisation. Islets derived from GLP-1R KO mice displayed elevated secretin-mediated insulin secretion and calcium responses compare to those derived from the wild-type animals.
Discussion. Evidence that SecR/GLP-1R hetero-dimerisation reduces the ability of secretin to promote downstream signalling could have physiological implications given the two receptors are co-expressed in pancreatic β-islet cells.

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Context-dependent allosteric modulation of the human calcium sensing receptor (CaSR). Implications for drug discovery

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Introduction. The CaSR is expressed at high levels in the parathyroid gland, where it negatively controls parathyroid hormone (PTH) secretion in response to elevated extracellular calcium (Ca^{2+}_o). CaSR negative allosteric modulators (NAMs) are indicated for the treatment of numerous disorders, and have been trialled as anti-osteoporosis drugs due to their ability to stimulate PTH release, which enhances bone formation when transient. However, to date, NAMs have failed to improve bone mass and density, which may be due to their inability to maximally stimulate the release of appropriately efficacious levels of PTH. An understanding of how NAMs act via the CaSR can afford the opportunity to develop therapeutically superior NAMs.
Aims. To evaluate CaSR negative allosteric modulation by previously classified NAMs, calhex231 and NPS-2143.
Methods. Agonist (Ca^{2+}_o) stimulation of intracellular calcium mobilisation and inositol phosphate accumulation in FlpIn HEK TRex CaSR cells, was evaluated in the absence and presence of each NAM. PTH release from primary human parathyroid cells natively expressing the CaSR was measured in the presence of each NAM using an ELISA.
Results. As expected, NPS-2143 partially inhibited CaSR signalling mediated by Ca^{2+}_o. Surprisingly, calhex231, exhibited mixed negative and positive allosteric modulation depending on its concentration, the assay paradigm and the ambient ion content of the buffer. Nonetheless, although 10μM NPS2143 had a significantly greater negative effect on Ca^{2+}_o potency than calhex231 ($P>0.05$ t-test), 10μM calhex231 had a significantly greater effect on Ca^{2+}_o Emax than NPS2143 ($P>0.05$ t-test). In parathyroid cells, NPS-2143 robustly stimulated PTH release, whereas calhex231 both weakly inhibited and weakly stimulated PTH release depending on its concentration.
Discussion. We have unraveled a novel mode of CaSR allostery that has implications for drug screening, discovery and development. A greater understanding of CaSR negative allosteric modulation will inform the development of novel NAMs with greater ability to inhibit CaSR signalling.

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The TM1/N-terminal domain interface of the GLP-1R is crucial for agonist affinity and biased agonism

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Introduction. The glucagon-like peptide-1 receptor (GLP-1R) is a Class B GPCR that can activate multiple signalling pathways and is a major therapeutic target for type 2 diabetes and obesity. Activation of the GLP-1R by discrete ligands can promote distinct signalling profiles, an effect termed biased agonism. Recently, we revealed three biased peptides engage distinct regions within the extracellular loops of the GLP-1R to promote downstream signalling linked to biased agonism (Wootten *et al*, 2016).
Aims. To extend our previous study by assessing the importance of the TM1/N-terminal domain interface and extreme N-terminus of the GLP-1R in the function of 3 biased agonists; GLP-1, exendin-4 and oxyntomodulin
Methods. Wild-type (WT) and mutant human GLP-1Rs were stably expressed in FlpInCHO cells and cell surface expression of mutant GLP-1Rs relative to WT were determined by ELISA. The affinity of each ligand for the WT and mutant GLP-1Rs were determined by competition radioligand binding. The ability of each ligand to activate each receptor variant was assessed using 3 signalling endpoints; cAMP accumulation, ERK1/2 phosphorylation and Ca^{2+} mobilisation. Advanced analytical methods were applied to separate effects on signalling from those on agonist affinity. The functional consequence of each mutation on each ligand was mapped onto a model of the GLP-1R.
Results. Multiple residues within the TM1-N-terminal domain interface crucial for affinity and efficacy of all 3 peptide agonists were revealed. In addition, residues with ligand-specific effects on receptor function were also identified.
Discussion. Taken together with our previous study, we have now mapped the contribution of the entire extracellular surface of the GLP-1R for affinity and signalling of 3 distinct biased agonists. This yields novel molecular insights into GLP-1R activation and biased agonism that may provide a foundation for the rational design of novel biased ligands for this receptor.
Wootten *et al*, 2016. *Cell* 165(7):1632-43

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Purification of active human Calcitonin receptor and Gs heterotrimer complex

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Introduction. G protein coupled receptors (GPCRs) play vital roles in a range of biological processes and are attractive targets for therapeutic drugs. In recent years, there have been significant advances in both structural and biophysical techniques that have lead to high-resolution structures of an increasing number of GPCRs. To date, only two Class B GPCR structures have been reported. The human calcitonin receptor (hCTR) is a Class B GPCR that mediates responses to the peptide hormone calcitonin. To advance our understanding of the mechanism of Class B GPCR activation by peptide ligands, we have optimised conditions to over-express and purify the active state ternary complex of hCTR bound to the agonist salmon calcitonin (sCT) and Gs heterotrimer for structure studies.
Aims. To over-express and purify sCT-hCTR and Gs heterotrimer complex for structural studies
Methods. hCTR and heterotrimeric Gs were co-expressed in insect cells. hCTR and Gs complex were solubilised by detergent maltose-neopentyl glycol (MNG). Purification was performed by FLAG antibody affinity chromatography and size exclusion chromatography. The agonist sCT was present during the purification process at a concentration of 100nM. The sCT-hCTR and Gs complex was examined by single particle electron microscopy.
Results. Yield of sCT, hCTR and Gs complex from 1L of insect cells was approximately 1mg, purity of complex from size exclusion chromatography was found to be >95% assessed by SDS-PAGE. 2D Class averages from single particle electron microscopy reveal that the CTR N-terminal domain is flexible in the presence of a peptide agonist adopting at least three metastable conformations, while more than one conformation is also observed in the Gs. Conclusion. The ability to produce large amount of high quality agonist bound receptor in complex with G protein for structural studies, such as electron microscopy, will aid in understanding Class B GPCR structure and function.

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A novel PAM that selectively boosts the action of agonists at the dopamine D2 receptor

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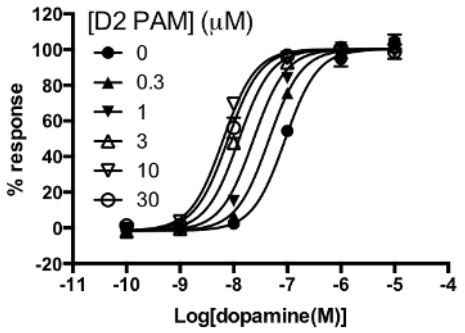
Introduction. The dopamine D_2 receptor agonists have been utilized for the treatment of Parkinson's disease. However, the available D_2 agonists also activate the dopamine D_3 and D_4 receptors, leading to potential undesired side effects. Selective activation of the D_2 receptor may be achieved by targeting the allosteric ligand binding site of the D_2 receptor
Aims. This study aimed to characterize the *in vitro* pharmacology of a novel dopamine D_2 receptor positive allosteric modulator (PAM).
Methods. Cellular cAMP levels were assessed with CAMYEL biosensor, ERK phosphorylation was determined with AlphaScreen® kit (PerkinElmer) and [^{3}H]raclopride and [^{3}H]spiperone were used in radioligand binding assays. All assays were performed using CHO heterologously expressing the human dopamine D_2, D_3, and D_4 receptors.



Results. The novel PAM increased the potency of dopamine at the D_2 receptor, decreased the maximum effect of dopamine at the D_3 receptor and did not affect the dopamine response at the D_4 receptor. The PAM did not only enhance the potency of partial agonists, but also increased their maximum effects at D_2 receptor. In binding assay, the PAM potentiated the affinity of dopamine at both D_2 and D_3 receptor, but did not modulate binding of dopamine at the D_4 receptor.
Discussion. We describe a novel PAM that selectively enhances the action of D_2 receptor agonists. Such selectivity and other advantages of PAMs, such as maintenance of spatio and temporal effects of endogenous agonist in the body, warrant the promising potentials of this PAM for future investigation and improvement in the discovery of new treatment of Parkinson's diseases.

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Agonist-mediated regulation of the striatal orphan receptor, GPR52

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Introduction. GPR52 is an orphan GPCR that is highly enriched in the striatum and it has been suggested that its cell surface regulation alters the clearance of the mutant huntingtin in Drosophila and may play a role in Huntington's disease (Yao et al., 2015). Thus, understanding the mechanisms underlying GPR52 cell surface regulation may provide information on how to target GPR52 for the treatment of Huntington's disease.
Aims. To study the molecular mechanisms of GPR52 agonist-induced trafficking and internalisation using a synthetic, small molecule agonist, 3-BTBZ (Compound 7m, Setoh et al., 2014).
Methods. N-terminally SNAP- and C-terminally NanoLuc-tagged GPR52 were separately and transiently expressed in HEK293T cells, and assays were performed after treatment with 3-BTBZ (1 μM). SNAP-GPR52 was labelled with BG-AlexaFluor488 and imaged using live-cell confocal microscopy to investigate receptor cycling under resting and 3-BTBZ-activated conditions. GPR52-NanoLuc was transiently co-expressed with β-arrestin1-Venus, β-arrestin2-Venus, endosomal markers Rab5a-Venus (early endosome) or Rab7-Venus (late endosome), studies were performed using BRET technology under resting and 3-BTBZ-activated conditions.
Results. SNAP-GPR52 undergoes constitutive receptor cycling that is seemingly unaltered in the presence of 3-BTBZ (1μM). However, the same concentration of 3-BTBZ elicited a robust recruitment of both β-arrestin1-Venus and β-arrestin2-Venus (t_{max} ~8mins), Rab5a-Venus (t_{max} ~13mins) and Rab7-Venus (t_{max} >20mins).
Discussion. These data suggest GPR52 may recruit arrestins and be targeted to endosomes in an agonist-dependent manner. Further studies are required to determine the role of GPR52 regulation in a physiological system.

Yao et al., (2015) Elife 4;4
Setoh et al., (2014) J Med Chem. 26;57(12):5226-37

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Extracellular loop 2 of the GLP-1R is a crucial domain for controlling agonist affinity and biased agonism

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Introduction. The glucagon-like peptide-1 receptor (GLP-1R) is a Class B GPCR that is activated by large peptide ligands via a two-domain mode. The C-terminus of the peptide interacts within the N-terminus of the receptor, while the N-terminus of the peptide contacts the receptor transmembrane domain to promote activation of downstream signalling. GLP-1 (the native ligand) and exendin-4 (a clinically approved peptide) are highly conserved within their N-terminal sequence. Extracellular loop 2 (ECL2) within the GLP-1R is a crucial domain for interaction of these ligands and subsequent receptor activation. Recently, novel GLP-1R peptide agonists have been reported; exendin-P5 that contains the C-terminus of exendin-4, but with a distinct N-terminal activating domain, and a series of cyclic 11mer peptides that are structurally distinct from GLP-1. These peptides display biased signalling profiles relative to exendin-4 and GLP-1.
Aims. Identify the importance of the GLP-1R ECL2 in the ability of novel peptide ligands (exendin-P5 and two 11mers) to activate intracellular signalling, and determine if ECL2 contributes to their distinct bias profiles.
Methods. FlpInCHO cells stably expressing either GLP-1R WT or GLP-1R with Ala mutations incorporated within ECL2 were pharmacologically assessed using GLP-1, exendin-4, exendin-P5, and 2 novel 11mer peptides. The ability of each ligand to activate each receptor variant was assessed in 3 signalling endpoints; cAMP accumulation, intracellular calcium mobilisation and ERK1/2 phosphorylation. Operational modelling was applied to concentration response data to determine the impact of each receptor mutation on the efficacy and functional affinity of each ligand for each signalling endpoint. These data were mapped onto a model of the activated GLP-1R.
Results. We identified regions within ECL2 that are critical for activation transition of all ligands assessed, however there were multiple residues where mutations had differential effects on the efficacy of signalling pathways when activated by exendin-P5 or the 11mer peptides relative to GLP-1 and/or exendin-4.
Discussion. This study demonstrates key roles for ECL2 within the GLP-1R in propagating activation transition in a ligand-dependent manner providing further evidence for the critical role of ECL2 in driving biased agonism.

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Use of BRET to demonstrate AT1R-EGFR complexes and AT1R-mediated EGFR transactivation

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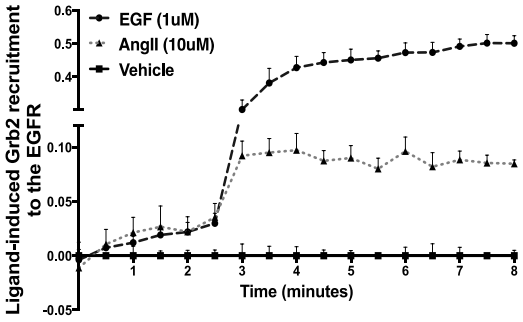

Introduction. The angiotensin II Type 1 Receptor (AT_1R) can stimulate signalling pathways downstream of growth factor receptors, thereby enabling AT_1R to regulate cell growth, differentiation and apoptosis. The Thomas laboratory has used a functional genomics approach to identify potential novel proteins involved in the 'cross-talk' between AT_1R and the epidermal growth factor receptor (EGFR). The screen identified a number of novel proteins, including, Choline Kinase Alpha (CHKA), the key enzyme involved in the synthesis of the membrane lipid, phosphatidylcholine.
Aims. Identify and characterise the molecular, temporal and spatial aspects of AT1R-EGFR transactivation and to define the potential role of CHKA in AT_1R-EGFR transactivation.
Methods. Bioluminescence Resonance Energy Transfer (BRET), GPCR-HIT assays and western blotting were used to functionally characterise the molecular basis of EGFR transactivation and validate CHKA involvement.
Results. Both AngII and EGF stimulated recruitment of Grb2 to the EGFR, indicating EGFR transactivation (see figure). Moreover, GPCR-HIT revealed a complex between the AT_1R and EGFR following AngII stimulation. Furthermore, addition of CHKA shows an increase in 'cross-talk' between AT_1R-EGFR.
Discussion. BRET was validated as a tool to characterise transactivation in living cells, and to our knowledge, this is the first to demonstrate transactivation in living cells in real-time. Data suggests that following AngII treatment a close complex forms between the two receptors, thus facilitating transactivation. Introducing CHKA enhanced EGFR transactivation following AngII stimulation; the mechanism forms part of ongoing investigations.

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Expression and signalling of calcitonin family receptors in four patient-derived GBM cell lines

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Introduction. Glioblastoma multiforme (GBM) is the most common and aggressive type of primary brain cancer. The calcitonin family of receptors, calcitonin (CTR), amylin (AMY; CTR/RAMP), adrenomedullin (AM_1, CLR/RAMP2; AM_2; CLR/RAMP3) and CGRP ($CGRP_1$; CLR/RAMP1) receptors are expressed in many cancers, including GBM. Studies involving breast cancer, prostate cancer and glioma cell lines show that tumor invasiveness, metastatic potential and angiogenesis can be modulated by stimulation of CTR/CLR with their agonists or antagonists.
Aims. Pharmacological characterization of 4 new patient-derived GBM cell lines that recapitulate the original disease when injected intracranially into mice, to identify which calcitonin family receptor subtypes are expressed.
Methods. We used cAMP accumulation assays to pharmacologically characterize these cell lines and qPCR to confirm mRNA expression of receptors and RAMPs.
Results. We detected functional CTR receptor in the Sb2b cell line (classical GBM subtype), with potent response to sCT and hCT but only weak response to CGRP, amylin or adrenomedullin. In this cell line sCT and hCT had distinct profiles with sCT demonstrating higher potency but lower Emax than hCT, a profile that is not routinely observed in study of CTRs. Investigation of this differential efficacy may provide insight into control of signaling in this patho-physiologically relevant system. The other three cell lines (Pb1, JK2, and WK1, representing classical, proneural and mesenchymal subtypes of GBM) have no functional CTR as assessed by cAMP accumulation assay. They display a potent cAMP accumulation response to αCGRP and less potent response to adrenomedullin, indicative of $CGRP_1$ receptors.
Discussion. We have shown activation of the cAMP pathway by CTR/CLR receptors in all of the four cell lines. cAMP is known to regulate various transcription factors that can be involved in tumor cell physiology. There are numerous genes and signaling pathways that are dysregulated in glioblastoma multiforme. Deciphering these pathways and identifying the receptors upstream of these pathways will provide us with better understanding of cancer biology and will help us to identify the new targets for cancer therapy.

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High Affinity α1B-adrenoceptor or Low Affinity β2-adrenoceptor Resting States Stabilised by Disrupting the Hydrogen Bond Network between the NPxxY Motif and TMH5

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Introduction. G protein-coupled receptor (GPCR) transmit extracellular stimuli to intracellular responses to control numerous physiological processes. Recent crystallographic and biophysical advances in GPCR structure-function have dramatically increased our understanding of these dynamic receptors, but the available crystal structures are only limited to a few receptors and the molecular mechanism of activation and signalling is still not well understood.
Aims. This study aimed to stabilize the α_{1B}-adrenoceptor (α_{1B}-AR) and the β_2-AR, in resting state conformations by mutating the residues involved in the formation of a water-mediated hydrogen bonded activation switch between the conserved $NPxxY^{7.53}$ sequence on TMH7 and a conserved tyrosine in TMH5 ($Y^{5.58}$) (Ballesteros et al. 1995), to characterize the signalling and ligand binding properties of these resting state receptors.
Methods. The conserved $Y^{5/58}$ and $Y^{7/53}$ residues were mutated to phenylalanine ($Y^{5/58}F$ and $Y^{7/53}F$), either singly or as a double mutant ($Y^{5/58}F/Y^{7/53}F$), and we examined the signalling properties of these receptors to confirm resting state using an inositol 1-phosphate assay (α_{1B}-AR) and cAMP assay (β_2-AR). We also determined agonist and antagonist affinities, including the α_{1B}-AR allosteric antagonist ρ-TIA, using radioligand binding assays.
Results. We report largely reduced agonist potency (EC_{50}) at resting state α_{1B}-ARs (24–123-fold) and β_2-ARs (13–114-fold), in addition to reduced signalling efficacy for both α_{1B}-ARs (169–705-fold) and β_2-ARs (2–17-fold) compared with wild type receptor. Antagonist affinity at resting state receptors was comparable with wild type receptor and we confirm a previously described low-affinity agonist β_2-AR resting state (Staus et al. 2016) but in contrast reveal a surprising high-affinity agonist α_{1B}-AR resting state with 2–15-fold increased affinity for NE.
Discussion. For the first time, we report that GPCRs can adopt low-affinity or high-affinity agonist resting state conformations depending on the GPCR.

Ballesteros J et al. (1995) Methods in Neuroscience 25:366-428.
Staus DP et al. (2016) Nature 535:448-452.

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Characterising the signalling of amylin receptors

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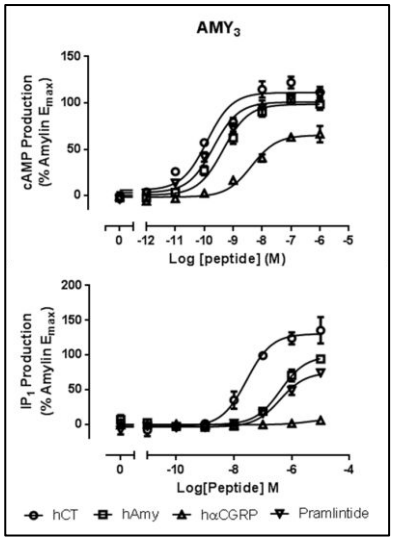

Introduction. Amylin is a peptide hormone released from the pancreas in response to food intake, resulting in meal ending satiation and lower blood glucose levels. It is because of these properties that amylin receptors offer therapeutically relevant targets for the treatment of diabetes and obesity. Pramlintide, an amylin analogue, is currently approved for the treatment of diabetes. Despite the promising effects of amylin and pramlintide, very little is known about how amylin receptors signal or which of the three known amylin receptors is of therapeutic relevance. Amylin receptors are comprised of a core calcitonin receptor (CTR) and one of three receptor activity-modifying proteins (RAMPs). Understanding how amylin receptors signal may allow for better targeted drug design.

Aims. To characterise the pharmacology and signalling profiles of amylin receptors in response to endogenous agonists and pramlintide.

Methods. Cos7 cells were transfected with CTR and the relevant RAMP or a bicistronic vector containing both the CTR and the RAMP. The responses to different agonists was measured using antibody-based functional assays for each of the different pathways.

Results. The three amylin receptors show subtle differences with agonist (see figure) and RAMP dependent signalling profiles for each pathway measured.

Discussion. There are emerging differences in signalling and pharmacology at each amylin receptor for each pathway measured; indicating possible RAMP dependent biased agonism. RAMPs may contribute to this bias directly, by interacting with G proteins via the C terminus, or indirectly, through modulation of the CTR structure and agonist binding site.

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The use of Fluorescence Correlation Spectroscopy to investigate ligand-receptor interaction at the Neuropeptide Y Y1 receptor

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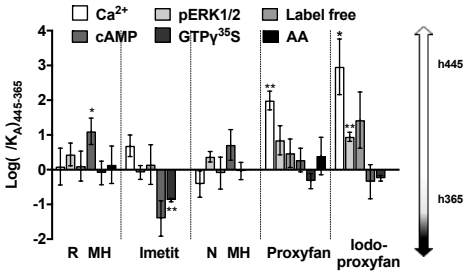

Introduction. Fluorescent ligands allow insight into ligand-receptor interactions when used in combination with Fluorescence Correlation Spectroscopy (FCS) to measure free and receptor-bound diffusion properties (Ayling et al, 2012). Here we describe this approach to study novel Neuropeptide Y Y1 receptor (NPY; Y1R) fluorescent monomeric antagonists BIDA84 (([Lys^2-sulphatedCy5,Arg^4]BVD15), BIDA81 (([Lys^2-RhoB, Arg^4]BVD15), and BIDB13, a rhodamine derivative of a BVD15 dimer (Mountford et al, 2014; Liu et al, 2016).

Aims. We characterised the behaviour of each fluorescent peptide by FCS and autocorrelation analysis, to obtain their diffusion co-efficients (*D*) in solution, and when bound to living cells expressing the Y1 receptor.

Methods. FCS solution measurements were taken at 1-100nM peptide in HBSS/0.1%BSA at room temperature (RT; 21°C). In receptor binding, HEK293 cells expressing the SNAP-tagged Y1R were incubated with 10nM peptide in HBSS/BSA (30mins; 37°C; 0%CO_2). Measurements were taken as previously described (Kilpatrick et al, 2012).

Results. In solution, at 10nM peptide, *D* values were 128.63±8.19µm^2/s (n=4) for BIDA84, 10.84±0.52µm^2/s (n=4) for BIDA81 and 10.42±0.59µm^2/s (n=4) for BIDB13. Cell membrane measurements showed receptor-bound and free ligand, by the detection of slower diffusing species. BIDA84 exhibited a faster bound *D* of 0.62±0.06µm^2/s (n=26 cells: 4 experiments) compared to BIDA81, 0.18±0.02µm^2/s (n=56:4), BIDB13 0.37±0.06µm^2/s (n=43:4) or 0.2µM SNAPsurface AF488/647 labelled Y1R (0.22±0.01µm^2/s n=59:4; 0.59±0.05µm^2/s, n=32:4).

Discussion. FCS detects Y1R fluorescent ligand binding. For both Cy5- and Rho-labelled ligands, the diffusion co-efficients of receptor-bound species closely reflect those of SNAP fluorophore labelled receptors

Ayling LJ et al (2012) J Cell Sci 125: 869-886; Kilpatrick LE et al (2012) Biochim Biophys Acta 1823: 1068-1081; Liu M et al (2016) Bioconjugate Chem [Epub ahead of print]; Mountford SJ et al (2014) Org Biomol Chem 12: 3271-81

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Isoform-specific bias of histamine H3 receptor agonists

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Introduction. The histamine H_3 receptor (H_3R) is widely expressed in human brain and has been implicated in various pathologies, including Alzheimer's disease, schizophrenia, dementia and Parkinson's disease (Barbier et al, 2007; Esbenshade et al. 2008). The H_3R is subject to extensive gene splicing that gives rise to a large number of functional and non-functional isoforms. G protein-coupled receptors (GPCRs) can adopt different ligand-induced conformations that give rise to biased signalling (Kenakin T, and Christopoulos A, 2013), however, this has not been studied for the H_3R; furthermore, it is unknown whether splice variants of the same receptor engender the same or differential biased signalling.
Aims & Methods. Five histamine receptor agonists were profiled at two abundant hH_3R splice variants stably expressed on CHO cells (hH_3R_{445} and hH_3R_{365}) using seven signalling endpoints. Agonist-response data were analysed using transducer ratios ($Log(\tau/K_A)$) to estimate ligand bias.
Results. Both isoforms engender biased signalling, although hH_3R_{365} was more permissive: imetit, proxyfan and iodoproxyfan were all markedly biased away from calcium signalling with $\Delta Log(\tau/K_A)$ values of -0.31 ± 0.54, -3.0 and -3.0, respectively. However, most interesting was the identification of differential biased signalling between the two isoforms. Strikingly, hH_3R_{365} was completely unable to stimulate GSK3 phosphorylation, an endpoint robustly activated by the full-length receptor, which, for proxyfan, resulted in a 260-fold bias. Individual one-sample T-tests revealed a number of significant examples of isoform bias (see Figure). Proxyfan and iodoproxyfan only promoted calcium mobilization *via* the hH_3R_{445} isoform $(Log(\tau/K_A)_{445\text{-}365}$ values of 2.0 ± 0.3 and 3.0 ± 0.8, equating to 98 and 912-fold bias, respectively); whereas RαMH was approximately 13-fold more effective at inhibiting cAMP levels at the hH_3R_{445} isoform.
Discussion. Herein we have demonstrated that differential biased signalling *via* isoforms of the same GPCR that are simultaneously expressed *in vivo* exist. Given the subtle differences in localisation and function of the two major isoforms of the H_3 receptor, choice of agonist could influence the observed response(s) and may represent a novel route to selective pharmacology.
Barbier AJ, Aluisio L, Lord B et al (2007) Eur J Pharmacol 576:43–54.
Esbenshade TA, Browman KE, Bitner RS et al (2008) B J Pharmacol 154:1166–1181.
Kenakin T, and Christopoulos A (2013). Nat Rev Drug Discov 12:205–216.

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GLP-2R signalling is modulated by RAMPs

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Introduction. The GLP-2 receptor (GLP-2R) is a Family B GPCR expressed in epithelial cells of the gut. Activation by glucagon-like peptide 2 (GLP-2) leads to crypt cell proliferation in the small intestine and increased nutrient absorption (Moore BA *et al*, 2010). It is targeted to treat patients with short bowel syndrome (Walsh N *et al*, 2003). The GLP-2R signals through $G\alpha_s$ leading to cAMP production, however it is unknown whether the receptor couples to other pathways such as $G\alpha_q$. We have demonstrated that the GLP-2R is able to interact with receptor activity modifying proteins (RAMPs), however the full impact of these interactions is not fully understood.
Aims. We determined the effects of transfecting cells with increasing ratios of RAMP DNA in relation to GLP-2R upon $G\alpha_s$ signalling. We also investigated whether the GLP-2R is able to couple to $G\alpha_q$.
Methods. The receptor was transiently transfected into HEK293S cells and cAMP production measured using a PerkinElmer LANCE assay kit (Weston C *et al*, 2015). Intracellular calcium (Ca^{2+}_i) mobilisation was measured using a Molecular Probes Fluo-4 direct calcium assay kit, and G_q coupling inhibited using compound YM.
Results. Upon increasing both RAMP1 and RAMP3 to receptor ratios, we found a decrease in E_{max} and rightward shift in cAMP response, suggesting that increased expression of RAMP1 or 3 attenuated signalling. Conversely, when RAMP2 to receptor ratio was increased, we saw no significant change to E_{max} until RAMP2 was transfected at 5:1 with GLP-2R, resulting in an increase. We observed a dose-dependent Ca^{2+}_i response when GLP-2R was stimulated with GLP2 1-33, which was abolished upon treatment with $G\alpha_q$ inhibitor YM, suggesting that Ca^{2+}_i mobilisation was as a result of signalling through $G\alpha_q$.
Discussion. Currently, the stoichiometry of RAMP-receptor complexes is largely unclear, however this data suggests that increasing the ratio of RAMP to receptor can affect signaling of the GLP-2R. In addition, the GLP-2R can couple to $G\alpha_q$ upon stimulation by GLP-2 1-33. This information provides novel insights into the signalling of this receptor. Moore BA *et al* (2010) *J. Pharm. Exp. Ther.* **333**: 574–583; Walsh N *et al* (2003) *Endocrinology* **144**: 4385 – 4392; Weston C *et al* (2015). *J Biol Chem* **290**: 23009-23022.

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Desensitization of CB1 receptor signaling by valinate and tert-leucinate synthetic cannabinoids.

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Introduction. Synthetic cannabinoids (SCs) with indole or indazole cores and featuring L-valinate or L-tert leucinate groups are epidemic recreational drugs in many parts of the world, and can be associated with severe toxicity. Despite this, there is little known about their pharmacology and its role in desensitization of cannabinoid receptor.
Aims. To evaluate the cannabimimetic activity of these SCs on human cannabinoid type 1 (CB1) and type 2 (CB2) receptors. We examined 5F-MDMB-PICA (most potent of these SCs) induced desensitization of CB1 receptors.
Methods. We used a fluorescence-based membrane potential assay to measure the potassium channel-mediated cellular hyperpolarization of AtT-20 cells expressing CB1 or CB2. Compound 101, Trametinib and SCH772984 were used to study the involvement of G Protein-Coupled Receptor Kinase2/3 (GRK), Mitogen activated protein kinase enzyme (MEK) and extracellular signal regulated kinase (ERK) in CB1 receptor desensitization.
Results. All 16 indole and indazole SCs tested activated CB1 and CB2, with a modest preference for CB1. The most potent was 5F-MDMB-PICA, with an EC_{50} of 0.45 nM at CB1 receptor. The desensitization of the CB1 mediated hyperpolarization produced by EC_{50} and EC_{90} concentrations of 5F-MDMB-PICA was 65±5% and 78±2% after 30 min (n=6). The hyperpolarization to a subsequent application of somatostatin (100 nM) was unchanged. In cells treated with Cmpd101 (10 µM), we did not observe any difference in the desensitization evoked by the EC_{90} concentration of 5F-MDMB-PICA, but a concentration of 10 µM reduced the desensitization from 97±3.9% to 77±3.5%. Inhibition of MEK or ERK had no effect on CB1 receptor desensitization.
Discussion. All SCs tested in this study have greater potency and maximum effect than Δ^9-THC. Desensitization was largely homologous, with little effect on the native somatostatin receptor responses. Our data demonstrate a role of GRK2/3 in CB1 receptor desensitization to high concentrations of agonist in AtT20 cells, but suggest that other mechanisms may by recruited by lower concentrations of drug.

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Biased agonism at the CC chemokine receptor CCR1

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Introduction. Chemokines are small soluble proteins that stimulate leukocyte recruitment by activation of chemokine receptors, a subfamily of G protein-coupled receptors (GPCRs) expressed on leukocytes. Chemokine-receptor interactions play critical roles in a wide range of inflammatory diseases, such as multiple sclerosis and atherosclerosis, as well as in HIV, cancer and diabetes. Among the chemokine receptor family, CCR1 is particularly interesting because it is already recognized as a drug target for multiple sclerosis and rheumatoid arthritis, and is a highly promiscuous receptor, as it can recognize more than 8 CC chemokines, including HCC-2/CCL15, MCP-3/CCL7 and MCP-2/CCL8. The existence of multiple ligands for the same receptor was once considered to be redundancy. However, it is now recognized that receptors can exhibit biased agonism, the ability to give different signalling responses depending on which ligand it is bound to. CCR1 also contains two tyrosine residues in its extracellular N-terminal region that are potential sites of sulfation, an important post-translational modification for chemokine receptor signalling.
Aims. To characterize the biased agonism at CCR1 and study the influence of tyrosine sulfation on CCR1 biased agonism.
Methods. We measured binding and activation of several pathways by three CCR1 cognate ligands. Binding affinities were determined using a radioligand displacement assay and signalling was observed by measurement of ERK phosphorylation, cyclic AMP production, β-arrestin recruitment and G protein activation. Bias factors were then calculated and the same experiments were performed with chlorate-treated cells to investigate the role of receptor tyrosine sulfation.
Results. CCR1 exhibits biased agonism towards cyclic AMP inhibition relative to other pathways and tyrosine sulfation does not influence CCR1 biased agonism.

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Novel allosteric modulators of M_1 muscarinic acetylcholine receptors

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Introduction. Positive allosteric modulators (PAMs) that target the M_1 muscarinic acetylcholine receptor (M_1 mAChR) are an encouraging avenue for the treatment of the cognitive deficits seen in diseases such as Alzheimer's and schizophrenia. We have recently discovered novel M_1 mAChR PAM scaffolds with the potential to display different modes of allosteric modulation and/or agonism (Mistry et al. 2016), but their molecular mechanisms of action remain undetermined. Aims. To compare the pharmacology of three novel allosteric ligands based on a 4-pheylpyridin-2-one scaffold with the prototypical M_1 mAChR PAM, BQCA. Methods. Agonist-mediated receptor activation of IP_1 accumulation, ERK 1/2 phosphorylation or β-arrestin 2 recruitment, in the absence or presence of allosteric modulator, was determined in FlpInCHO cells stably expressing the M_1 mAChR. Data were fitted to an operational model of allosterism to determine modulator affinity and cooperativity values. Results. Using an IP_1 accumulation assay, very low receptor reserve was identified in our cell line, allowing the determination of a rank order of orthosteric agonist efficacies as Iperoxo>ACh>oxotremorine-M>>xanomeline. In all instances, interaction of each agonist with each of our novel PAMs or BQCA revealed an allosteric potentiation consistent with modulation of agonist affinity but not efficacy. The greatest degrees of positive cooperativity was observed against the higher efficacy agonists, whereas minimal potentiation was observed when the modulators were interacted with xanomeline. When the effect of each PAM on the endogenous agonist, ACh, was determined at either ERK1/2 phosphorylation or β-arrestin 2 recruitment, similar degrees of potentiation were noted, suggesting no evidence of pathway bias in the allosteric effects. Discussion. Despite the elaboration and identification of novel allosteric scaffolds targeting the M_1 mAChR, the molecular mechanism of action of these compounds is consistent with a simple two-state model of allosteric activity, as previously described for BQCA (Canals et al. 2012).

Canals M et al (2012) J Biol. Chem 287:650-659
Mistry S.N. et al (2016) J. Med. Chem 59:388-409

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Arrestin biased GPCR agonism induces acute catecholamine secretion through TRPC3 coupling

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Introduction. Activation of GPCRs underlie the reactions of cells to extracellular stimulis. Two general signaling pathways, mediated by either specific G protein subtypes or β-arrestins, are shared by most of the GPCRs. In a traditional view, the receptor/G protein complex residing at the plasma membrane initiates acute signaling events, which is often called first-wave signaling. Compared with the receptor/G protein signaling at the plasma membrane, the second wave of GPCR signaling mediated by arrestin, commonly occurs 2–3 min later and plays critical roles in long-term GPCR functions.

Aims. we investigate the role of β-arrestin or G protein subtype-mediated GPCR signaling in catecholamine secretion after activation of AT1R, CCKAR and MachR etc, which may mimic clinical conditions, such as hypertension or neuro-endocrine interactions

Methods. we used specific G protein or β-arrestin-biased GPCR agonists, the β-arrestins , specific G protein subtype and specific TRP subtype knock out mouse models, combined with electrophysiology, cell biology and biochemical methods.

Results. Here, we revealed that β-arrestin-1-biased agonism stimulates acute catecholamine secretion through TRPC3 coupling and extracellular calcium influx. The β-arrestin-biased agonist promoted the formation of the AT1R/β-arrestin-1/TRPC3/PLCγ signaling complex localized in the plasma membrane and caused specific conformational change of TRPC3, which required not only the recognition of the C-terminal IP3R-binding motif of TRPC3 by C-terminal of the β-arrestin-1, but also the specific interaction between the SH3 domain of PLCγ and the poly-proline region-1 of β-arrestin-1.The receptor-β-arrestin-1-TRPC3 signaling contributed almost equally compared to the classic receptor-Gq-IP3R pathways in the regulation of calcium mobilization and vesicle secretion in the adrenal medulla by endogenous hormones, including AngII and Mch

Discussion. We demonstrated that the receptor-β-arrestin-1-TRPC3 signaling constitutes an important component after the activation of endogenous AT1R or muscarinic acetylcholine receptors. Taken together, the GPCR/arrestin complex initiated non-desensitized "first-wave" signalling not only in endosomes but also on the plasma membrane through fast communication to ion channels could be a general mechanism in many cellular processes.

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mTOR has a key role in α1-adrenoceptors mediated cardioprotection in hypoxic neonatal rat cardiomyocytes

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Introduction. α1-adrenoceptors (α1-ARs) activate pleiotropic downstream signalling to mediate important protective and adaptive functions in the heart failure (O'Collen et al., 2014; Shi et al., 2016).
Aims. We investigated signalling pathways utilized by the α1-AR to mediate cell survival and glucose uptake in neonatal rat ventricular myocytes (NRVM).
Methods. Primary cultured NRVM were incubated in the hypoxic chamber (1% O2) for 18h, then the role of α1-ARs was investigated by [3H]-prazosin binding, [3H]-glucose uptake, Ca2+ mobilization assay, α-screen kinase assays, western blot, and propidium iodide (PI) staining which measures apoptotic cells.
Results. After the incubation of cells in the hypoxic chamber, α1-ARs expression was significantly increased (Bmax; Hypoxic 102.5±14.6, Normoxic 62.3±3.2 fmol/mg protein). α1A-AR stimulations have no effect on Akt phosphorylation but increased phosphorylation on Erk1/2 and S6rp. The general mTOR complex 1/2 inhibitor KU0063794 significantly inhibited A61603-mediated glucose uptake by 70% and cell survival 58%, whereas mTOR complex 1 inhibitor rapamycin had no effects, indicating that mTOR complex 2 has a key role.
Discussion. Data indicate that α1A-ARs mediate protective signalling in hypoxic NRVM. α1A-ARs are proportionally increased under hypoxia, and mediate insulin-independent glucose uptake and cell survival via mTOR.

O'Connell et al., (2014) Pharmacol Rev. 66:308-333 Shi et al., (2016) J Recept Signal Transduct Res. 36(3):261-70

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Probing ligand binding modes and allosterism at ultra-stable α1-adrenergic receptors with NMR

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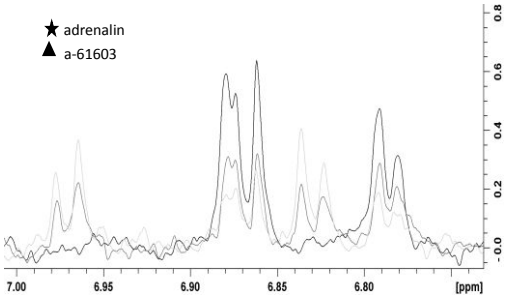
Introduction. α_1-Adrenergic receptor (AR) subtypes (α_{1A}, α_{1B}, α_{1D}) maintain a complex balance in modulating the sympathetic and CNS. The activation of individual subtypes can be either detrimental or protective. Selective drug development for such receptors is hindered by a lack of structural information.
Aims. Develop rapid atomic resolution NMR methods to gain a molecular insight into ligand binding to the α_1-AR subtypes.
Methods. The directed evolution method has been used to evolve detergent-stable α_{1A} and α_{1B}-AR mutants. We are developing NMR techniques including Saturation-Transfer Difference (STD-NMR), Water-LOGSY (Water-Ligand Observed via Gradient SpectroscopY), Transferred NOESY and INPHARMA (Interligand Noes for PHARmacophore Mapping) to understand how ligands bind to these receptors.



Results. Data will be presented comparing the binding of the native agonists, adrenaline and noradrenaline, an α_{1A}-AR selective agonist a-61603, as well as allosteric modulators, benzodiazepines (BZs). Approaches include epitope mapping by STD NMR, Tr-NOESY that provides conformational data of the bound state of ligands and Water-LOGSY to optimize conditions for studying BZs in solution.
Discussion. We have shown NMR is a powerful approach for gaining structural insights into ligand interactions with GPCRs. This study will play a critical role in shaping the research of α_1-AR subtype selective drug development.

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Investigating relaxin family peptide receptor 1 (RXFP1) signalling using a novel cAMP signalling biosensor

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Introduction. The relaxin hormone is the endogenous ligand for relaxin family peptide receptor 1 (RXFP1), a G protein-coupled receptor. In a Phase III clinical trial, relaxin demonstrated clinical benefits for acute heart failure including organoprotection and reduced mortality. ML290 is a novel small molecule agonist at RXFP1 with potential therapeutic benefits. However, the mechanisms of relaxin- and ML290-induced RXFP1 signalling have not been fully understood. Novel signalling biosensors that can measure spatiotemporal aspects of signalling with high sensitivity in living cells may help to better understand the physiological effects resulting from RXFP1 activation.
Aims. To optimise the conditions for use of the novel bioluminescence resonance energy transfer (BRET)-based biosensor CAMYEL (cAMP sensor using YFP-Epac-RLuc) in HEK-RXFP1 cells, and to examine relaxin- and ML290-induced cAMP production via RXFP1 using this sensor.
Methods. Agonist-induced cAMP production was measured in real-time in HEK cells expressing RXFP1 or its mutants. Both HEK cells transiently transfected with the CAMYEL biosensor as well as lentivirally-transduced HEK-CAMYEL stable cell lines were tested.
Results. We confirmed that the CAMYEL biosensor is a useful tool to detect cAMP production through RXFP1, allowing detection in real-time 96-well plate-based assays. Time-courses and concentration-response curves were generated for relaxin and ML290, showing robust cAMP signalling. cAMP production at a number of RXFP1 mutants was also examined. A HEK-CAMYEL stable cell line was also prepared, transiently transfected with RXFP1, and successfully tested for agonist-induced cAMP production.
Discussion. The optimised CAMYEL cAMP assay will allow easy testing of novel drugs at RXFP1. The CAMYEL biosensor demonstrated advantages over traditional end-point assays by providing detailed kinetics with a high sensitivity. The sensor may now also be used in primary cell lines that natively express RXFP1, as the newly-developed CAMYEL lentiviral vector will allow the transduction of primary cell lines to fully assess the kinetics of cAMP signalling for RXFP1 and other GPCRs.

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Identification of a potent adenosine A2B receptor agonist that stimulates cardiac anti-fibrotic signalling

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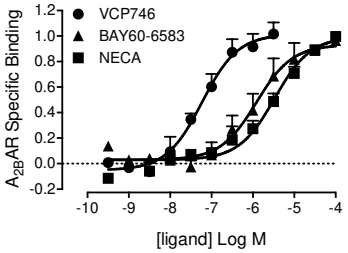

Introduction. The adenosine A_{2B} receptor (A_{2B}AR) has a key role in cardiac fibrosis. However, effective modulation of A_{2B}AR signalling has been limited by a lack of potent agonists. Recent screening revealed VCP746, a biased A_1AR agonist, also had significant and previously unappreciated A_{2B}AR activity.
Aims. To characterise VCP746 A_{2B}AR pharmacology and examine its anti-fibrotic activity.
Methods. The affinity and efficacy of VCP746 was examined in FlpInCHO cells stably expressing the human A_{2B}AR. VCP746 concentration response curves were generated across multiple functional pathways and compared to the conventional A_{2B}AR agonists NECA and BAY60-6583. The ability of VCP746 to modulate fibrotic signalling and inhibit TGFβ- or angiotensin II- mediated collagen synthesis was assessed in neonatal rat cardiac fibroblasts (NCFs).
Results. VCP746 had significantly higher (30-100 fold) affinity than NECA or BAY60-6583 at the A_{2B}AR ($P<0.01$; one-way ANOVA, Dunnett's post hoc; n=3). Functional assays in A_{2B}AR-FlpInCHO cells demonstrated VCP746 stimulated a robust increase in cAMP accumulation, ERK1/2 phosphorylation, IP_3 accumulation and Ca^{2+} mobilization. In NCFs, VCP746 stimulated anti-fibrotic cAMP signalling and inhibited pro-fibrotic IP_3 signalling, which manifested as potent inhibition of both TGFβ- and angiotensin II- mediated collagen synthesis (pIC_{50} 7.6 ± 0.4 and 7.8 ± 0.4, respectively; n=4-6). The influence of VCP746 on collagen synthesis was selectively reversed in the presence of an A_{2B}AR antagonist, demonstrating that the effects were mediated through A_{2B}ARs endogenously expressed in NCFs.
Discussion. VCP746 was found to be the highest affinity and highest potency A_{2B}AR agonist identified to date. Furthermore, VCP746 stimulated potent anti-fibrotic signalling in NCFs, thus we believe that VCP746 provides a novel tool to modulate cardiac fibrosis and to further investigate the role of the A_{2B}AR in cardiac (patho)physiology.

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Development of fluorescent ligands as tools for studying cannabinoid type 2 receptor

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Introduction. Cannabinoid type 2 receptor (CB2R) is found primarily in the peripheral tissue and is implicated in the pathologies of various diseases including osteoporosis, inflammatory bowel disease, atherosclerosis and cancer. However, there is a significant lack of understanding of the specific role of CB2R in disease, as well as the variation of receptor expression levels between cell types and healthy and diseased tissue. There are a variety of existing methods available for studying CB2R such as fluorescent immunohistochemistry, radioligands and reporter gene assays, however each have limitations. Fluorescent ligands are excellent tools to study receptor structure and function in live cells and as such have been successfully developed for other GPCRs.
Aims. To develop a selective, high affinity fluorescent antagonist for CB2R.
Methods. A library of compounds containing the aminoalkylindole pharmacophore was synthesised with various substituents and linkers in positions predicted to be tolerable of chemical change and steric bulk. A fluorophore was then attached to a subset of this library. The affinity of these fluorescent compounds and key synthetic intermediates for CB2R was measured with HEK 293-hCB_2 cells using a [^{3}H]CP55,940 radioligand binding assay, and the K_i of the compounds were determined. For lead compounds, real-time cAMP BRET measurements were made to determine compound function at CB2R and compound affinity for cannabinoid type 1 receptor was measured using a radioligand binding assay with HEK 293-hCB_1 cells.
Results. The affinities of the six fluorescent compounds for CB2R were all K_i = >10 μM. The lead pharmacophore-linker compound without a fluorophore had an affinity of K_i = 4.8 nM for CB2R. Many of the compounds showed agonism of CB2R in the cAMP functional assay.
Discussion. Several very interesting structure-activity-relationships (SAR) were established and very minor changes in linker position on the aminoalkylindole pharmacophore gave significantly altered affinity for CB2R. Although none of the fluorescent compounds had acceptable binding to CB2R, the SAR relationships established have informed the design and synthesis of a follow up chemical library. This work is currently underway.

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The effects of cannabinoid ligands on the gene expression profile in murine adipocytes

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Introduction. The cannabinoid receptor 1 (CB1) inverse agonist rimonabant was effective in promoting weight loss in humans but was taken off the market due to increasing depression/suicidal thought in a small percentage of patients. While its effects on weight loss were thought to be due to actions in the central nervous system, newer studies with other ligands indicate that CB1 ligands may have effects directly on adipose tissue (Tam et al., 2010).
Aim. To examine the effects of several cannabinoid ligands on the gene expression profile in mouse adipocytes.
Method. Mouse primary brown, inguinal white, or epididymal white adipocytes from 3-4 week old C57Bl/6J mice were cultured in the presence/absence of the CB1 inverse agonist rimonabant, the CB1 antagonist AM251, the peripherally restricted CB1 antagonist AM6545, the CB2 agonist AM1241or the CB2 antagonist AM630, acutely (24h, 1μM) or chronically (7 days, 1μM). The expression of brown adipocyte genes (UCP1, Prdm16, Cpt1b), white adipocyte genes (Hoxc9, Tcf21), glucose transporters (GLUT1, GLUT4) and receptors (CB1, CB2, β_3-AR) were measured by qPCR. Adipocyte function (oxygen consumption) was measured using the Seahorse xF96.
Results. Several CB ligands affected the gene expression profile in mouse adipocytes and affected basal and maximally stimulated cellular respiration. There were no major changes in the gene expression profile in brown adipocytes treated either acutely or chronically with CB ligands, except for AM630 increasing UCP1 mRNA levels to a similar degree to the β-AR agonist isoprenaline. GLUT4 and Cpt1B mRNA levels increased following chronic AM630 treatment of epididymal white adipocytes. Acute AM6545 treatment increased GLUT4, GLUT1 and Prdm16 mRNA levels in epididymal white adipocytes. AM630 treatment increased GLUT4 mRNA acutely, and increased. UCP1, Prdm16, GLUT4 and GLUT1 mRNA chronically in inguinal white adipocytes.
Discussion. This data shows that CB ligands can have direct effects on gene expression in adipocytes.

Tam J et al (2010). J Clin Invest, 120:2953-2966.

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Using BRET-based Rluc8-EKAR-Venus pERK1/2 biosensors to characterise RXFP1 signalling

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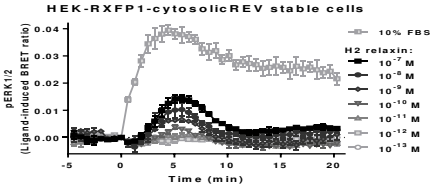
Introduction. Relaxin, a peptide hormone in a Phase IIIb clinical trial for treatment of Acute Heart Failure, shows particular promise for its organ-protective anti-fibrotic effects. These effects are known to involve Relaxin Family Peptide 1 (RXFP1) receptor activation and subsequent ERK1/2 phosphorylation, however the precise mechanism requires further elucidation. BRET-based biosensors such as Rluc8-EKAR-Venus (REV) allow spatial measurements of real time signalling in live cells.



Aims. To better understand RXFP1 signalling using nuclear and cytosolic REV pERK1/2 biosensors.

Methods. Firstly, relaxin responses were assessed in HEK-RXFP1 cells transiently transfected with REV sensors. Secondly, lentiviral constructs encoding the REV constructs were cloned and lentiviruses were produced to transduce REV into HEK-RXFP1 cells. Time course and concentration response assays of pERK1/2 activation were conducted in the HEK-RXFP1-cytoREV and HEK-RXFP1-nucREV stable cell lines in a 96 well assay.

Results. No pERK1/2 response was detected for RXFP1 ligand stimulation following REV transient transfection. However, HEK-RXFP1-cytoREV and HEK-RXFP1-nucREV stable cell lines demonstrated robust signalling in response to relaxin stimulation with a time course of activation similar to that measured using AlphaScreen pERK1/2 assays. Furthermore both cell lines demonstrated a concentration-dependent activation of pERK1/2 upon stimulation with relaxin with a pEC50 consistent with previous work (Figure shows HEK-RXFP1-cytoREV cells).

Discussion. The establishment of HEK-RXFP1-cytoREV and HEK-RXFP1-nucREV stable cell lines will allow detailed analysis of pERK1/2 signalling in these cells and allow easy testing of novel relaxin peptide analogs. Additionally the lentiviral constructs that have been developed will allow the transduction of primary cell lines to fully assess the kinetics and compartmentalisation of pERK1/2 signalling for RXFP1 and other GPCRs.

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Quantifying biased agonism of novel, adenosine and NECA-based, adenosine A_1 receptor agonists.

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Introduction. 4 adenosine receptors (ARs) subtypes exist; A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R. All are activated by the purinergic nucleotide, adenosine. We have developed novel, AR agonists, based upon adenosine and NECA, some of which are A_1R-selective (Knight *et al.* 2016).

Table 1. Pathway potency of novel A1R agonists

Cmpd	Pathway – pEC_{50}			
	↓cAMP	↑cAMP	$Ca^{2+}{}_i$	pERK1/2
5	6.9±0.2	5.5±0.3	7.6±0.1	7.2±0.2
6	9.2±0.2	6.7±0.5	7.4±0.1	8.7±0.3
16	7.7±0.3	6.2±0.3	7.0±0.1	7.6±0.3
18	8.7±0.4	7.1±0.3	8.0±0.1	7.7±0.2
20	10.5±0.3	7.1±0.2	7.9±0.1	9.5±0.2
21	8.2±0.2	6.7±0.2	6.7±0.2	8.4±0.2

Aims. Quantify agonist bias for A_1R-selective compounds.

Methods. We stimulate CHO-K1, cells stably expressing the A_1R, with NECA, adenosine, CCPA and our novel A_1R agonists. We then measure cAMP inhibition and production (through pre-treatment with pertussis toxin (PTX)), $Ca^{2+}{}_i$ mobilisation, and pERK1/2 activation. Using this data we then quantify the degree of agonist bias.

Results. Treating CHO-A_1R cells with PTX allowed us to measure an increase in cAMP levels upon agonist stimulation (Table 1). In addition we show that all our novel compounds are able to act via the canonical $G\alpha_{i/o}$ pathway, as well as leading to mobilisation of $Ca^{2+}{}_i$, and pERK1/2 activation (Table 1).

Discussion. Our novel compounds present the first instance of wholly selective A_1R agonists (Knight *et al* 2016). During the process of screening these compounds we also identified non-selective compounds, some of which we have characterized here. We also identify a potential $G\alpha_s$ signaling component, whilst not entirely novel (Baker and Hill, 2007)), it has not been observed to such levels as we see with our compounds.

Knight A, Hemmings J, Winfield I *et al* (2016) *J. Med. Chem* **59**:947-64, Baker J and Hill S (2007) *J. Pharmacol. Exp. Ther.* **320**:218-82

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Identifying novel triazoloquinazolines as dual target A_{2A}R/PDE10A compounds, with a potential therapeutic application in Huntingdon disease

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Introduction. Multi-target A_{2A}R ligands provide a potentially highly beneficial method in the treatment of many pathological disorders, such as Huntingdon disease, Parkinson and schizophrenia. Compounds targeting both A_{2A}R and PDE10A are promising prospects for therapeutic intervention for Huntingdon disease (Latini and Pedata, 2016 , Giampà *et al*. 2009) .
Aims. Utilise ligand and structure-based virtual screening to identify novel dual A_{2A}R/PDE10A targeted ligands, and validate in both yeast and mammalian cells.
Methods. We used an *in silico* docking strategy to identify A_{2A}R agonists from a database of known PDE10A inhibitors. Potential compounds were initially screened for receptor activation in yeast cells expressing the A_1R, A_{2A}R or A_{2B}R (3). Active compounds were then validated in CHO-K1 cells expressing each of the four adenosine receptors subtypes, for their ability to modulate cAMP production (3).
Results. We have developed a computational strategy that has allowed us to identify dual-target ligands compounds, which identified 3 triazoloquinazolines. *In vitro* experiments demonstrate all 3 compounds to be A_{2A}R selective. Generation of dose response curves in CHO-K1 cells show compounds 1, 2 and 3 to be full agonists with pEC_{50} values of; 6.95 ±0.3, 5.58 ±0.4 and 6.11 ±0.3, respectively.
Discussion. We have successfully developed and utilised a ligand and structural based virtual screening strategy that has allowed us to identify three potential dual A_{2A}R/PDE10A targeting compounds. Thus far we have identified that these are wholly A_{2A}R selective. We will now attempt to establish if they also further elevate cAMP levels, through activating the A_{2A}R and inhibiting PDE10A. by using cell lines expressing the A_{2A}R in conjunction with PDE10A.

Latini and Pedata (2001) *J. Neurochem* **79**:463-84, Giampà *et al*. (2009) *Neurobiol. Dis.* **34**:450–56, Knight *et al* (2016) *J. Med. Chem* **59**:947-64.

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Intracellular loop 1 of family B GPCRs determines G protein specificity

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Introduction. No active state crystal structures exist for family B GPCRs; thus our understanding of their activation has come from mutagenesis and molecular modelling. This has identified residues at the junction between intracellular loop 1 (ICL1) and transmembrane bundle (TM) 2 along with residues in TM3 that influence G protein binding (Vohra *et al,* 2013, Wooten *et al*, 2016). However, characterisation ICL1 has not been undertaken.
Aims. To understand the contribution of ICL1 in modulating signalling bias at family B GPCRs.
Methods. We performed saturation mutagenesis on ICL1 (Y165-Q172) of the CLR, and co-expressed each mutant with RAMP1 in HEK-293T cells. To discern the role each residue has upon receptor activation, we stimulated with CGRP and measured; cAMP production, intracellular calcium (Ca^{2+}_i) mobilisation and pERK1/2 activation. This was also performed for the CRF1a and 1b receptors to determine the role of ICL1 inserts
Results. Mutation of residues in ICL1 to alanine reveals minimal effects for pERK1/2 activation whilst L169, C171 and Q172 play key roles in $G\alpha_s$ activation. Residues F166, L169 and C171 appear to play critical roles in the mobilisation of Ca^{2+}_i ($G\alpha_q$ mediated (Weston *et al*, 2016)). Analysis of bias reveals that residues towards TM2 are more important for $G\alpha_q$ signalling. Further, we characterise these pathways at the CRF 1a and 1b receptors. The addition of an insert into ICL1 of CRF1b (at the point where a switch in bias towards $G\alpha_q$ occurs) significantly alters its ability to couple to $G\alpha_q$ whilst having minimal effects upon $G\alpha_s$ signalling and pERK1/2 activation.
Discussion. The differing effects observed for $G\alpha_s$ and $G\alpha_q$ signalling with each mutant CLR, and CRF1a and 1b suggest that ICL1 plays a key role in determining G protein binding. The mechanism of how this occurs is unclear.
Vohra S, Taddese B, Connor A *et al*. (2013) *J. R. Soc. Interface*, 10, 20120846; Wooten D, Reynolds CA, Koole C *et al.* (2016) *Mol. Pharmacol.* **89**:335; Weston C, Winfield I, Harris M *et al* (2106) *J. Biol. Chem.* Pii: jbc.M116.751362

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Understanding 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. 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, H3 (B1-22)R which retains the core binding residues but with the C-terminal activation residues replaced by a non-native Arginine residue, Arg23. Importantly this Arginine contributes to a novel high affinity binding interaction with 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.
Aims. To identify the sites of agonist and antagonist binding in RXFP3 using mutagenesis and homology modelling.
Methods. Preliminary mutagenesis and modelling on RXFP3 have successfully identified interaction sites for relaxin-3 B chain R12, R16 and R26. The RXFP3/relaxin-3 model is used to further predict potential ligand interactions with the transmembrane domains of RXFP3 for activation. The potential residues involved are mutated to alanine and the effects of mutations are tested in binding assays using Europium (Eu)-labelled agonist, R3/I5 and antagonist, H3 B1-22 R. Mutant receptors are also tested in cAMP activity and pERK1/2 phosphorylation 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 mutations (W138A, T162A, L246A, L248A, K271A, F364A and T346A) resulted in markedly decreased agonist binding and activity. Antagonist binding assays also suggested a few of these residues to be involved with binding to H3 (B1-22)R.
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. There are overlapping binding sites between the double chain agonist and the single chain antagonist of RXFP3 and our current model suggests the binding site to be in the extracellular loops whereas the activation domain is in the transmembrane domains.

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Characterisation of the internalisation and compartmentalised signalling of the GLP-1R

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Introduction. The glucagon-like peptide-1 receptor (GLP-1R) is a therapeutic target in the treatment of type 2 diabetes, neurodegenerative and cardiovascular diseases. Emerging evidence suggests that cells respond to GLP-1R activation, via compartmentalised signalling; the specific localisation of signalling mediators and receptors in discrete regions of the cell.
Aims. To explore the internalisation and trafficking of GLP-1R and its role in signaling upon activation by multiple peptide agonists.
Methods. Flp-In-Chinese Hamster Ovary cells were transiently transfected with GLP-1R and co-localisation of the receptor and cyclic adenosine monophosphate (cAMP) with subcellular compartments were assessed via bioluminescence and fluorescence resonance energy transfer, respectively. Global cellular cAMP accumulation and extracellular signal -related kinases 1/2 phosphorylation (pERK1/2) were measured through Perkin Elmer's LANCE™ cAMP and AlphaScreen® SureFire®, respectively.
Results. Upon ligand binding, the GLP-1R internalises rapidly and co-localises with early, late and recycling endosomes. Internalisation is inhibited by dominant-negative forms of dynamin and caveolin-1, and this inhibition of internalisation reduces pERK1/2 and cAMP signaling. Different profiles of cAMP signaling were detected in plasma membrane (PM) and cytosolic compartments, with a more sustained cytosolic response. Glucagon-like peptide-1 was 10-fold more potent in producing cytosolic cAMP relative to PM cAMP despite being equipotent in global assays.
Discussion. Ligand stimulation promotes GLP-1R internalisation, via a dynamin and caveloae-mediated mechanism, and subsequently promotes trafficking through both degradative and recycling pathways. Inhibiting this process reduces cAMP and pERK1/2 levels, suggesting a potential role of signalling from an intracellular receptor. This is supported by the determination of distinct compartments of cAMP at the PM and in the cytosol.

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Role of conformational dynamics on the functional selectivity of α_1-adrenoceptor.

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Introduction. G protein-coupled receptors (GPCRs) are the largest class of signalling proteins in humans. They typically bind to an extracellular ligand and following a conformational change transduce the signals intracellularly by coupling to G proteins. Although recent crystallography studies have advanced our understanding of the structure of GPCRs, the structural dynamics underlying the signal transducing process remain poorly understood. In principle, such dynamics could be obtained by NMR. The α_{1A} and α_{1B} adrenergic receptors are essential to hypertrophic responses and neuromodulation. However, like all GPCRs these receptors are unstable in detergent micelles. We have engineered detergent-stable variants of human α_{1A}AR and α_{1B}AR using directed evolution methods [(1)] which has enabled biophysical characterization by techniques such as NMR spectroscopy.
Aims. To characterize and compare the conformational dynamics of $\alpha_{1A/1B}$AR in the apo and ligand bound states.
Methods. The methyl groups of the amino acids have favourable relaxation properties. As these residues are well dispersed in proteins they serve as excellent probes for monitoring subtle conformational changes. As an initial experiment we have labelled α_{1A}AR with $^{13}CH_3$-methionine.
Results and Discussion. We obtained $^{13}C,^{1}H$ two dimensional spectra of unliganded receptor as well as receptor bound to an inverse agonist and agonist. Through single site mutations, we have assigned all six methionines in α_{1A}AR. Importantly, methionines distant from the orthostatic binding site showed significant changes on binding agonist, including line broadening suggesting an increase in protein motion whereas binding of antagonist showed line narrowing suggesting a reduction in motion. Also, methionines near the ligand binding pocket undergo peak shifting when bind to different ligands. So current results show valuable dynamics information of α1-adrenoceptor.

(1) Scott D. J., (2013) J Mol Biol 425:662-67

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Structural determinants of binding the seven-transmembrane domain of the glucagon-like peptide-1 receptor

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Introduction. The glucagon-like peptide-1 receptor (GLP-1R) belongs to the secretin-like (class B) family of G protein-coupled receptors (GPCRs). Members of the class B family are distinguished by their large extracellular domain (ECD), which works cooperatively with the canonical seven-transmembrane (7TM) helical domain to signal in response to binding of various peptide hormones. Although GLP-1R and glucagon receptor (GCGR) share over 50% sequence identity, they have opposing physiological roles.
Aims. The objectives of the current study were to identify the structural determinants of GLP-1 binding in the 7TM domain of GLP-1R and to elucidate the molecular mechanism of selective recognition of GLP-1.
Methods. Combined structure-based site-specific mutational studies with molecular dynamics (MD) simulations of a full-length model of GLP-1R bound to multiple peptide ligand variants
Results. Despite the high sequence similarity between GLP-1R and GCGR, nearly half of the 62 stably expressed mutants affected GLP-1R in a different manner than the corresponding mutants in GCGR. The MD simulations of wild-type and mutant GLP-1R-ligand complexes provided molecular insights into GLP-1R specific recognition mechanisms for the N-terminus of GLP-1 by residues in the 7TM pocket, and explained how glucagon mimicking GLP-1 mutants restored binding affinity for (GCGR mimicking) GLP-1R mutants.
Discussion. The molecular mechanism of class B GPCR peptide ligand selectivity in the 7TM domain demonstrates the complexity of peptide ligand recognition by their GPCRs, and illustrates how full-length receptor models complemented by extensive mutation and simulations of the conformational dynamics of receptor-ligand complexes can be used to investigate class B GPCR structure-activity and –selectivity relationships.

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Mechanisms involved in PKC mediated regulation of μ opioid receptor desensitization.

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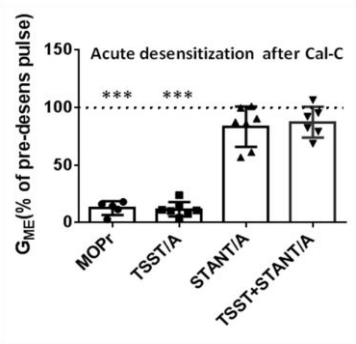
Introduction. Utility of opioid analgesics is hampered by tolerance and dependence. Agonist induced desensitization of μ-opioid receptor (MOPr) is widely considered to be an initial step in the development of tolerance. Different opioids acting on MOPr can activate different regulating pathways leading to desensitization but exact mechanisms are still uncertain.

Aims. The aim of this study was to determine the role of PKC in regulating μ opioid receptor desensitization

Methods. Three different mutations of mouse MOPr C-terminal tail i.e. ^{355}TSST^{357}/A, ^{375}STANT^{379}/A, TSST+STANT/A were transfected and expressed stably in AtT20 cells. The effects of MOPr activation and desensitization were examined using activation of GIRK channels with whole cell and perforated patch clamp electrophysiology at 37^{o}C.



Results. Acute MOPr desensitization produced by 5 minutes exposure to Met-Enkephalin (10μM) did not differ from wild type for all three mutants in perforated patch mode but was abolished in STANT/A and TSST+STANT/A in whole cell mode. Using the selective PKC blocker, Calphostin-C (30nM) in perforated patch clamp mode, desensitization by met-enkephalin was reduced in STANT/A but completely blocked in TSST+STANT/A. By contrast, desensitization was maintained in wild type MOPr and all mutants upon 5 minutes exposure to morphine (10μM) in both perforated and whole cell patch mode. The PKC inhibitor Calphostin-C reduced desensitization by morphine in perforated patch mode by ~50% in wild type and TSST/A but it was maintained in STANT/A and TSST+STANT/A mutants.

Discussion. Taken together these results indicate that phosphorylation in the region including ^{375}STANT^{379} of MOPr is crucial for switching the mechanism of desensitization from a GRK/β-arrestin to PKC mediated process.

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Analysis of the C-terminus of the human amylin peptide to inform the development of new treatments for diabetes and obesity

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Introduction. Amylin is a peptide hormone involved in glucose regulation and satiety, offering significant potential for the treatment of obesity and diabetes. Amylin is a Family B G protein-coupled receptor (GPCR) ligand, proposed to bind *via* the two-domain model, whereby the peptide *C*-terminus is important for receptor binding. Pramlintide, an amylin mimetic, lacks the complicated aggregative properties of human amylin, rendering it a useful therapeutic drug. Despite its obvious benefits, pramlintide still suffers from a number of pharmacokinetic problems, leaving a window of opportunity for the development of novel amylin-based pharmacotherapies.

Aims. To investigate the critical determinants of amylin affinity and selectivity for its receptor(s) by performing an alanine (Ala) scan and residue swapping with related peptides, focussing on the *C*-terminal region of human amylin.

Methods. 9-fluorenyl methoxycarbonyl (Fmoc) solid phase peptide synthesis, oxidation and purification techniques were used for the synthesis of amylin, calcitonin gene-related peptide and amylin analogues. Pharmacological evaluation was carried out in transfected COS-7 cells, and cyclic adenosine monophosphate (cAMP) was measured using LANCE™ cAMP assay techniques.

Results. Modification of three residues to Ala in the *C*-terminal region of human amylin significantly reduced receptor activity (Thr-30, Val-32 and Gly-33). However, amylin receptor activity was retained with a number of Ala scan mutants. Some peptides showed dependency on receptor activity-modifying proteins, a subunit of amylin receptors, giving insights into receptor selectivity.

Discussion. *C*-terminal residues of human amylin have differential impacts on receptor activation. These results aim to inform ideal sites for future chemical modification to create novel and selective amylin mimetics.

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Regulation of Compartmentalized Protease-Activated Receptor-2 Signaling by Receptor Trafficking

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Introduction. Protease-activated receptor-2 (PAR_2) can be activated by a number of endogenous proteases that cleave the receptor at distinct sites. This has been shown to be critical for the spatial and temporal control of PAR_2 signaling and trafficking. The contribution of compartmentalized signaling to the physiological role of PAR_2, however, remains unknown.
Aims. To investigate the molecular mechanisms of compartmentalized PAR_2 signaling and its contribution to pain and inflammation.
Methods. Receptor trafficking was studied through bioluminescence resonance energy transfer (BRET) assays in conjunction with a range of pharmaceutical and genetic inhibitors that disrupt normal trafficking patterns. Förster resonance energy transfer (FRET) biosensors specific to several downstream signaling molecules, and targeted to different subcellular locations, were used to determine the signaling responses of biased proteases in both HEK cells and dorsal root ganglia (DRG) neurons. Finally, endocytic inhibitors were employed to test whether internalization is required for PAR_2-mediated pain response.
Results. Trypsin, but not elastase or cathepsin-S, induces clathrin- and dynamin-dependent PAR_2 internalization, which resulted in ERK activation in both cytosolic and nuclear cell compartments. While both these signals occurred in a $G\alpha_q$ and EGFR dependent manner, receptor internalization was found to be a major contributor to cytosolic ERK activation. Importantly, inhibition of receptor trafficking significantly reduced trypsin-induced mechanical hyperalgesia, but had little or no effect on elastase or cathepsin-S mediated pain.
Discussion. The data suggest that differences in receptor internalization and subsequent compartmentalized signaling underpin protease induced PAR_2-mediated pain. As such, therapeutic agents that selectively target PAR_2-derived endosomal signals can be an effective alternative to alleviate inflammatory pain with limited side effects.