

Pharmacovigilance and computational chemistry approaches to predicting torsade de pointes

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Introduction. Drug-induced QT interval prolongation has been linked to a rare, yet fatal tachyarrhythmia torsade de pointes (TdP). The majority of drugs associated with QT prolongation and TdP, including antiarrhythmics, antihistamines and antibiotics have a common pharmacologic action on potassium ion channel (hERG) inhibition. Early identification of QT liability and torsadogenic risk of drugs is a critical aspect of the drug development process.

Aims. To combine pharmacovigilance and computational chemistry methods to predict drug mediated torsadogenic risk.

Methods. Case reports of TdP were retrieved from the Australian pharmacovigilance database. A list of drugs with suspected TdP liability from literature was used as a reference to define drug searches. The reporting odds ratio as a measure of disproportionality was calculated for each drug using binary logistic regression to signal active drugs. These were used to develop a pharmacophore hypothesis and were docked into a homology model of the hERG channel.

Results. A significant disproportionality was observed for amiodarone, sotalol, erythromycin, azithromycin, linezolid, thioridazine, citalopram, cisapride, methadone, terfenadine, fluconazole, ribavirin, zidovudine and levosimendan. The generated pharmacophore model contained three hydrogen bond acceptors and one aromatic ring feature. Docking of the drugs into the homology model revealed that amino acids THR623, SER624, SER649, TYR652 and PHE656 were consistently involved in interactions with the ligands, consistent with the spatial distribution of the pharmacophoric features.

Discussion. Amino acids identified to interact with ligands particularly TYR652 and PHE656 are consistent with alanine-scanning mutagenesis which have identified TYR652 and PHE656 as crucial for hERG blockade by cisapride and terfenadine. Pharmacovigilance databases contain a wealth of information which can be utilised to guide *in silico* methods of predicting torsadogenic risk.

Tocomin® restores endothelium-dependent relaxation in diabetic rat aorta.

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Introduction: Tocotrienols, a component of vitamin E with structural similarities to tocopherols, may have beneficial effects on the vascular function particularly in pathologies involving oxidant stress such as diabetes. Tocomin® is an extract of palm oil with a high tocotrienol content.

Aims: To determine the effect of tocotrienol rich tocomin® (composition: tocotrienol rich fraction:40%, α -tocopherol:11% and palm olein:38%) treatment on endothelium-dependent and -independent relaxation in the diabetic rat aorta.

Methods: Male wistar rats were randomly assigned to 4 groups (control, control+tocomin®, diabetic and diabetic+tocomin®). Diabetes was induced by a single injection of streptozotocin (50 mg/kg iv). Rats were by treated with tocomin® (40 mg/kg per day s.c.) or vehicle (100% peanut oil) for a period of 4 weeks commencing 6 weeks after induction of diabetes. Acetylcholine (ACh)-induced endothelium-dependent and sodium nitroprusside (SNP)-induced endothelium-independent relaxation was measured in rat aortae using standard organ bath techniques.

Results: STZ increased blood glucose (control, 7.1 ± 0.4 mmol/L, STZ 29 ± 2.3 mmol/L, $n=15-17$) and glycated haemoglobin (HbA1c control, $5.5 \pm 0.5\%$, STZ $12.5\% \pm 2.8\%$, $n=15-17$). Neither parameter was affected by tocomin® treatment in diabetic rats (BGL control+tocomin® 7.3 ± 0.7 mmol/L, diabetic+tocomin® 26.3 ± 4 mmol/L, HbA1c control+tocomin® $5.5 \pm 0.2\%$, diabetic+tocomin® $11.8 \pm 1.3\%$, $n=8-10$). Diabetes impaired ACh induced endothelium-dependent relaxation (R_{max} control $95 \pm 2\%$ vs diabetic $82 \pm 4\%$, $n=6-8$, $p<0.01$, pEC_{50} 7.50 ± 0.23 vs 6.6 ± 0.13 diabetic, $n=6-8$, $p<0.01$) without affecting SNP-induced relaxation. Tocomin® treatment significantly improved endothelium-dependent relaxation (pEC_{50} diabetic+tocomin®, 7.2 ± 0.13 $n=6-8$, $p<0.05$).

Discussion: These findings demonstrate that 4-week treatment of diabetic rats with tocotrienol rich tocomin® significantly improves endothelium-dependent relaxation in diabetic rat aorta without affecting blood glucose levels.

Resistin, a novel adipokine, increases renal SNA and reduces BAT SNA using PI 3-kinase or ERK 1/2 mediated mechanisms

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Introduction. A characteristic of obesity is a marked elevation of sympathetic nerve activity (SNA) to the skeletal muscle blood vessels and to the kidney which contribute to obesity induced hypertension. The causes of the increase in SNA are not known. Adipose tissue is now recognised as a major endocrine organ that releases many hormones, including leptin, adiponectin and, more recently, resistin. Leptin and adiponectin have cardiovascular and metabolic effects that involve actions in the central nervous system that influence SNA. Leptin, for example, increases SNA to the kidney and brown adipose tissue (BAT), a metabolic organ, but little is known of the effects of resistin.

Aims. To investigate the effect of centrally administered resistin (i) on SNA targeting the kidney and BAT, and (ii) the intracellular signalling pathways mediating those changes.

Methods. Rats were anaesthetised (urethane 1.4 g/kg iv) and renal or BAT SNA was recorded using standard methodology. Resistin (7ug) was injected into the lateral cerebral ventricles (icv) in the presence or absence of inhibitors of the enzymes P I3-Kinase (LY294002, 5ug) or ERK 1/2 (U0126, 7ug). The changes in SNA, blood pressure and heart rate were monitored and compared to control groups.

Results. Resistin induced a significant increase in renal SNA by approximately 40%. This response was prevented when PI 3-Kinase was inhibited but was unaffected by ERK 1/2 inhibition. In contrast, resistin reduced BAT SNA by about 50% and this response was delayed by 150 minutes when ERK1/2 was inhibited but was unaffected by inhibition of PI 3-Kinase.

Discussion. The findings indicate that resistin increases renal sympathetic nerve activity via PI 3-kinase but reduces BAT SNA via ERK1/2. Since the plasma levels of resistin are elevated in obesity, resistin may contribute to the cardiovascular and metabolic dysfunction in obesity.

Head to head comparison of the relative anti-fibrotic efficacy of H2 relaxin to a clinically used ACE inhibitor (Enalapril)

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Introduction: Organ scarring (excess matrix/collagen accumulation) is the final endpoint of numerous cardiovascular diseases, for which there is currently no effective cure.

Aims: This study aimed to compare a novel anti-fibrotic (relaxin) to that of a clinically used angiotensin converting enzyme inhibitor (ACEi; enalapril) in a mouse model of isoproterenol (ISO)-induced ischemic heart disease/fibrosis.

Methods and Results: Repeated s.c administration of isoprenaline hydrochloride (ISO) for 5 consecutive days induced a 2-fold increase in aberrant collagen deposition and 11-12-fold increase in picrosirius red-stained interstitial collagen, 12 days later (day17) (both $P < 0.001$ vs untreated controls). Increasing doses of relaxin (0.5, 1, 2mg/kg/day) and enalapril (200, 300, 500mg/L) ($n=5-6$ mice/treatment group), delivered via osmotic mini-pumps and drinking water, respectively, were first evaluated for their ability to prevent ISO-induced collagen levels. All doses of relaxin tested significantly prevented total collagen concentration (hydroxyproline assay) and picrosirius-red stained interstitial collagen (morphometry) by 55-60% (all $P < 0.01$ vs ISO alone), without affecting blood pressure (tail-cuff). The lowest dose of enalapril (200mg/L) also prevented ISO-induced collagen levels by 25-40% ($P < 0.05$ vs ISO alone) while lowering blood pressure; but higher doses of the ACEi significantly decreased body weight (BW), left ventricular (LV) weight and LV/BW ratio, without further reducing aberrant collagen deposition. Based on these findings, a combination of relaxin (0.5mg/kg/day) and enalapril (200mg/L) was found to significantly prevent fibrosis progression by 62-67% when administered at the onset of injury, and remarkably also reduced established collagen deposition (by 31-34%), when administered after injury onset. Relaxin was more effective as an anti-fibrotic due to its ability to markedly inhibit the pro-fibrotic influence of TGF- β 1/Smad2 phosphorylation, over its effects on collagen degrading-matrix metalloproteinases (MMP-2, MMP-13).

Discussion: These findings demonstrate that relaxin has improved anti-fibrotic efficacy compared to enalapril alone and may have clinical potential as an adjunct therapy to currently used ACEi.

Comparing the anti-fibrotic actions of relaxin versus an angiotensin AT1 receptor blocker and AT2 receptor agonist

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Introduction: Fibrosis is a hallmark of several forms of cardiovascular disease, current front-line treatments such as angiotensin receptor blockers (ARBs) only modestly prevent its progression.

Aims: This study aimed to compare the efficacy of emerging anti-fibrotics (relaxin and an AT2 receptor agonist, CGP42112) against a clinically used ARB (candesartan cilexetil), in a mouse model of isoproterenol (ISO)-induced ischemic heart disease/cardiac fibrosis (Brooks and Conrad, 2009).

Methods and Results: Hydroxyproline analysis (total collagen concentration) and morphometry (picrosirius red-stained interstitial collagen) showed that increasing doses of relaxin (0.5, 1, 2mg/kg/day, via mini-pumps) similarly prevented ISO-induced fibrosis progression (by 55-60% vs ISO alone, $P < 0.001$); and to a greater extent than candesartan (0.5, 2, 5mg/kg/day via drinking water) or CGP42112 (1.44mg/kg/day via mini-pumps) ($n=5-6$ animals/treatment group), neither of which significantly affected ISO-induced collagen deposition. Unexpectedly, combination of the optimal dose of relaxin (0.5mg/kg/day) and candesartan (0.5mg/kg/day; which had no effect on blood pressure) inhibited the anti-fibrotic actions of relaxin (on aberrant TGF-1, Smad2 phosphorylation (pSmad2) and collagen concentration) back to ISO alone-induced levels. On the other hand, combination of CGP42112 (1.44mg/kg/day) and relaxin (0.5mg/kg/day) equivalently prevented fibrosis to that of relaxin alone ($P < 0.01$ vs ISO alone), without inducing any additive effects over either treatment alone. The enhanced anti-fibrotic efficacy of relaxin over candesartan and CGP42112 was associated with its ability to more effectively reduce TGF-1 expression/staining ($P < 0.05$ vs candesartan or CGP42112 treatment alone). However relaxin did not demonstrate any significantly improved effects on pSmad2 (immunohistochemistry and morphometry) or on matrix-degrading metalloproteinases: MMP-13 (immunohistochemistry) and MMP-2 (gelatin zymography).

Discussion: Although further studies are required to assess the anti-fibrotic efficacy of these treatments in other models/strains of injury/disease, these findings suggest that relaxin is a more effective anti-fibrotic compared to candesartan or CGP42112 in a model of ischemic heart disease.

Brooks WW & Conrad CH (2009) *Comp Med* 59:339-343.

Is insulin-mediated sensitization of platelets to nitric oxide in diabetics thioredoxin-interacting protein-dependent?

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Introduction. Acute coronary syndrome (ACS) in diabetics is associated with substantial mortality, which is reduced by correction of hyperglycaemia¹. We have previously shown that rapid reversal of hyperglycaemia with IV insulin is associated with suppression of O_2^- release and restoration of normal platelet responsiveness to NO². We now wish to determine whether falls in BSL induce associated rapid (and potentially crucial) reductions in platelet expression of the hyperglycaemia-sensitive redox inducer thioredoxin-interacting protein (TxNIP).

Method. Patients were selected on the basis of concomitant ischaemia and BSL > 11.1 mmol/L¹. Investigations were performed before and after 12 hours of insulin infusion. These included:- platelet responsiveness to NO (whole blood aggregometry), whole blood O_2^- content (EPR), platelet TxNIP expression (immunostaining) and PMA stimulated leukocyte O_2^- release (EPR).

Results. Results of pilot study ($n=4$) are reported. BSL fell by 32 ± 22 (SD) % over 12 hours. This was associated with an increase in platelet NO response from 14 ± 14 (SD) to 52 ± 26 % ($p < 0.05$), and a decrease in whole blood O_2^- content by 25 ± 17 %. However, there was no consistent change in neutrophil O_2^- release ($\delta = 10 \pm 50$ %), nor in platelet TxNIP content ($\delta = 72 \pm 120$ %).

Conclusion. While these pilot data confirm the previous findings that treatment of hyperglycaemia with IV insulin reduces O_2^- content and potentiates platelet response to NO, the PMA and TxNIP data do not provide insights into the mechanism(s) underlying the changes in O_2^- generation. It is possible that despite the glucose-responsive element in genes for TxNIP expressions modulation of such changes in (non-nucleated) platelets is inherently slow.

1. Malmberg K et al (1999) *Circulation* 99: 2626-32

2. Worthley et al (2007) *Journal of the American College of Cardiology* 49: 304-310

Utilisation of antithrombotics for secondary stroke prevention at hospital discharge

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Introduction. Stroke is a leading cause of death and disability yet it is both preventable and treatable. Significant proportions of stroke presentations are by patients with a previous stroke. For this reason, the clinical guidelines for stroke management recommend an appropriate antithrombotic drug therapy (either anticoagulant or antiplatelet) for the secondary prevention of stroke.

Aims. To determine the rates of utilisation of antithrombotic drug therapy for stroke patients and to identify factors associated with use of treatment at discharge.

Methods. A retrospective clinical audit was conducted in five metropolitan hospitals in NSW, comprising two tertiary referral centres and three district hospitals. Patients discharged with a principal diagnosis of ischaemic stroke during a 12-month time period (July 2009-2010) were identified and the medical records of a systematically chosen sample reviewed.

Results. In total, 521 records were reviewed (48.8% females; mean age 74.4±14 years; range 5-102). Overall, 97.6% of eligible patients were prescribed an antithrombotic at discharge; of these 68.5 % were prescribed monotherapy. The univariate analysis identified hypercholesterolemia as the only variable associated with the utilisation of any antithrombotic therapy at discharge. For patients with atrial fibrillation (AF), 97.0% were prescribed an antithrombotic at discharge, however, only 57.4% were prescribed an anticoagulant. Multivariate logistic regression analysis identified male gender, heart disease, discharge destination (home) and functional status at discharge (independent) as significant predictors for the utilisation of anticoagulants in AF patients. As for non-AF patients 95.2% were prescribed an antithrombotic at discharge, of whom 87.9% were prescribed an antiplatelet agent.

Discussion. The majority of stroke patients received an antithrombotic at discharge. However, the utilisation of anticoagulant therapy for the secondary prevention of stroke in patients with AF is suboptimal. Additional efforts are needed to increase the utilisation of evidence-based, guideline-recommended therapies for the secondary prevention of stroke.

Blood Pressure lowering therapy for stroke patients at hospital discharge

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Introduction. Elevated blood pressure (BP) is a major risk factor for recurrent stroke. Contemporary evidence shows that lowering BP, even in the absence of hypertension, significantly reduces recurrent stroke and cardiovascular events in stroke patients. Furthermore, commencement of BP lowering therapy prior to hospital discharge significantly improves rates of adherence post discharge. Therefore, the clinical guidelines for stroke management recommend that all stroke patients (normotensive and hypertensive) receive a BP lowering therapy unless contraindicated by symptomatic hypotension.

Aims. To determine the use of BP lowering therapy for stroke patients on discharge and to identify factors associated with utilisation at discharge.

Methods. A retrospective clinical audit was conducted in five metropolitan hospitals in NSW, comprising two tertiary referral centres and three district hospitals. Patients discharged with a principal diagnosis of ischaemic stroke during a 12-month time period (July 2009-2010) were identified and the medical records of a systematically chosen sample reviewed.

Results. In total, 521 records were reviewed (48.8% females; mean age 74.4±14 years; range 5-102). Overall 75.4% of eligible patients were prescribed BP lowering therapy at discharge. The majority were prescribed either monotherapy (39.7%) or dual therapy (23.8%). In addition 40 patients were prescribed triple therapy and 15 patients were prescribed 4 antihypertensive drugs or more. Univariate analysis identified 6 independent variables as having a potential impact on the utilisation of BP lowering therapy: age ≥65years, hypertension, ischaemic heart disease, diabetes, atrial fibrillation and discharge destination. However, multivariate logistic regression analysis showed that only hypertension, atrial fibrillation and discharge destination (home) were significant predictors for the utilisation of BP lowering therapy.

Discussion. Rates of BP lowering therapy post stroke in this population are suboptimal and consistent with the literature. Additional efforts are needed to increase the utilisation of evidence-based, guideline-recommended therapies for the prevention of cardiovascular and cerebrovascular events.

AT₂ and Mas receptor agonists as neuroprotective drugs for stroke

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Introduction: Direct stimulation of the angiotensin II type 2 receptor (AT₂R) and/or the Mas receptor (MasR) may be neuroprotective in experimental stroke (Lee et al., 2012; McCarthy et al., 2009; Mecca et al., 2011) however the cell types involved remain to be elucidated.

Aims: To test the effectiveness of activating these two receptors with classical and novel agonists to reduce neuronal death *in vitro* or *in vivo*.

Methods: Classical AT₂R and MasR agonists, CGP42112 and AVE0991, respectively, were tested as were the respective novel agonists, β-proline-angiotensin III and β-isoleucine-angiotensin III. Primary cortical neurons were cultured from FVB/N mouse E17 embryos for 9 d, subjected to glucose deprivation for 24 h alone or with drugs, and cell death determined using trypan blue exclusion. Adult male FVB/N mice were subjected to cerebral ischemia for 1 h, administered with vehicle (n=10) or AVE0991 (0.55mg/kg ip; n=10) at the commencement of 23 h reperfusion. Neurological assessment was performed and then infarct volume analysed.

Results: Following glucose deprivation classical agonists, CGP42112 (0.1 and 1 μM; n=7) and AVE0991 (0.1 μM; n=3) reduced cell death by ~60% and ~45% respectively. By contrast, novel agonists had no effect on glucose deprivation-induced cell death. Furthermore, AVE0991 was unable to improve functional outcome or reduce infarct volume following stroke *in vivo*.

Discussion: The data support the concept that protection by classical AT₂R and MasR agonists following injury involves direct actions on neurons. The non-classical agonists tested may lack sufficient potency and/or efficacy to exert similar protection in this model. The neuroprotection achieved by AVE0991 *in vitro* was unable to translate into an improved outcome following stroke *in vivo*.

Lee S, et al (2012). *Exp Transl Stroke Med* 4:16

McCarthy C et al (2009). *Stroke*, 40: 1482-1489

Mecca A et al (2011). *Exp Physiol*, 96:1084-1096

Capsaicin relaxes arteries via NO-dependent and independent mechanisms.

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Introduction. Capsaicin, the active constituent in chilli, is known to stimulate the release of sensory neuropeptides via transient receptor potential vanilloid type 1 (TRPV1) receptors. Capsaicin also relaxes blood vessels however the mechanisms responsible are not fully understood.

Aims. In light of recent studies suggesting dietary capsaicin may lower blood pressure, we sought to further investigate the mechanisms responsible for vasorelaxation, using porcine coronary arteries as a model.

Methods. Anterior descending coronary artery segments from porcine hearts were mounted in organ baths in physiological saline. Capsaicin dose responses were performed in the presence of receptor antagonists and enzyme blockers. Nitric oxide (NO) release from tissues was quantified using a nitrate/nitrite fluorometric assay.

Results. Low concentrations of capsaicin (0.01-1 μM) evoked small amplitude relaxations and release of nitric oxide into the bath effluent which were abolished by N^ω-nitro-L-arginine (L-NNA) but were unaffected by indomethacin. High doses of capsaicin (10 μM-100 μM) evoked large amplitude relaxations which remained unaffected by both L-NNA and indomethacin. The purportedly selective TRPV1 antagonist capsazepine had no effect on relaxations to high concentrations of capsaicin but unexpectedly enhanced relaxations to low concentrations by 10-20% (*P*<0.05 cf control) and also increased NO release. Furthermore in calcium-free, high-potassium physiological saline, capsazepine and high concentrations of capsaicin, inhibited contractions evoked by the reintroduction of calcium.

Discussion. These results suggest that low doses of capsaicin, in the physiological range following dietary consumption of chilli, produce vasorelaxations due to endothelial-dependent release of NO which is independent of TRPV1 receptors. This may account for the antihypertensive effects reported in other studies. High doses of capsaicin relax blood vessels by a non-selective mechanism, probably due to interference with calcium influx. Capsazepine while not causing a direct relaxation of arteries, potentiates the effects of low doses of capsaicin.

Gupta S et al (2007) *Naunyn Schmiedeberg Arch Pharmacol* 375:29-38.

Vanilloid-like agents inhibit platelet aggregation *in vitro*

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Aim. Vanilloids exert their effects primarily through activation of transient receptor potential vanilloid 1 (TRPV1). These agents inhibit platelet aggregation and may protect against the development of cardiovascular disease. The aim was to investigate the effects of a range of vanilloid-like agents on *in vitro* platelet aggregation.

Methods. Collagen-, ADP- and arachidonic acid (AA)-induced platelet aggregation (%Max, %AUC, slope) was determined in the absence and presence of capsaicin (CAP), dihydrocapsaicin (DHC), *N*-oleoyldopamine (OLDA) and *N*-arachidonoyl-dopamine (NADA). Lactate dehydrogenase (LDH) release was measured to determine the direct toxic effects of vanilloids on platelets. Finally, PF4 and β -TG release were measured to determine the effects of vanilloids on alpha granule release.

Results. ADP-induced aggregation was inhibited in a concentration-dependent manner by CAP (%Max, mean \pm SEM; 0 vs 100 μ mol/L, 83.8 \pm 0.9 vs 45.2 \pm 2.4, p <0.001); OLDA (71.6 \pm 8.2 vs 9.4 \pm 1.4, p <0.001); NADA (71.5 \pm 5.9 vs 38.2 \pm 1.4, p <0.008). OLDA (89.3 \pm 1.4 vs 45.5 \pm 12.5, p <0.001) and NADA (87.7 \pm 0.8 vs 28.5 \pm 8.2, p <0.001) inhibited aggregation induced by collagen. AA-induced aggregation was inhibited by CAP (89.6 \pm 0.9 vs 11 \pm 0.8, p <0.001); DHC (88.3 \pm 2.1 vs 18.7 \pm 6.9, p <0.001); and NADA (84 \pm 1.8 vs 21.9 \pm 4.7, p <0.001). The rate of aggregation (slope) was not affected. As LDH release was not affected by vanilloids, inhibition of aggregation was not due to a direct toxic effect. The TRPV1 antagonist, SB-452533, did not affect inhibition of ADP-induced aggregation by OLDA or CAP, suggesting that inhibition of aggregation by vanilloids is not TRPV1 mediated. Preliminary experiments suggest that ADP-stimulated PF4 release from platelets is impaired by CAP, DHC and OLDA whereas NADA enhances ADP-stimulated PF4 release.

Discussion. CAP, DHC, OLDA and NADA inhibit *in vitro* platelet aggregation, a mechanism that is not TRPV1 mediated nor due to a direct toxic effect on platelets. Vanilloids may inhibit platelet aggregation by interfering with granule release. Further studies are warranted.

Psychotropic drugs inhibit the acetylcholine receptor-operated potassium current ($I_{K,ACh}$) by different mechanisms in guinea-pig atrial myocytes.

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Introduction. Although psychotropics have a high therapeutic index and are generally safe agents, some of them produce cardiovascular complication by direct actions and indirect actions through nervous influences. Chlorpromazine, an antipsychotic drug, depressed cardiac repolarization and prolonged QTc resulting in increasing risk of malignant arrhythmia through inhibiting voltage-gated potassium channel. However, influences of psychotropics on ligand-gated potassium channel were not well examined.

Aims. Influences of six antipsychotics and three antidepressants on the acetylcholine receptor-operated potassium current ($I_{K,ACh}$) were examined. And mechanism of anticholinergic action was explored.

Methods. Whole-cell patch clamp method in freshly isolated guinea-pig atrial myocytes was used. $I_{K,ACh}$ was induced by carbachol (CCh) or by an intracellular application of guanosine 5'-[thio] triphosphate (GTP γ S). To elucidate the mechanism for inhibitory effect, the ratio of IC₅₀ values for inhibition of GTP γ S-activated $I_{K,ACh}$ to CCh-induced $I_{K,ACh}$ was calculated using following equation;

$$IC_{50} \text{ ratio} = [IC_{50} \text{ for GTP}\gamma\text{S-activated current}] / [IC_{50} \text{ for CCh-induced current}]$$

Results. Antipsychotics and antidepressants inhibited CCh-induced $I_{K,ACh}$ in a concentration-dependent manner. The IC₅₀ values (μ mol/L) were as follows; chlorpromazine 0.53, clozapine 0.06, fluphenazine 2.69, haloperidol 2.66, sulpiride 42.3, thioridazine 0.07, amitriptyline 0.03, imipramine 0.22, and maprotiline 1.81. The drugs, except sulpiride, inhibited GTP γ S-activated current with following IC₅₀ values (μ mol/L); chlorpromazine 1.71, clozapine 14.9, fluphenazine 3.55, haloperidol 2.73, thioridazine 1.90, amitriptyline 7.55, imipramine 7.09, and maprotiline 5.93. IC₅₀ ratio for fluphenazine and haloperidol was close to unity. IC₅₀ ratio for chlorpromazine, clozapine, thioridazine, amitriptyline, imipramine, and maprotiline was much higher than unity.

Discussion. Psychotropics studied suppressed $I_{K,ACh}$. Chlorpromazine, clozapine, thioridazine, amitriptyline, imipramine, and maprotiline are preferentially acting on muscarinic receptor. Fluphenazine and haloperidol may act on G protein and/or potassium channel.

Effect of diabetes on the production and vasoactivity of hydrogen sulfide in rat middle cerebral arteries.

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Introduction. Hydrogen sulfide is produced endogenously in vascular tissue by cystathionine- γ -lyase (CSE), and has both vasoregulatory and anti-oxidant effects. Little is known of H₂S production or physiological role in cerebral vasculature, particularly in disease states such as diabetes, where there is increased oxidative stress.

Aims. To examine the effect of diabetes on H₂S production and function in rat middle cerebral arteries.

Methods. Diabetes was induced with streptozotocin (50mg/kg, iv). Middle cerebral artery (MCA) function was examined using myography and superoxide anion (O₂⁻) generation measured using NADPH(100 μ M)-dependent lucigenin-enhanced chemiluminescence. CSE mRNA expression was measured via RT-PCR and plasma sulfide and CSE activity were measured using a spectrophotometric assay.

Results. Diabetic rats had elevated blood glucose (P<0.05) and significantly reduced MCA endothelial function (Relaxation to the endothelium-dependent dilator bradykinin (100nM): Control: 48 \pm 7% Diabetic: 30 \pm 4%, n=5, P<0.05). Vasorelaxation to exogenous H₂S was unaffected in diabetic MCA and was elicited via a combination of K⁺, Cl⁻ and Ca²⁺ channel modulation. Vasorelaxation to the H₂S precursor L-cysteine was significantly enhanced in diabetic MCA (%R_{max}: Control: 83 \pm 4 Diabetic: 97 \pm 3 n= 7, P<0.01). Plasma sulfide, CSE activity and CSE mRNA were elevated in diabetes. MCA O₂⁻ production was increased in diabetes (O₂⁻ counts/mg (x10³): Control: 14.6 \pm 2.3, Diabetic: 43.0 \pm 9.4, n=9, P<0.05). This increase was attenuated by incubation with exogenous H₂S (25.8 \pm 3.0, n=9, P<0.05).

Discussion. These data suggest that endogenous H₂S production is upregulated in this model of diabetes. Vasorelaxation responses to exogenous H₂S are preserved and exogenous H₂S attenuates the enhanced MCA-generated O₂⁻ observed in the diabetic group. These data suggest that upregulation of endogenous H₂S in diabetes may play a vasoprotective role.

Contractile recovery induced by pharmacological conditioning with GTN & cariporide at cardioplegia is associated with mitochondrial protective signaling in a model of donor heart preservation.

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Introduction: Activation of pro-survival pathways by pharmacological conditioning agents represents a novel approach to enhance post-storage function of donor hearts.

Aim: To audit the activation of the ERK, Akt and STAT3 pathways by the nitric oxide donor, glyceryl trinitrate, (GTN), and the sodium hydrogen exchange inhibitor, cariporide, after cold storage and warm reperfusion.

Methods: After baseline function was measured in isolated working rat hearts, they were arrested and stored for 6h in either Celsior, Celsior supplemented with 0.1mg/ml GTN, 10 μ M cariporide or both agents combined. After reperfusion, cardiac function was re-measured then the tissue processed for immunoblotting or histology.

Results: GTN and cariporide alone or in combination significantly improved post-storage cardiac function (69 vs 20% of baseline cardiac output GTN+cariporide vs Celsior; P<0.05). Recovery was inhibited by statin, an inhibitor of STAT3 phosphorylation. Significant increases in post-reperfusion necrosis and apoptosis in the Celsior group were abolished by inclusion of GTN, cariporide or both. Increased phosphorylation of ERK and its downstream target, Bcl2, after reperfusion was seen in groups stored in GTN cariporide or both along with increased phospho-STAT3 levels in the GTN/Cariporide group. No phospho-Akt increase was seen in any treatment.

Discussion: Phosphorylation of ERK and Bcl2 has been associated with activation of mitophagy (1), an important endogenous strategy for selection of highly functional mitochondria post reperfusion. The interaction of phospho-STAT3 with cyclophilin D has also recently been implicated in decreasing the open probability of the mitochondrial permeability transition pore (2). Both processes are crucial for functional recovery of the heart after ischemia reperfusion injury. Importantly for potential application of this approach to clinical donor heart retrieval, the conditioning supplement(s) need only be added to the cardioplegic/storage solution.

1. Kang R et al (2011) Cell Death Differ 18:571-80.

2. Boengler K et al (2010) Basic Res Cardiol 105:771-85.

Relaxation of rat isolated pulmonary arteries by a novel vasodilator, BAY 41-8543.

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Introduction: Pulmonary hypertension (PH) is a debilitating disease characterised by increased pulmonary vascular resistance due to arterial vasoconstriction and remodelling, leading to eventual right heart failure and death. Current treatments for PH target the nitric oxide pathway to induce pulmonary vasorelaxation and relieve pressure on the right ventricle; however they are unable to prevent disease progression. A number of compounds targeting pulmonary vascular tone, including BAY 41-8543, are currently being evaluated as new therapeutic options.

Aims: The aims of this study were to compare the effectiveness of two current pulmonary vasodilator agents, sildenafil (PDE5 inhibitor) and milrinone (PDE3 inhibitor), to BAY 41-8543, a novel soluble guanyl cyclase (sGC) activator.

Methods: The effects of sildenafil, milrinone and BAY 41-8543 were assessed in rat isolated pulmonary arteries. Vessels were pre-contracted with U46619 and concentration-relaxation response curves to vasodilator drugs were performed.

Results: Sildenafil (1 nM-30 μ M) and milrinone (10 nM-30 μ M) caused concentration-dependent relaxation of U46619 pre-contracted pulmonary arteries by $66\pm 8\%$ (pEC_{50} , 8.14 ± 0.19 ; $n=5$) and $82\pm 4\%$ (pEC_{50} , 5.69 ± 0.20 ; $n=5$) of maximum, respectively. The maximum relaxation response to BAY 41-8543 (1 nM-30 nM; $-90\pm 5\%$) was significantly greater in comparison to sildenafil ($P<0.05$; $n=5$), however it was less potent (pEC_{50} , 7.22 ± 0.15 ; $P<0.05$).

Discussion: These data demonstrate that BAY 41-8543 caused greater maximum relaxation than either sildenafil or milrinone, as BAY 41-8543 is able to stimulate sGC independently of nitric oxide. This suggests it may be an effective treatment for PH by eliciting pulmonary vasorelaxation. Comparative studies in human isolated vessels are in progress.

Pharmacological profiling of angiotensin II and bradykinin receptor heteromers.

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Introduction: The renin-angiotensin system and the kallikrein-kinin system constitute two regulatory systems involved in the maintenance of blood pressure. Interactions between the two systems are numerous and have been extensively studied for decades. With the establishment of the concept of G protein-coupled receptor (GPCR) heteromerisation a new avenue of investigation has emerged. Several heteromers have already been identified including the heteromer between the type-1 and type-2 angiotensin II receptors (AT₁R and AT₂R) as well as the heteromer between the AT₂R and the bradykinin type-2 receptor (B₂R). Although the AT₁R-B₂R heteromer has also been described (AbdAlla et al, 2001), its existence remains contentious after a second study was unable to detect it in a variety of systems (Hansen et al, 2009).

Methods: This study has investigated interactions between the AT₁R, the AT₂R and the B₂R using various bioluminescence resonance energy transfer (BRET) techniques. The novel GPCR-heteromer identification technology (GPCR-HIT) enables detection of heteromers through ligand-dependent recruitment of GPCR interacting proteins such as arrestin. To investigate interactions between all three receptors, a modified GPCR-HIT assay which includes Venus complementation was employed. HEK293 cells were transfected with receptor or arrestin cDNA fused to either Rluc8 (variant of *Renilla* luciferase), Venus (variant of green fluorescent protein), or split Venus fragments 1 or 2.

Results: The results of GPCR-HIT studies between the AT₁R and the B₂R suggest that an interaction may cause an inhibition of angiotensin II-induced arrestin recruitment to the AT₁R. The modified GPCR-HIT studies between all three receptors further support this, while also confirming the existence of the AT₁R-AT₂R heteromer and the AT₂R-B₂R heteromer.

Discussion: These studies highlight the complex interactions that occur between all three receptors, and suggest that these interactions likely lead to competition for formation of the heteromers.

AbdAlla S et al (2001) Nat Med 7(9):1003–1009.

Hansen J et al (2009) J Biol Chem 284(3):1831–1839.

Region-dependent contribution of the T-type voltage-operated calcium channel to rat renal vascular contraction

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Introduction. T-type voltage-operated calcium channels (VOCC) have been shown to be expressed in the systemic vasculature including the renal vasculature (Hansen et al, 2001). However, unlike the L-type VOCC, their contribution to smooth muscle tone and contraction is debated.

Aims. This study aimed to pharmacologically characterise the role of T-type VOCC in different regions of the renal vasculature.

Methods. Vascular contraction was assessed via wire myography in ring segments of medulla interlobar arteries (internal diameter = $371 \pm 15 \mu\text{m}$) and extrarenal arteries (anterior and posterior division, internal diameter = $629 \pm 21 \mu\text{m}$) isolated from male Sprague Dawley rats. Vessels were exposed to graded, non-cumulative, sequential increases in depolarising potassium solution (10, 20, 40, 62 mM K^+) in the absence and presence of the selective T-type VOCC antagonist NNC 55-0396 (0.01-0.1 μM , 30 min). To ascertain the role of T-type VOCC in agonist-induced contraction, endothelin-1 cumulative concentration-response curves were generated in the absence/presence of NNC 55-0396.

Results. NNC 55-0396 caused concentration-dependent inhibition of potassium-induced contraction; 0.1 μM decreased the maximum contraction in both interlobar (113 ± 8 to $78 \pm 11\%$, $n=4$, $P<0.05$) and extrarenal arteries (95 ± 4 to $80 \pm 2\%$, $n=5$, $P<0.05$). NNC 55-0396 (0.1 μM) also decreased the maximum contraction elicited by endothelin-1 (125 ± 3 , $n=7$ to $110 \pm 3\%$, $n=3$, $P<0.05$) and increased the vasoconstrictor threshold (30-fold) in the interlobar but not extrarenal arteries. Basal tone was unaltered with the selective antagonist.

Discussion. T-type VOCC may contribute to vascular contraction in the renal vasculature. The extent of this contribution varies between regions. The contribution of T-type VOCC to the actions of other physiological vasoconstrictor and vasodilator agents remains to be characterised.

Hansen PB et al (2001) Circ Res 89(7):630-638.

Brain infarct volume after permanent focal ischemia is not dependent on Nox2 expression

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Introduction. Reactive oxygen species (ROS) generated by Nox2 oxidase are reported to contribute to infarct damage following cerebral ischemia-reperfusion. Experimental studies investigating mechanisms of post-stroke brain injury have mostly utilized models of ischemia with reperfusion. Hence, they are most relevant for elucidating the pathology occurring in ischemic stroke cases receiving clot-buster therapy or where there is spontaneous reperfusion. It is thus important to clarify if Nox2 oxidase is also a valid therapeutic target in ischemia without reperfusion.

Aims. We examined the role of Nox2 expression in outcome following permanent focal cerebral ischemia.

Methods. Male wild-type (WT) C57Bl6/J and Nox2^{-/-} mice were anesthetized with i.p. ketamine-xylazine (80 and 10 mg/kg, respectively). Cerebral ischemia was induced using a monofilament to cause middle cerebral artery occlusion (MCAO) for 24 h. Regional cerebral blood flow was reduced by ~85 % in all mice. Neurological deficit and hanging wire grip time was assessed, and infarct and edema volumes were estimated using thionin-stained brain sections at 24 h. In separate mice, Nox2 expression was measured in the ischemic hemisphere using Western blotting.

Results. Nox2 expression was increased in the ischemic versus non-ischemic hemisphere of WT mice 24 h after MCAO (1.77 ± 0.31 versus 1.00 ± 0.09 ; $n=9-10$, $P<0.05$). However, genetic deletion of Nox2 had no effect on any outcome measures at 24 h after permanent MCAO. WT and Nox2^{-/-} mice had similar neurological deficit scores and hanging times. Similarly, the edema volume and total, cortical, and subcortical infarct volumes did not differ between WT and Nox2^{-/-} mice.

Discussion. ROS production by Nox2 oxidase activity plays no significant role in the pathophysiology of cerebral ischemia in the absence of reperfusion. The findings may highlight the importance of ROS production by Nox2 oxidase-containing leukocytes that enter the ischemic brain specifically if reperfusion is instituted.

Neuroprotective effect of an angiotensin receptor-2 agonist following cerebral ischemia in vitro and in vivo

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Introduction. The renin-angiotensin system is known to be important in the development of a number of cardiovascular diseases. Intracerebral administration of the angiotensin II type 2 receptor (AT2R) agonist, CGP42112, is neuroprotective in a rat model of ischemic stroke.

Aims. To explore further its possible cellular target(s) and therapeutic utility, we firstly examined whether CGP42112 may exert direct protective effects on primary neurons following glucose deprivation in vitro. Secondly, we tested whether CGP42112 is effective when administered systemically in a mouse model of cerebral ischemia.

Methods. Primary cortical neurons were cultured from E17 C57Bl6 mouse embryos for 9 d, exposed to glucose deprivation for 24 h alone or with drug treatments, and percent cell survival assessed using trypan blue exclusion. Ischemic stroke was induced in adult male C57Bl6 mice by middle cerebral artery occlusion for 30 min, followed by reperfusion for 23.5 h. Neurological assessment was performed and then mice were euthanized and infarct and edema volume were analysed.

Results. During glucose deprivation, CGP42112 (1×10^{-8} M and 1×10^{-7} M) reduced cell death by ~30%, an effect that was prevented by the AT2R antagonist, PD123319 (1×10^{-6} M). Neuroprotection by CGP42112 was lost at a higher concentration (1×10^{-6} M) but was unmasked by co-application with the AT1R antagonist, candesartan (1×10^{-7} M). By contrast, Compound 21 (1×10^{-8} M to 1×10^{-6} M), a second AT2R agonist, had no effect on neuronal survival. Mice treated with CGP42112 (1 mg/kg i.p.) after cerebral ischemia had improved functional outcomes over vehicle-treated mice as well as reduced total and cortical infarct volumes.

Discussion. These results indicate that CGP42112 can directly protect neurons from ischemia-like injury in vitro via activation of AT2Rs, an effect opposed by AT1R activation at high concentrations. Furthermore, systemic administration of CGP42112 can reduce functional deficits and infarct volume following cerebral ischemia in vivo.

Endothelin and endothelin receptor antagonism in rat isolated cerebral arteries: relevance for subarachnoid haemorrhage

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Introduction. Delayed cerebral vasospasm following subarachnoid haemorrhage (SAH) is characterized by sustained narrowing of cerebral arteries. The pathological mechanism leading to vasospasm is not completely understood. Endothelin-1 (ET-1) is a key vasoconstrictor agent mediating development of vasospasm, where its concentration is increased in the cerebrospinal fluid (CSF) of SAH patients. Further, animal studies have revealed an increased sensitivity of cerebral arteries to ET-1 after SAH. There are two types of ET receptors - ET_A and ET_B. ET_A receptors are expressed on vascular smooth muscle cells and mediate vasoconstriction, while ET_B receptors, mainly located on vascular endothelium, mediate vasodilatation, except in pathological states when they may mediate vasoconstriction.

Aims. The aim of this study was to investigate regional variation in ET-1 responses in rat cerebral arteries.

Methods. Rat anterior cerebral (ACA), middle (MCA), posterior communicating (Pcom) and basilar (BA) arteries with respective internal diameters of 340 ± 8 , 320 ± 9 , 314 ± 10 and 428 ± 13 μ m were dissected and ET-1 concentration-response curves were analyzed using wire myography.

Results. ET-1 caused potent contraction in all four cerebral arteries. The pEC_{50} of ET-1 in BA was 8.39 ± 0.15 , with no significant difference in ACA, MCA and Pcom. However, ACA, MCA and Pcom had a lower threshold than BA. Bosentan (a dual ET_A and ET_B receptor antagonist) shifted the ET-1 concentration-response curve to the right in all arteries except ACA at low concentration (0.1 μ M); the rightward shifts were 1.4, 3.8, 4.0 and 3.2 fold in ACA, MCA, Pcom and BA, respectively. The selective ET_B receptor agonist, sarafotoxin 6C, did not cause vasoconstriction, but induced concentration-dependent relaxation that was similar in all vessels.

Discussion. Rat cerebral arteries are highly sensitive to ET-1 vasoconstriction with some regional variation in the response to bosentan. Potent relaxant effects of sarafotoxin 6C indicate the presence of ET_B receptors in intact cerebral vascular endothelium.

Chronic NaHS treatment protects vascular function by reducing oxidative stress in streptozotocin-induced diabetes in mice.

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Introduction. Hydrogen sulfide (H₂S) is endogenously produced in vascular tissue (Kimura 2011). H₂S is an antioxidant (Kimura 2011) and may be a useful therapeutic agent under conditions of increased oxidative stress.

Aim. The aim was to investigate whether chronic treatment with the H₂S donor NaHS could elicit a vasoprotective effect in diabetes, where there is known to be increased oxidative stress (Shen 2010).

Methods. Diabetes was induced in male C57 mice with streptozotocin (60mg/kg daily, ip for 2 weeks) and confirmed by elevated blood glucose and HbA_{1c} levels. Following a further 3 weeks, mice were then treated with NaHS (100μmol/kg/day) for 4 weeks, then tissues collected. Myography was employed to examine vascular reactivity and NO and H₂S bioavailability in thoracic aortae. Vascular superoxide levels were determined by the NADPH-dependent lucigenin-enhanced chemiluminescence assay. Plasma H₂S concentration and the activity of H₂S synthesising enzyme, cystathionine-γ-lyase (CSE) were measured by spectrophotometric assay.

Results. Vascular superoxide levels were significantly increased in diabetic aortae compared to control aortae (P<0.01). Daily NaHS treatment for 4 weeks in diabetic mice reduced superoxide production in diabetic aortae. ACh-mediated, endothelium-dependent vasorelaxation was significantly inhibited in diabetic aortae (P<0.05), but NaHS treatment restored the maximal relaxation to ACh. Vascular H₂S bioavailability and plasma H₂S concentration were reduced in diabetes, while liver CSE activity was significantly increased in diabetes (P<0.0001), however none of these parameters were affected by chronic NaHS treatment.

Discussion. These data suggest that NaHS acts as a scavenger of NADPH-induced superoxide and protects endothelial function *in vivo* in this model of oxidative stress.

Kimura H (2011), *Exp Physiol*, 96(9):833-5.

Shen G (2010), *Can J Physiol Pharmacol*, 88(3):241-8.

Review of Epidemiology and Management of Atrial Fibrillation in Developing Countries

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Introduction. Atrial fibrillation (AF) is the commonest sustained cardiac arrhythmia with a substantial impact on mortality and morbidity (Wolf P et al, 1999). Pharmacologic management of AF includes treatment of the underlying cause, anticoagulation to reduce the risk of stroke and systemic thromboembolism, and rate or rhythm control for symptomatic relief. Relatively little is known about AF in the developing world.

Aims. To examine in developing countries the prevalence of AF, the medical conditions associated with AF and the pharmacological management of AF.

Methods. A literature search was conducted via MEDLINE and EMBASE (1990-2012).

Results. A total of 70 articles were included in the review. The prevalence of AF in the general population ranged from 0.03% to 1.25% while the prevalence of AF in hospital-based studies varied according to the patient population studied (from 0.7% to 55.7%). Hypertension was the most common associated disease (10.3%-71.9%). The utilization of anticoagulants (coumarins) was highly variable (from 2.7% to 72.7%). No study was identified on the use of newer oral anticoagulants. Approximately half of AF patients using warfarin had therapeutic INR levels. There was a high prevalence of using rate control therapies (55.3% to 87.3%).

Discussion. The limited studies available suggest that in the developing world there is a significant prevalence of AF. Highly variable use of anticoagulants may be related to different health care and socioeconomic settings. More studies are needed to improve understanding of the epidemiology and management of AF in developing countries.

Wolf P et al (1991) *Stroke* 22:983-8.

An allosteric enhancer of the adenosine A₁ receptor improves cardiac function following ischaemia in isolated murine hearts.

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Introduction. Adenosine is released from tissues during the conditions of low oxygen tension. Stimulation of the adenosine A₁ receptor following an ischaemic episode has been shown to be cardioprotective (Butcher et al., 2007).

Aims. The effect of an allosteric enhancer of the adenosine A₁ receptors was investigated using an ischaemia-reperfusion protocol in murine isolated hearts.

Methods. Isolated hearts were perfused with Krebs-Henseleit solution gassed with 95%O₂; 5%CO₂ in Langendorff mode and electrically paced at 480 bpm. Following 20 mins equilibration and 20 mins global normothermic ischaemia, the allosteric enhancer VCP333 (1 µmol/L) or the partial adenosine A₁ receptor agonist VCP102 (10 µmol/L) were infused after 5 mins of reperfusion for 15 mins. Upon termination of the drug treatment, reperfusion continued for a further 40 mins.

Results. At the end of 60 mins reperfusion treatment with VCP333 or VCP102 the recovery of the left ventricular developed pressure was higher when compared to control group responses ($P < 0.05$). Neither compound affected end diastolic pressure, coronary flow or dp/dt_{max} values when compared to control tissues during reperfusion ($P > 0.05$). The infusion of VCP102 or VCP333 during reperfusion reduced cardiac troponin I efflux to 6.7% and 25% respectively of control heart efflux ($P < 0.05$).

Conclusion. This data indicates that the allosteric enhancer of the adenosine A₁ receptor (VCP333) has similar characteristics to the adenosine receptor partial agonist VCP102 as it improves cardiac function and reduces myocardial cell death following an ischaemic episode.

Butcher, A et al (2007) Drug Dev Res 68: 529-537.

The DPP-4 inhibitor linagliptin improves endothelium-dependent relaxation of rat mesenteric arteries in the presence of high glucose

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Introduction. The dipeptidyl peptidase-4 (DPP-4) inhibitors are a novel class of pharmacological agents used to treat hyperglycaemia that causes impairment of vascular endothelial function in type 2 diabetes. Both experimental and clinical studies indicate that DPP-4 inhibition may exert beneficial effects on the cardiovascular complications of diabetes independently of their glucose lowering effects.

Aim. To investigate the effect of linagliptin, a DPP-4 inhibitor, on the mechanism(s) of endothelium-dependent relaxation in rat mesenteric arteries in the absence and presence of high glucose (40 mM).

Methods. Endothelium-dependent and -independent relaxation to acetylcholine (ACh) (0.1 nM–10 µM) and sodium nitroprusside (SNP) (0.1 nM–10 µM) was determined in mesenteric arteries from Wistar rats pre-contracted with phenylephrine (10–100 nM) and exposed to normal (11 mM) or high (40 mM) glucose concentrations.

Results. Incubation of mesenteric rings with high glucose (40 mM) for 2 h caused a significant impairment of endothelium-dependent relaxation (ACh pEC_{50} glucose: 11 mM = 7.76 ± 0.20 , 40 mM = 6.32 ± 0.21 , $p < 0.05$), but did not affect SNP-induced relaxation. Co-incubation with linagliptin prevented the impairment of endothelium-dependent relaxation caused by high glucose (ACh pEC_{50} 40 mM glucose + 1 µM linagliptin = 7.20 ± 0.07). When the contribution of NO was abolished by N-nitro-L-arginine (L-NNA, 100 µM) plus a soluble guanylate cyclase inhibitor (ODQ, 10 µM), or the contribution of endothelium derived hyperpolarising factor (EDHF) was inhibited with TRAM-34 (1 µM) plus apamin (1 µM), the ACh-induced relaxation was significantly impaired by high glucose under both conditions suggesting that the contributions of both NO and EDHF were affected. Linagliptin significantly improved ACh-induced relaxation in the presence of both groups of inhibitors.

Discussion. Endothelium-dependent relaxation was impaired by high glucose but was significantly improved by linagliptin which preserved the actions of both NO and EDHF demonstrating that the vasoprotective actions are independent of any glucose lowering activity.

Direct stimulation of AT2R and the MasR prevents TNF α -induced endothelial inflammation.

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Introduction. Activation of the angiotensin II type 1 receptor (AT1R) mediates pro-inflammatory effects with recent evidence demonstrating that activation of the angiotensin II type 2 receptor (AT2R) and Mas receptor (MasR) counteract some of the pro-inflammatory effects of AT1R stimulation such as cytokine release. Importantly, the potential anti-inflammatory properties of direct AT2R or MasR activation on the adhesion cascade remain unknown.

Aims. We aimed to examine whether direct activation of the AT2R and MasR elicits anti-inflammatory effects; specifically by reducing TNF α -induced leukocyte-endothelial cell adhesion and adhesion molecule expression.

Methods. Leukocyte-endothelial cell adhesion was examined *in vitro* (cultured HUVECs) and *ex vivo* (intact mouse thoracic aorta). Adhesion molecule (Intercellular Adhesion Molecule 1 (ICAM-1) and E-selectin) protein expression was assessed using flow cytometry.

Results. Direct stimulation of the AT2R (using Compound 21; C21 100 μ M) and MasR (using AVE 0991; 100 μ M) reduced TNF α -induced endothelial-leukocyte adhesion *in vitro* resulting in 54 \pm 5% (n=5, P<0.005) and 69 \pm 5% (n=4, P<0.01) of TNF α -induced adhesion (100%), respectively. These effects were abolished following concurrent treatment with the respective receptor antagonists (MasR agonist: A779, 10 μ M, 110 \pm 5%, n=4 and AT2R agonist: PD 123319, 10 μ M, 93 \pm 2%, n=4). Similarly, TNF α increased adhesion from 10 \pm 2 to 30 \pm 4 adhered leukocytes/field of view (FOV) in intact mouse aortae (n=12, P<0.001); which was completely prevented in aorta treated with C21 (10 μ M, 11 \pm 4 adhered leukocytes/FOV, n=6, P<0.01). Furthermore, C21 reduced TNF α -induced ICAM-1 and E-selectin protein expression (n=3, P<0.005).

Discussion. This study provides the first evidence that direct activation of endothelial AT2R and MasR attenuates the inflammatory effects of TNF α in the setting of the vascular adhesion cascade.

Review of antithrombotic risk assessment tools for stroke prevention in atrial fibrillation patients

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Introduction. Clinical guidelines advocate stroke prevention therapy in persons with Atrial fibrillation (AF), recommending antithrombotic agents (*e.g.*, warfarin). However, the decision to initiate treatment is based on the risk (*e.g.*, side-effects such as bleeding) versus benefit (*prevention of stroke*) of therapy, and this is often difficult to assess.

Aim. To identify and review available risk assessment tools to facilitate the optimal use of antithrombotic therapy for stroke prevention in AF.

Methods. Review of literature (1992-2012) via key databases and online clinical resources.

Result. Overall, 18 tools were identified: 11 addressing stroke risk, 7 addressing bleeding risk. Among the stroke risk assessment tools (*e.g.*, Framingham, SPAF, AFI, Birmingham), CHADS₂ and CHA₂DS₂-VASc were the most commonly advocated tools, sharing common risk factors: age, hypertension, diabetes, previous stroke/transient ischaemic attack. Among the bleeding risk assessment tools (*e.g.*, OBRI, ATRIA), HEMORR₂HAGES and HAS-BLED were most commonly advocated, sharing common risk factors such as: age, previous bleeding, renal and liver impairment. Overall, all but 1 tool targeted the separate aspects of the risk versus benefit equation; only the Computerised Antithrombotic Risk Assessment Tool (CARAT) brings together individual risk assessment for both stroke and bleeding. None of the other tools consider other key factors in decision-making regarding antithrombotic therapy, particularly those increasing the risk of medication misadventure with treatment (*e.g.*, function, cognition, drug interactions, medication adherence, medication management capabilities).

Discussion. Although, separate tools are available to assess stroke risk and bleeding risk independently, but they do not estimate the relative risk versus benefit of available treatment options in an individual patient. Also, these separate tools seldom consider key medication safety aspects of prescribing treatment. More effort is needed to synthesise these separate risk assessments, and integrate key medication safety issues, particularly in view of the introduction of new anticoagulants into practice.

Allosteric Modulation of a Chemogenetically Modified G Protein-Coupled Receptor

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Introduction. DREADDs (Designer Receptors Exclusively Activated by Designer Drugs) are chemogenetically modified muscarinic acetylcholine receptors (mAChRs) that have minimal responsiveness to ACh, but are potently and efficaciously activated by an otherwise inert synthetic ligand, clozapine-N-oxide (CNO). DREADDs have been used as tools for selectively modulating signal transduction pathways *in vitro* and *in vivo*. Recent comprehensive studies have validated how the pharmacology of a CNO-bound DREADD mirrors that of an ACh-bound wild-type (WT) mAChR. However, nothing is known about whether this equivalence extends to the allosteric modulation of DREADDs by small molecules.

Aims. To investigate the actions of an M₁ DREADD of BQCA, a positive allosteric modulator of ACh binding and function that is known to behave according to a simple two-state mechanism at the WT receptor.

Methods. Radioligand binding studies and a range of intracellular functional assays were performed on Chinese Hamster Ovary (CHO) cell expressing either WT or DREADD mAChRs.

Results. 1. Allosteric modulation of the CNO-bound DREADD receptor is not equivalent to the corresponding modulation of the ACh-bound WT receptor. 2. BQCA engenders stimulus bias at the M₁ DREADD, having differential types of cooperativity depending on the signaling pathway. 3. The modulation of ACh itself by BQCA at the DREADD is not compatible with the two state model that has been previously applied to the M₁ WT.

Discussion. The results indicate that caution must be exercised when interpreting studies of allosteric modulation using DREADDs.

The effect of morphine on the growth and dissemination of breast tumour in mice

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Introduction. Appropriate pain management during and after cancer surgery may play a role in prevention of tumour recurrence and metastasis. Opioids are proven to be highly effective perioperative analgesics and are widely used in cancer surgery patients.

Aims. Using a mouse syngeneic model of breast cancer, we studied the effect of morphine on tumour growth and dissemination to lungs. We investigated in an *in vitro* co-culture model the effect of morphine on the interaction of breast tumour cells with non-malignant cells present in the tumour microenvironment, namely macrophages and endothelial cells.

Methods. Morphine was injected intraperitoneally (10mg/kg) to mice (n=8) every 12h for 3 days and its effect on murine 4T1 breast tumour cell dissemination to the lungs was measured 18 days after tumour inoculation. In the *in vitro* studies, 4T1 breast cancer cells, RAW264.7 macrophages, H5V endothelial cells and co-cultures of 4T1 with either macrophages or endothelial cells were treated with morphine (0.1-10 μ M). The level of extra-cellular matrix (ECM) degrading enzymes, matrix metalloproteinase-9 (MMP-9) and urokinase-like plasminogen activator (uPA) as well as tissue inhibitors of matrix metalloproteinases (TIMPs) were measured in the conditioned media using in-gel zymography.

Results. Morphine treatment caused a reduction in breast tumour growth and tumour cell dissemination to the lungs. Morphine treatment also caused a reduction in circulating MMP-9 and uPA. In co-cultures of 4T1 cells with endothelial cells or macrophages, the level of matrix proteases was increased, and so was the level of TIMPs. Morphine treatment reduced the level of MMP-9 and increased its endogenous inhibitor, TIMP-1 in co-cultures but not cells grown individually.

Discussion. Our data suggest that morphine treatment could decrease tumour growth and dissemination in mice and that this anti-tumour effects are mediated at least in part through modulation of paracrine communication between cancer cells and tumour infiltrating cells.

Altered purinergic receptor calcium signalling associated with hypoxia in MDA-MB-468 breast cancer cells

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Introduction. Hypoxia is a common feature of the microenvironment of some breast cancers. Hypoxia can induce epithelial-mesenchymal transition (EMT) a process that can convert breast cancer cells into a more invasive phenotype. We have previously shown an association between epidermal growth factor (EGF)-mediated EMT and alterations in purinergic receptor-mediated calcium signalling and levels of purinergic receptor mRNA (e.g. up-regulation of P2X5).

Aims. 1. To compare changes in ATP-induced calcium transients in MDA-MB-468 breast cancer cells in normoxia and hypoxia. 2. To assess mRNA levels of a panel of purinergic receptors in MDA-MB-468 cells in normoxia and hypoxia.

Method. MDA-MB-468 cells were placed in a hypoxic incubator (0.1% O₂) for 24 hours at 37°C. Cytosolic free Ca²⁺ ([Ca²⁺]_{CYT}) levels were assessed using a Fluorescent Imaging Plate Reader (FLIPR). Changes in mRNA levels were evaluated by quantitative real-time RT-PCR.

Results. Hypoxia-induced EMT was confirmed by changes in EMT markers including vimentin, N-cadherin, snail, and CD24. The nature of the cytosolic calcium response to ATP was altered with hypoxia (e.g. EC₅₀ 0.5 μM in normoxia vs 1.3 μM in hypoxia). Significant changes in mRNA levels of some purinergic receptors in response to hypoxia were observed, however, these were often different from those previously observed with EGF-induced EMT.

Discussion. Hypoxia-induced EMT alters ATP-mediated calcium signalling and mRNA levels of purinergic receptors in MDA-MB-468 breast cancer cells. Further studies are required to assess the significance of altered purinergic receptor-mediated Ca²⁺ signalling associated with the acquisition of EMT.

Functional selectivity at the adenosine A₁ receptor: Implications for cytoprotection.

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Introduction. Adenosine A₁ receptor (A₁AR) stimulation is protective in a number of cardiovascular and neuronal conditions, however, current therapeutic targeting is limited due to bradycardia (Jacobson & Gao, 2006). Remarkably, the novel A₁AR agonists, VCP28 and VCP746, retain cytoprotective signaling in the absence of bradycardia, a phenomenon suggestive of functional selectivity (Kenakin et al., 2012; Urmaliya et al., 2010).

Aims. To delineate A₁AR-mediated cytoprotective signal transduction and quantify the ability of the A₁AR ligands, NECA, R-PIA, VCP28 and VCP746 to promote cytoprotective signal transduction.

Methods. A propidium iodide-based assay assessed A₁AR agonist-mediated cytoprotection of CHO cells stably expressing the human A₁AR (CHO-A₁) after 24-hour serum starvation in the absence or presence of pharmacological inhibitors. Phosphorylation of ERK1/2 and AKT, calcium mobilization and cAMP accumulation were determined using fluorescence and luminescence approaches. Functional selectivity was quantified as described previously (Kenakin et al., 2012).

Results. A₁AR agonist stimulation of CHO-A₁ cell survival (n=9) was dependent on G_{i/o} protein stimulation, phosphorylation of ERK1/2 and AKT1/2/3 and PKC activation (n=3). Functional assays demonstrated that each A₁AR agonist mediated a robust increase in ERK1/2 and AKT1/2/3 phosphorylation, calcium mobilization and inhibition of cAMP accumulation (n=3-6). In contrast to R-PIA and NECA, VCP28 and VCP746 showed functional selectivity with respect to calcium mobilization (p<0.05, one-way ANOVA, Tukey Post Hoc test).

Discussion. A₁AR agonists stimulate CHO-A₁ cell protection via G_{i/o} protein, ERK1/2, AKT1/2/3 and PKC dependent mechanisms. Functionally selective signalling may underlie the preferential physiological profile observed for VCP28 and VCP746.

Jacobson K & Gao K (2006) Nat Rev Drug Discov 5:247-264

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

Urmaliya V (2010) J Cardiovasc Pharm 56:282-292

Can interference of GIP activity modulate GPCRs for therapeutic gains?

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Introduction: GPCRs are a large family of cell surface proteins participating in signal transduction where the C-terminus recruits GPCR Interacting Proteins (GIPs) that regulate GPCR function. The 5-HT₄ (a, d, e, f and g) receptor splice variants possess canonical type 1 or type 2 PDZ domains predicted to interact with various GIPs which could influence their modulatory and prokinetic functions in the intestine (Coupar et al. 2007).

Aims: To investigate the distribution of 5-HT₄ receptors and GIPs in the guinea pig intestine and secondly to determine if human 5-HT₄ receptors and specific GIPs interact using an in vitro cell system.

Methods: RT-PCR, western and immunofluorescence analysis were used to investigate transcript and protein expression. N-terminal FLAG tagged 5-HT₄ receptor splice variants and Lin 7 homologues with C-terminal tagged V5, c-Myc and HA constructs were generated and expressed in COS-7 cells to study protein interactions.

Results: 5-HT₄ receptors, GRKs and Lin 7 homologues were expressed in the guinea pig intestine. Lin 7 homologues and 5-HT₄ receptors co-localized in cell lines and the 5-HT_{4a} receptor and Lin 7 were co-immunoprecipitated.

Discussion: We have previously shown that 5-HT₄ receptors and the GIPs, GRKs and Lin7 homologues, are found in the human colon (Chetty et al. 2009). Our data here indicates that this also occurs in the guinea pig and we demonstrate that it may be possible for 5-HT₄ receptor and Lin 7 to interact in the same cells. The results suggest that Lin 7 could be a potential target to modulate 5-HT₄ receptor function. This would be of particular relevance when 5-HT₄ splice variant expression is altered as occurs in cancerous tissue (Cartier et al. 2005).

Cartier D et al. 2005 Eur J Endocrinol 153:939-47

Chetty N et al. 2009 Neurogastroenterol Motil 21:551-58.e15

Coupar IM et al. 2007 Curr Neuropharmacol 5: 224-31

Activation and acute desensitization of μ -opioid receptor wild-type and mutants with deleted phosphorylation sites

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Introduction: Opioids are widely used clinically because of their unique analgesic properties, largely mediated by activation of the μ -opioid receptor (MOR). Understanding the molecular mechanisms underlying MOR regulation is important for developing pain relieving drugs that will decrease unwanted effects, dependence and tolerance.

Aims: To investigate the mechanisms of acute MOR desensitization with an emphasis on receptor phosphorylation.

Methods: Wild-type mouse MOR (MOR-WT) and MOR with mutations of c-terminus phosphorylation sites were stably transfected in AtT-20 cells and grown in 96 well plates. MOR signalling was measured using a proprietary membrane potential dye (Molecular Devices) in a Flexstation 3. MOR desensitization was quantified using a high "challenge" concentration of agonist added after the start of the desensitizing stimulus. Heterologous desensitization was assessed with somatostatin.

Results: Activation of MOR hyperpolarized AtT-20 cells, and the hyperpolarization waned over time. Morphine and [D-Ala²,N-MePhe⁴,Gly-ol]-enkephalin (DAMGO) activated each MOR variant with a similar potency and signaling at all variants was inhibited by prolonged exposure to morphine. In cells expressing MOR-WT, morphine (1 μ M) produced a 71 \pm 2% inhibition of the hyperpolarization caused by a subsequent application of 10 μ M morphine (t_{1/2} 343s, 95% CI 264-490s). Morphine (1 μ M) inhibited the response to somatostatin (1 μ M) by 29 \pm 1%, with a similar timecourse. Met-enkephalin (1 μ M) produced a maximum 58 \pm 2% inhibition of a subsequent application of ME (10 μ M), and a 47 \pm 3% decrease in the response to somatostatin (n=5). Pretreatment of MOR-WT cells with the protein kinase C activator phorbol 12-myristate 13-acetate (1 μ M) or the protein kinase inhibitor staurosporine (1 μ M) did not significantly affect morphine potency or desensitization (n=5).

Discussion: These results support the idea that MOR desensitization is rapid and not necessarily dependent on protein kinase C. In addition, desensitization persists after deletion of several key phosphorylation sites in the c-terminal tail of the receptor.

Selective GPCR signalling opens TRPV4 expressed in HEK293 cells

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Introduction. Activation of muscarinic receptors in mouse vascular endothelial cells leads to the opening of the ion channel TRPV4 (Adapala et al, 2011). We hypothesized that other GPCRs might also be able to open TRPV4.

Aims. To investigate the ability of endogenous or exogenous GPCRs expressed in HEK293 cells to open TRPV4.

Methods. We generated HEK293 cell lines expressing wild type human TRPV4 (TRPV4) and compared the rise in intracellular calcium ($[Ca^{2+}]_i$) evoked by agonists for different GPCRs expressed in HEK293 cells (e.g. PAR₁ and PAR₂ and P2Y receptors) with non transfected cells (Nt), using a FURA-2 calcium influx assay in a Fluorimeter. Angiotensin II (ANGII) receptor 1 (AT₁R) is not expressed in HEK293 cells, so it was transiently transfected into Nt and TRPV4 and responses to angiotensin, measured using fluorescence microscopy.

Results. PAR₁ and PAR₂ receptors were activated with SFLLR-NH₂ and SLIGRL-NH₂, respectively and evoked a transient increase of $[Ca^{2+}]_i$ in Nt that returned to baseline 35s after stimulation, whereas, in TRPV4 cells, there was a sustained increase in $[Ca^{2+}]_i$ measured 60s after stimulation ($p < 0.01$, $n = 4$, t test). The AT₁R receptor showed a similar difference between Nt and TRPV4 with the sustained response in TRPV4. We did not observe opening in response to P2Y activation by ATP.

Discussion. We show that GPCR opening of TRPV4 in HEK293 occurs with activation of PAR₁, PAR₂ and AT₁R but not P2Y receptors, showing that a transient increase in $[Ca^{2+}]_i$ alone, is not sufficient to open TRPV4.

Adapala et al (2011) AmJ Physiol 301:H757-H765

The binding mode of SB269652: a novel bitopic ligand at the dopamine D2 receptor

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Introduction. To date all clinically effective antipsychotics target the D2 dopamine receptor (D2R) by competing with the neurotransmitter dopamine for the 'orthosteric' binding site. SB269652 was recently identified as the first negative allosteric modulator of the D2R (Silvano et al. 2010). We have progressively truncated fragments of this ligand, demonstrating that SB269652 has a 'bitopic' (simultaneous allosteric/orthosteric) mode of interaction with the D2R.

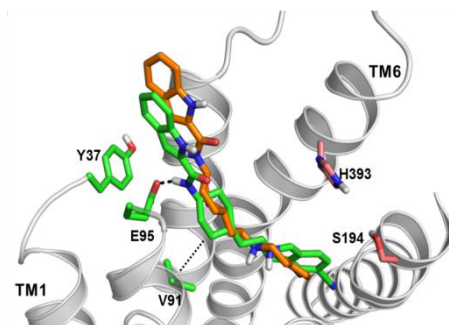
Aim. To gain further insight as to the specific receptor residues important for binding and function of this ligand

Methods. We selected and mutated residues in both orthosteric and putative allosteric sites of the D2R and screened these mutants in both functional (D2R mediated ERK1/2 phosphorylation) and radioligand binding assays ($[^3H]$ -spiperone)

Results. Mutation to alanine of two residues predicted to interact with the allosteric moiety of SB269652, Glu95 and Val91, caused a significant decrease in the negative cooperativity exerted by SB269652 upon dopamine ($\log\alpha_{Glu95Ala} = -0.32 \pm 0.14$; $\log\alpha_{Val91Ala} = -0.48 \pm 0.16$; $n = 3$, $P < 0.05$) as compared to the wild type receptor ($\log\alpha_{WT} = -1.20 \pm 0.12$). Similarly, mutation of Ser194 within the orthosteric site to alanine caused a significant decrease in negative cooperativity ($\log\alpha_{Ser194Ala} = -0.52 \pm 0.09$, $n = 3$, $P < 0.05$).

Discussion: These data demonstrate that SB269652 makes key interactions both within the orthosteric site and in an allosteric site at the top of transmembrane domain 2. As such this study provides validation of a bitopic mechanism of action for SB269652 and reveals the location of a novel allosteric site within the D2R.

Silvano E et al (2010). Mol Pharmacol 78(5): 925-934



Antitumour actions of the synthetic ω -3 17,18-epoxyeicosanoic acid in breast cancer cells

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Introduction. Unlike ω -6 polyunsaturated fatty acids (PUFA), which promote the growth and spread of tumours, ω -3 fatty acids decrease tumourigenesis. We have found previously that the CYP-derived ω -3 epoxide of the naturally occurring eicosapentaenoic acid inhibits cell proliferation by down-regulating cyclin D1 (Cui et al., 2011). In contrast, isomeric epoxides formed by the action of CYPs on non- ω -3 olefinic bonds in ω -3 and ω -6 PUFA stimulated cell growth.

Aims. This study evaluated the anti-tumour actions of ω -3 17,18-epoxyeicosanoic acid (ω -3 EEA), a saturated monoepoxide that was synthesized to circumvent the formation of protumourigenic PUFA epoxides, in breast cancer cells.

Methods. ω -3 EEA was synthesized from 17,18-eicosenoic acid (Cui et al., 2012) by treatment with *m*-chloroperbenzoic acid. MDA-MB 231 and MDA-MB 468 cells were treated with several concentrations of ω -3 EEA for varying times. Endpoints relating to proliferation (MTT reduction, cell cycle kinetics and cyclin D1/E expression) and apoptosis (caspase-3 activity, caspase-3/9 and PARP cleavage, sub-G1 analysis, annexin V staining, and expression of cytochrome c and several Bcl family proteins) were estimated.

Results. ω -3 EEA decreased proliferation and activated apoptosis in MDA-MB 231 and, to a lesser extent, the less aggressive MDA-MB 468 cells in a concentration- and time-dependent fashion. The cell cycle was arrested at G0/G1 phase due to a decrease in cyclin D1 expression. The Bak/Bcl-2 ratio was increased in treated cells.

Discussion. These findings suggest that the mechanism of breast cancer cell growth inhibition by ω -3 EEA involves activation of the intrinsic (mitochondrial) death pathway and cell cycle arrest. ω -3 EEA may be the prototype of a novel class of anticancer agents based on ω -3 PUFA epoxides.

Cui et al. (2011) Br J Pharmacol 162, 1143-1155

Cui et al. (2012) J Med Chem 55, 7163-7172

Identification of novel allosteric modulators of the α_{1A} and β_2 adrenoceptors

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Introduction. Current adrenoceptor (AR) allosteric modulators are either peptidic or have significant off-target affinity, such as the β_2 AR positive allosteric modulator (PAM), the "MA" fragment of the bitopic ligand THRX-198321, which is also a muscarinic receptor antagonist (Steinfeld et al, 2011).

Aim. To identify novel small molecule allosteric modulators of the α_{1A} and β_2 AR.

Methods. Allosteric modulators were docked into a β_2 AR crystal structure (3P0G) (MA, THRX-198321) or an α_{1A} AR homology model (C9). A receptor-ligand pharmacophore was generated based the MA/ β_2 AR docking data and a structure-based pharmacophore were developed in Discovery Studio and used to screen the SPECS database. COS-1 membranes expressing α_1 or β_2 AR were used in radioligand dissociation assays.

Results. Docking studies predicted H296, K305 and Y308 to form interactions with MA. H296A, K305A and Y308A mutations resulted in an increased dissociation rate of [³H]dihydroalprenolol (DHA) (k_{off}) compared to wildtype. Y308A also altered MA's (1mM) ability to decrease [³H]DHA dissociation (wildtype k_{obs}/k_{off} 56±2.5%; Y308A k_{obs}/k_{off} 75±2.7%, n=3 p<0.05). Seven compounds were chosen from the SPECS database. Preliminary testing has shown that compound 4 displays properties of a β_2 AR PAM, decreasing the k_{off} of [³H]DHA ([³H]DHA, 0.074 min⁻¹, + Compound 4 (100μM), 0.019 min⁻¹). We have also identified novel α_{1A} AR negative allosteric modulators; a symmetrical bis-4-aminoquinoline (C9) and 4-aminoquinoline (C9 30μM, k_{obs}/k_{off} 529±29%; 4-aminoquinoline 300μM, k_{obs}/k_{off} 206±15%, n=3). The docking studies suggest that C9 interacts with both the orthosteric binding site and residues at the top of transmembrane helix II.

Discussion. We have identified amino acids associated with allosteric effects in the extracellular regions of α_{1A} and β_2 AR. Novel allosteric compounds have been identified which can be further developed into pharmaceutical agents targeting the α_{1A} and β_2 AR.

References

Steinfeld T et al (2011) Mol Pharmacol 79:389-399

Inhibition of human haematological malignant cell line growth by capsaicin is not TRPV1-mediated

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Aim. Transient receptor potential vanilloid-1 (TRPV1) is a non-selective cation channel activated by a variety of endogenous and exogenous stimuli, including the major active component of 'hot chilli peppers', capsaicin. Recent evidence suggests that capsaicin induces apoptosis and inhibits cell proliferation, although this has not been extensively investigated in haematological malignancies. The aims of this study were to: 1) investigate the whether capsaicin kills human haematological malignant cells, and if so, 2) whether this action was TRPV1-mediated.

Methods. THP-1 (acute monocytic leukaemia), U266B1 (myeloma) and U937 (histiocytic lymphoma) cells were exposed to increasing concentrations of capsaicin (8-1000 μM) in the presence and absence of TRPV1, and cannabinoid 1 and 2 receptor (CB1, CB2; 0.1-100 μM) antagonists. Cell metabolic activity (indicative of viability) was measured after 24hrs using the alamarBlue® method (resazurin reduction assay).

Results. Capsaicin reduced viable THP-1, U266B1 and U937 cell numbers in a concentration-dependant manner. A biphasic effect was observed on THP-1 cells [EC_{50} and IC_{50} (95% CI) = 32.9 (19.9-54.3) and 219 (144-246) μM]. SB452533 and AM251 (100 μM) suppressed the capsaicin-induced increase in THP-1 cell activity ($P < 0.001$). U266B1 cells were more resistant to capsaicin than THP-1 and U937 cells. Cell activity was significantly inhibited by capsaicin in U937 compared to U266 cells (IC_{50} : 197 vs. 431 μM , respectively, $P < 0.008$). AM251 and SB452533 appeared to act as partial agonists and displayed a synergistic effect with capsaicin in U937 cells.

Discussion. THP-1, U266B1 and U937 cells responded differently to capsaicin. TRPV1, CB1 and CB2 antagonists did not affect capsaicin-induced changes in U266B1 cell activity although CB1 and CB2 receptors appeared to mediate an increase in cell activity in THP-1. We conclude that capsaicin inhibits the viability of haematological malignant cells through a non-TRPV1-dependent mechanism.

Investigation of activation mechanism of TRPA1 ion channel

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Introduction. The Transient receptor potential ion channel TRPA1 is expressed in a subset of nociceptive neurons. It can be activated by noxious cold and by pungent electrophilic compounds like allyl isothiocyanate (AITC), the pungent component of wasabi and mustard oil and non-reactive compounds such as menthol (Story GM et al. 2003). Recent studies demonstrated that electrophilic compounds activate TRPA1 by covalent binding to the N-terminal cysteine (C) and lysine (K) residues (Hinman et al., 2006; Macpherson et al., 2007).

Aims. The aim of this study is to further investigate the role of the implicated N-terminal C and K residues of in the activation of TRPA1 by both electrophilic and non-reactive compounds.

Methods. Cell lines expressing human TRPA1 or mutants of the N-terminal C and K residues were generated in TRex HEK293 cells (Invitrogen). Intracellular calcium imaging assays using FURA2 in a plate-reading fluorimeter (FlexStation 3, Molecular Devices) were used to characterize responses to compounds.

Results. Our data suggest that the N-terminal C and K residues are important for activation of TRPA1 by both electrophilic and non-electrophilic compounds. K708 is crucial for menthol activation.

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Hinman, A et al. (2006) TRP channel activation by reversible covalent modification. *Proc Natl Acad Sci U S A*, 103, 19564-8.

Macpherson LJ et al. (2007) Noxious compounds activate TRPA1 ion channels through covalent modification of cysteines. *Nature*, 445, 541-5

A methanolic extract of propolis collected from the Australian native stingless bee, *Tetragonula carbonaria*, scavenges free radicals and inhibits 5-lipoxygenase activity *in vitro*

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Introduction. Propolis is a resinous material produced by bees from plant exudates, beeswax and salivary secretions. Studies conducted on global sources of propolis have indicated its anti-inflammatory and anti-oxidant effects (Araujo et al, 2012; Kumazawa et al, 2004); however, similar properties within propolis collected from Australian native stingless bees (*Tetragonula carbonaria*) have not been extensively studied.

Aims. To examine the potential of a *T. carbonaria* propolis extract to scavenge free radical species and inhibit 5-lipoxygenase activity *in vitro*.

Methods. Propolis collected from 40 *T. carbonaria* hives in South-East Queensland was homogenized and extracted in 2:1 methanol:hexane. The methanolic extract was dried and reconstituted for use in bioassays. Anti-oxidant activity of the extract (1-500 µg/mL in methanol) was assayed colorimetrically using 1,1-diphenyl-2-picrylhydrazyl (DPPH; 100 µmol/L), and by HPLC analysis of the extract (2 mg/mL in acetonitrile) spiked with 2,2'-azobis-2-methyl-propanimidamide (AAPH; 80 mg/mL; 40°C; 8 h). The effect of the extract (1-500 µg/mL in dimethyl sulfoxide) on the oxidation of linoleic acid by 5-lipoxygenase was examined using a cell-free colorimetric assay and by examination of Michaelis-Menten kinetics.

Results. A methanolic extract of *T. carbonaria* propolis dose-dependently scavenged DPPH ($EC_{50}=27.0\pm2.34$ µg/mL; $n=3$). HPLC analysis of the AAPH-spiked extract revealed several polar compounds potentially responsible for this bioactivity. The extract also inhibited 5-lipoxygenase activity in a dose-dependent manner ($IC_{50}=70.0\pm19.84$ µg/mL; $n=3$). Linoleic acid oxidation by 5-lipoxygenase ($K_m=115.0\pm7.34$ µmol/L; $V_{max}=0.08\pm0.006$ absorbance units/min) was significantly altered in the presence of 100 µg/mL extract ($K_m=71.3\pm10.35$ µmol/L; $V_{max}=0.04\pm0.002$ absorbance units/min; $n=3$; $P<0.05$), suggesting mixed enzyme inhibition. Solvent controls had no activity in any assay.

Discussion. A polar extract of Australian native stingless bee propolis displayed free radical-scavenging activity and mixed inhibition of 5-lipoxygenase activity *in vitro*. Further bioactivity-guided fractionation and chemical analysis of the extract are required to isolate and identify the constituent compounds responsible for these properties.

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Kumazawa S et al (2004) Food Chem 84:329-339

Opioid inhibition of cAMP in an optimised cell based assay

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Introduction. Opioid peptides are capable of providing efficacious analgesia through their interaction with opioid receptors; activation of opioid receptors changes cell excitability through a number of downstream mechanisms. A major mechanistic pathway involves the inhibition of adenylyl cyclase which leads to decreased levels of cyclic adenosine monophosphate (cAMP). Therefore, evaluation of cAMP inhibition potencies of peptides can provide an insight to their analgesic potencies.

Aims. This study was aimed at optimising the experimental conditions for the determination of cAMP inhibition in a cell based system.

Methods. Beta endorphin (β -END) was used as a prototypical opioid peptide agonist and the study utilised a cAMP Alphascreen method (PerkinElmer) and cultured HEK cells overexpressing mu opioid receptors. The cell concentration and extent of activation by forskolin were examined and then the cAMP inhibition potencies of β -END and fentanyl were assessed. Finally, opioid specificity was confirmed by blockade with the non-selective opioid antagonist naloxone.

Results. 20,000 cells/well, 30 µM forskolin and 30 min incubation time were found to be the optimal conditions for cAMP assay. Fentanyl and β -END both inhibited forskolin increased cAMP with IC_{50} values of 0.01µM and 0.2µM respectively. Prior incubation with 100 µM of naloxone prevented the cAMP inhibition of both β -END and fentanyl.

Discussion: This study describes an efficient and accurate method for the comparison of both exogenous and endogenous opioid efficacy in a cell-based system. Fentanyl is a clinically utilised mu opioid agonist whilst β -END is one of the most ubiquitous endogenous peptides. The optimised assay conditions determined in this study allowed for the comparison of β -END potency with that of fentanyl and for the demonstration of opioid receptor specificity.

Activity of a novel alpha-conotoxin LsIA, isolated from *Conus limpusi*

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LsIA is an α -conotoxin that has been isolated and purified from the crude venom of the vermivorous conesail, *Conus limpusi*, found off the coast of south east Queensland, Australia. LsIA contains a 4/7 cysteine motif which is common among α -conotoxins and the Ser-Xaa-Pro-Xaa motif associated with this family of conopeptides. Taking into account the lineage of α -conotoxins it would be expected that LsIA antagonises neuronal nicotinic acetylcholine receptors (nAChRs). This α -conotoxin inhibits rat $\alpha_3\beta_2$ and α_7 nAChRs. Interestingly, native LsIA does not appear to block human $\alpha_3\beta_2$ while the analogue [R10N]LsIA gains activity at human nAChRs. Molecular modelling of the extracellular binding domain of the $\alpha_3\beta_2$ nAChR in conjunction with data from Everhart et al has revealed that a single amino acid situated just outside the C-loop may be responsible for the species specificity of certain α -conotoxins with activity at nAChRs. This finding is crucial when considering the potential development of these peptides for the treatment of diseases such as neuropathic pain.

Assessment of two-pore channels in MDA-MB-231 breast cancer cells.

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Introduction. Two-pore channels (TPCs) are calcium release channels localized to the endolysosomal system. The binding of the intracellular second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) to TPC1 or TPC2 releases calcium from endolysosomal calcium stores (Ruas et al, 2010). This suggests possible roles for these channels in calcium-dependent processes, including cell death. TPCs in breast cancer have not yet been assessed, despite reports of altered calcium signaling in breast cancer cells via other calcium channels.

Aims. To evaluate the potential roles of TPC1 and TPC2 in MDA-MB-231 breast cancer cells.

Methods. TPC1 and TPC2 silencing was performed using ON-TARGET^{plus} SMARTpool siRNAs and knockdown was confirmed using real time RT-PCR. MDA-MB-231 cells were treated with ABT-263, ionomycin or ceramide to induce cell death. Cells were stained with Hoechst 33342 and propidium iodide and then assessed using a high content imaging system (ImageXpress) to evaluate the consequences of TPC1 or TPC2 silencing on cell death.

Results. More than 75% knockdown was achieved for TPC1 or TPC2 in MDA-MB-231 cells. Silencing of TPC1 or TPC2 gene expression significantly attenuated ABT-induced cell death (3 and 10 μ M ABT-263; $P < 0.05$). In contrast, ionomycin-induced cell death was significantly augmented by TPC1 or TPC2 silencing (3 μ M Ionomycin; $P < 0.05$). However, TPC1 or TPC2 silencing did not alter ceramide-induced cell death at both submaximal (30 μ M) and maximal (100 μ M) concentrations.

Discussion. These data indicate that TPC1 and TPC2 may play a role in modulating cell death pathways. Future work is required to define the possible mechanisms involved.

Ruas M et al (2010) Curr Biol 20:703-9

TRPV4 channels in basal-like breast cancer cells

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Introduction. TRPV4 is a polymodal non-selective cation channel. TRPV4 is involved in processes including osmoregulation, mechanosensation and thermoregulation. Despite the established roles of other TRP channels in cancers (such as TRPV6), the role of TRPV4 in breast cancer has not been fully assessed.

Aims. 1) To assess TRPV4 mRNA levels in a panel of basal breast cancer cell lines; 2) to evaluate changes in TRPV4 mRNA levels with epithelial to mesenchymal transition (EMT) - a process implicated in cancer metastasis, in breast cancer cells; and 3) to assess the effects of activating and silencing TRPV4 on Ca²⁺ signalling in MDA-MB-468 breast cancer cells.

Methods. Real-time RT-PCR was used to assess TRPV4 mRNA levels in HCC1569, MDA-MB-468 and MDA-MB-231 basal-like breast cancer cells. Changes in TRPV4 levels associated with EMT were assessed using epidermal growth factor (EGF, 50 ng/mL) treatment for 48 h to induce EMT in MDA-MB-468 cells. Fluorescent imaging plate reader (FLIPR) Ca²⁺ assays were used to assess the effect of a TRPV4 pharmacological activator GSK1016790A (30 nM) in MDA-MB-468 cells. GSK1016790A-induced Ca²⁺ influx was assessed in MDA-MB-468 cells 72 h post-transfection with TRPV4 siRNA.

Results. TRPV4 mRNA levels were highest in MDA-MB-468 cells, followed by HCC1569 and MDA-MB-231 cells. Real-time RT-PCR analysis revealed no change in TRPV4 mRNA during EGF-induced EMT. Stimulation with GSK1016790A (30 nM) induced a robust and sustained Ca²⁺ influx in MDA-MB-468 cells. A dose-response curve of GSK1016790A in MDA-MB-468 cells showed an EC50 of approximately 3.9 nM. TRPV4 silencing in MDA-MB-468 cells decreased GSK1016790A-induced Ca²⁺ influx.

Discussion. MDA-MB-468 cells exhibit GSK1016790A-mediated Ca²⁺ influx that is sensitive to TRPV4 silencing. These results suggest that TRPV4-mediated Ca²⁺ influx is a feature of some breast cancer cells.

A novel analytical approach reveals a distinct pattern of stimulus bias for an antipsychotic drug at the dopamine D2 receptor

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Introduction. Most antipsychotics antagonize the dopamine D₂ receptor (D2R) subtype as their main mechanism of action. Unfortunately, this antagonism also leads to extrapyramidal side effects. More current development of antipsychotics is focused on D₂-selective partial agonists. Although several D₂-selective partial agonists have been tested for the treatment of schizophrenia, only aripiprazole has made it to the market so far. It remains unclear why certain partial agonists are efficacious as a treatment for schizophrenia whereas others are not. One emerging hypothesis is that aripiprazole displays 'stimulus bias', that is, it promotes unique conformations of the D2R that signal selectively via certain signalling pathways and not others, and that this bias underlies its unique antipsychotic activity.

Aims. To determine if aripiprazole displays stimulus bias at the D2R and if it has a distinct pattern of bias from partial agonists with limited anti-psychotic efficacy such as S-3PPP.

Methods. The effect of various D2R ligands with known *in vivo* efficacy and their derivatives on both the inhibition of forskolin-induced cAMP production and the phosphorylation of ERK1/2 was determined using FlpIn CHO cells expressing the D2R. Data was analyzed using a method based upon the Black and Leff operational model of agonism to allow the quantification of stimulus bias (Kenakin *et al.*, 2011).

Results. Aripiprazole is approximately 30-fold more biased towards the inhibition of cAMP production than towards ERK1/2 phosphorylation as compared to the typical D2R agonists dopamine or ropinirole and the partial agonist S-3PPP.

Discussion. Our novel analytical approaches have revealed that the antipsychotic aripiprazole displays a distinct pattern of stimulus bias as compared to the endogenous agonist dopamine or S-3PPP. This observation will provide a foundation for future studies aimed at relating *in vitro* activity with *in vivo* efficacy.

Kenakin T *et al* (2011) ACS chemical neuroscience 3(3): 193-203

Development of a real-time, fluorescence based assay of mu-opioid receptor mediated inhibition of adenylate cyclase activity in Chinese hamster ovary cells

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Introduction. Activation of the mu-opioid receptor (MOR) leads to inhibition of adenylyl cyclase (AC) and reduction of cyclic adenosine monophosphate (cAMP) levels. Modulation of AC activity is frequently used as an assay for measuring opioid ligand potency and efficacy. Most currently used AC assays are single time point, require multiple reagents and/or cell lysis, with lengthy development times.

Aims. To develop a simple, fluorescence based assay of MOR-mediated AC inhibition in Chinese hamster ovary (CHO) cells.

Methods: CHOK1 cells stably expressing human MOR were grown in 96-well microplates. Membrane potential was measured using the Molecular Devices FLIPR membrane potential dye. Dye emission intensity increases on membrane depolarization and decreases upon hyperpolarization.

Results. Treatment of CHO cells with the AC activator forskolin (FSK) resulted in membrane hyperpolarization. Forskolin hyperpolarized cells with a pEC₅₀ of 7.3±0.1 to a maximum of 52±2% from baseline. The hyperpolarization induced by FSK (300nM) was inhibited in a dose-dependent manner by the opioid agonists [D-Ala²,N-MePhe⁴,Gly-ol]-enkephalin (DAMGO, E_{max} 57±3%, pEC₅₀ 7.8±0.1), morphine (E_{max} 58±9%, pEC₅₀ 7.1±0.3) and buprenorphine (E_{max} 22±4%, pEC₅₀ 9.0±0.6). The effects of opioids were prevented by treatment with pertussis toxin (200ng/mL overnight), and blocked by naloxone (1µM). The FSK hyperpolarization was mimicked by Sp-8-CPT-cAMPS (100µM), a direct activator of protein kinase A. The FSK hyperpolarization was not blocked by K channel inhibitors tetraethylammonium chloride (10mM), 4-aminopyridine (300µM), glibenclamide (10µM), VU-591 (100µM) or charybdotoxin (100nM). However, increasing extracellular K⁺ from 2.5mM to 30mM and 75mM decreased the FSK response from 52±2% to 26±2 % and 1±0.2%, respectively, implying the involvement of a K channel.

Discussion. This assay is a novel method for rapid, no-wash, real-time measurement of AC inhibition by MOR ligands in intact CHO cells, which may be suitable for high-throughput screening.

In vitro assessment of ligand-mediated µ-opioid receptor interaction with G protein and β-arrestin 2

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Introduction. Strong opioid analgesics such as morphine are the mainstay for the relief of moderate to severe nociceptive pain but they also produce adverse effects including respiratory depression, nausea, vomiting, sedation and constipation. Although most clinically used opioid analgesics produce analgesia through activation of the µ-opioid (MOP) receptor, there are between-opioid differences within individuals with respect to efficacy and tolerability. Recently, ligand-mediated preferential signalling at (µ) MOP, δ (DOP) and κ (KOP) receptors via their molecular transducers has been proposed to contribute to between-opioid differences within individuals in analgesia and tolerability observed in the clinical setting (Molinari et al, 2010).

Aims. To investigate the extent to which various opioid ligands promote coupling of the MOP receptor to β-arrestin 2 and G protein.

Methods. Bioluminescence Resonance Energy Transfer (BRET) was used to assess opioid-mediated coupling of the MOP receptor to either β-arrestin 2 or G protein. Human embryonic kidney 293 (HEK293) cells co-transfected with chimeric plasmids expressing *Renilla* luciferase-tagged opioid receptors with either green fluorescent protein-tagged β-arrestin 2 or the Gβ₁ subunit. Various opioid ligands were added to transfected cells, and the resulting BRET signals were measured using a luminometer.

Results. Opioid ligands exhibit differential agonist and antagonist signaling via β-arrestin 2 and G protein interactions at the MOP receptor.

Discussion. *In vitro* profiling of the extent to which opioid analgesics induce biased signaling via β-arrestin 2 and G protein-coupled pathways has the potential to enhance knowledge of signalling pathways that are linked to analgesia rather than adverse effects.

Molinari P et al (2010) J Biol Chem 285:122522-122535

Taking advantage of kinetic data: an alternative approach to obtain affinity estimates from GPCR-mediated intracellular calcium mobilization.

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Introduction. G protein-coupled receptors (GPCRs) represent common therapeutic targets. Over the last decade, the primary high throughput-screening assays used for drug discovery have been calcium mobilization assays, which provide kinetic data at a resolution of 1-2 seconds. Despite the widespread use, the rapid and dynamic nature of calcium assays often introduce non-equilibrium artifacts upon the derivation of key pharmacological parameters for drug discovery, including antagonist equilibrium dissociation constants (Charlton & Vauquelin, 2010).

Aim. To investigate the influence of agonist and antagonist exposure time on human M₃ muscarinic acetylcholine receptor (M₃ mAChR) mediated calcium mobilization.

Methods. CHO cells stably expressing the human M₃ mAChR (M₃-CHO) were incubated in loading buffer containing Fluo-4AM. Cells were then washed and agonist-mediated changes in fluorescence measured every 1.52 seconds in the absence or presence of antagonist pre-treatment (30 minutes) or simultaneous addition.

Results. In M₃-CHO cells, the inhibition of agonist- (oxotremorine-M, carbachol, pilocarpine and bethanecol) mediated calcium mobilization by atropine (100 nM), NMS (10 nM) clidinium (100 nM) and ipratropium (100 nM) varied with both agonist and antagonist exposure time. However as a function of time, the change in agonist potency in the presence of antagonist was agonist independent and reached a common plateau that correlated well with previous equilibrium estimates of corresponding antagonist equilibrium dissociation constants.

Discussion. Kinetic analysis provides an alternative method to define antagonist equilibrium dissociation constants from non-equilibrium calcium mobilization data. Employing a kinetic analysis can generate robust affinity estimates and as such assist GPCR drug discovery programs.

Charlton & Vauquelin (2010) Br J Pharmacol, 161:1250-1265

The efficacy of kappa-opioid receptor agonists in pain and inflammation

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Introduction. The κ -opioid receptor (KOR) agonists have received increasing interest for the treatment of pain, due to their reduced side effect profile, reduced addiction and anti-inflammatory properties.

Aims. This study aimed to examine the endogenous KOR agonist Dynorphin 1-17 and the synthetic KOR agonist U50488H efficacy at the KOR receptor, in a unilateral model of pain and anti-inflammatory properties.

Methods. HEK-fKOP cells were stimulated to produce cAMP with L-858501 (300 μ M), followed by treatment with increasing concentrations of the KOR agonists. Intracellular cAMP levels measured by alphascreen. Male Wistar rats paws were unilaterally treated with FCA (150 μ L). After 4 days the inflamed paw was administered agonists (500 μ M, 50 μ L/paw) and paw withdrawal measured with an analgesimeter. A monocyte cell line (THP-1) were differentiated to macrophages with PMA (50nM) for 48h, and then treated with KOR agonists at increasing concentrations for 2 h, followed by LPS (1 μ g/ml). Media was collected after 4 h and analysed for IL-1 β by alphascreen.

Results. U50488H and Dynorphin 1-17 inhibited cAMP production, with an IC₅₀ of 2.7nM and 0.2nM respectively. U50488H resulted in a significantly increased paw withdrawal threshold in the inflamed paw, but this was not seen with equimolar concentrations of Dynorphin 1-17. Both U50488H and Dynorphin 1-17 produced a concentration dependent inhibition of IL-1 β release from THP-1 cells.

Discussion. U50488H and Dynorphin 1-17 potently activate the KOR receptor, but only U50488H inhibited nociception in the inflamed paw. This is likely due to increased metabolism of Dynorphin 1-17 as we described previously for inflamed tissue. The dose-dependent inhibition of TNF- α and IL-1 with U50488H has previously been described, and this study further substantiates those findings.

Anti-inflammatory kinase inhibitors attenuate invasiveness and chemoresistance of glioblastoma cells

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Introduction. Glioblastoma (GBM) is a fatal brain tumour. An important factor that drives the invasion of transformed cells, including human glioblastoma, is the development of an inflammatory microenvironment. Chronic inflammation in combination with infiltrated macrophages and oncogenic mutations contribute to up-regulate tumour-promoting cytokines which support migration, invasiveness and chemotherapy resistance in carcinogenesis. p38 mitogen-activated protein kinase (MAPK) plays a critical role in the development and amplification of inflammation accompanying various CNS disorders. However, it is yet unclear whether elevated inflammatory cytokine secretion and p38 MAPK activity are potential risk factors for the expansion of GBMs.

Aim: To determine the function of p38 MAPK signaling pathway in GBM pathophysiology.

Methods: p38 MAPK inhibitors were tested in multiple inflammation, migration, invasion and proliferation assays employing primary human microglia and GBM cells. We also tested p38 MAPK inhibitors in glioblastoma cells carrying epidermal growth factor receptor vIII mutation (EGFRvIII) as approximately 40-50% of glioblastomas are characterized by EGFR gene amplification and mutation; all of which are implicated in tumour growth, invasion and poor response to therapy.

Results: Inhibition of p38 MAPK pathway efficiently reduced the formation of an inflammatory glioblastoma microenvironment and attenuated aggressive phenotype of GBM cells. Investigation of the molecular mechanism revealed that inhibition of the p38 MAPK downstream kinase MK2 and regulation of HuR nuclear-cytoplasm shuttling are responsible for the anti-inflammatory activity. Importantly, anti-inflammatory kinase inhibitors significantly restored chemosensitivity of GBM cells to temozolomide, the standard chemotherapeutic used in GBM therapy.

Discussion: Together, our data suggest that regulation of inflammatory GBM microenvironment with anti-inflammatory kinase inhibitors may represent a useful approach to improve the current management of this serious disease. Development of follow-up kinase inhibitors and investigation of MK2 in human GBM specimens will also be discussed.

Yeung YT *et al* (2012) J Neurooncol 109:35-44

Yeung YT *et al* (2012) Br J Pharmacol doi: 10.1111/bph.12008

Assessing the cytotoxicity of natural products on squamous cell carcinoma and human keratinocyte cell lines

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Introduction. *In-vitro* cell culture studies are valuable tools for screening chemo-preventive potential of natural products, which is receiving increasing attention due to continuing increase in new cancer cases as well as cancer deaths, and the cost and side effects of current radiation or chemotherapy. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay quantifies viable cells in proliferation and cytotoxicity studies. Although the method is rapid and convenient, various parameters have been identified that can result in an under- or over-estimation of the tested drugs. Therefore, it is essential to refine the assay parameters for tested cell lines in order to have accurate and reliable results.

Aims. To establish parameters for cytotoxicity studies of natural products using MTT assay on cancerous and non-cancerous skin epithelial cells.

Methods. We used squamous cell carcinoma (SCC25) and non-cancerous human keratinocyte (HaCaT) cell lines and MTT assay with 595-nm reading in a 96-well plate format. We optimised cell seeding densities, serum content of media; determined the maximum solvent concentration and identified a suitable positive control for the assay.

Results. The optimised method required different seeding cell numbers for two cell lines and different serum concentrations for the duration of the assay in order to reach interpretable optical densities (0.4-0.8) at the end of experiment. With the objective of testing natural extracts prepared in aqueous ethanol, we determined that the optimal, final concentration of ethanol in media is 0.3%. Epigallocatechin-3-gallate (EGCG) was used as positive control and exhibited significantly more toxicity in SCC25 than in HaCaT. Control wells with medium only added with EGCG revealed the absence of intrinsic reducing activity of EGCG in our optimised assay.

Discussion. The method that we have refined can be applied to screening extracts from natural products selectively to identify candidates with anti-proliferative effects on SCC25 cancer but not on HaCaT non-cancer cell line.

Alteration of SKBR3 cancer cell proliferation by silencing specific calcium pumps, channels and channel modulators.

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Introduction. HER2-positive breast cancers are characterised by an overexpression of the growth factor receptor HER2 and represent about 20% of all breast cancers. Calcium transporters and modulators have been studied in several types of cancers, including breast cancer and their altered expression may contribute to the development and progression of some breast tumours. However, the expression and the effect of silencing of calcium transporters and modulators have not been fully evaluated in HER2-positive cells, such as SKBR3 cells.

Aims. To evaluate the effect of silencing of calcium transporters and modulators on the proliferation of SKBR3 cells.

Methods. Dharmacon siRNA was used to individually silence 16 calcium pumps, channels and channel modulators in SKBR3 cells. The effect on cell proliferation (144 h post siRNA) was evaluated using EdU-Alexa Fluor[®] 555 staining (Life Technologies) and high content imaging. Real time RT-PCR was used to confirm the silencing of the transporters and to evaluate their expression level.

Results. In SKBR3 cells there are greater levels of the stromal interaction molecule 1 (STIM1) mRNA compared to its related isoform STIM2; whereas two pore channel (TPC2) mRNA levels are lower compared to TPC1. Silencing of STIM1 and TPC2 significantly decreased the proliferation of SKBR3 cells. Calcium transporters such as plasma membrane calcium ATPase (PMCA) isoforms 1 and 4, secretory pathway calcium-ATPase (SPCA) isoforms 1 and 2 and the transient receptor potential (TRP) cation channel TRPV6 showed relatively high mRNA levels in SKBR3 cells, but silencing of these channels and pumps did not affect the proliferation of SKBR3 cells.

Discussion. These studies suggest that the proliferation of SKBR3 breast cancer cells is decreased by STIM1 and TPC2 silencing. Thus, STIM1 and TPC2 should be the focus of further investigation in HER2-positive breast cancers.

Immunohistochemical analysis of PMCA2 expression in normal and malignant human breast tissues

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Introduction. PMCA2 is an isoform of the plasma membrane Ca^{2+} ATPase that pumps Ca^{2+} from the cytosol into the extracellular space. Expression of PMCA2 is significantly increased in mouse mammary glands during lactation where it plays a major role in the excretion of Ca^{2+} into milk; however PMCA2 expression has not been assessed in human mammary glands during lactation. In breast cancer cells PMCA2 over-expression reduces apoptosis, but its assessment in breast cancer is still limited.

Aims. To compare PMCA2 expression in normal breast tissue and breast tissue exhibiting lactational change and; to assess PMCA2 expression in human malignant breast samples.

Methods. Formalin-fixed, paraffin-embedded samples of non-cancerous and malignant breast tissues assembled into tissue microarrays were assessed for PMCA2 by immunohistochemistry using a rabbit anti-PMCA2 ATPase polyclonal antibody. Samples which showed membranous staining were considered PMCA2 positive.

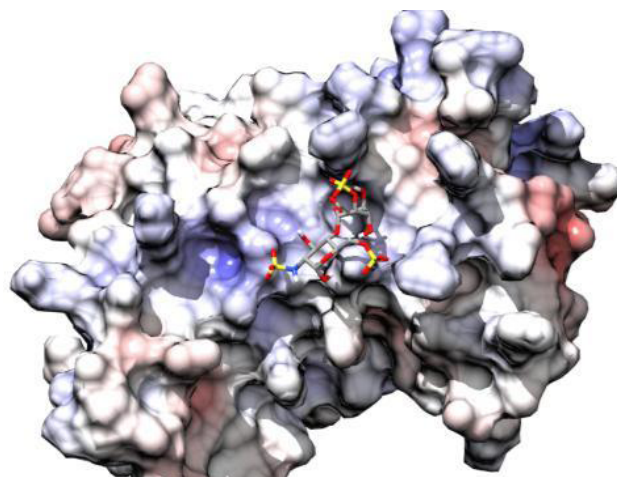
Results. Membranous PMCA2 expression was observed in luminal epithelium of breast tissue exhibiting lactational change. PMCA2 expression was observed in 9 of 96 breast tumours (9.4%). These preliminary studies suggest that there is no obvious significant correlation with estrogen, progesterone or HER2 receptor status. Our results also identify that PMCA2 expression is not lost during lymph node metastasis.

Discussion. The findings indicate that PMCA2 is up regulated in human lactation and is a feature of some breast cancers. Inhibitors of PMCA2 may represent a therapeutic strategy for women with breast cancers that overexpress this Ca^{2+} pump.

Prediction of the GAG-binding interactions of four-helical cytokines

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Cytokines are regulators of intracellular communication that orchestrate virtually every response to infection, injury and inflammation. The interactions of cytokines with cell surface carbohydrates known as glycosaminoglycans (GAGs) play a pivotal role in the activation of cytokine-mediated biological responses. While an increasing number of cytokines have been shown to bind specifically to GAGs, little is known about the structural specificities of the underlying interactions. Understanding the GAG-binding properties of cytokines is ultimately the cytokine activation process and realizing its therapeutic potential. Here we report a combined approach using the analysis of evolutionary amino acid conservation, the computation of electrostatic potential molecular surfaces and molecular docking simulations of representative small heparin fragments, all of which have been used to predict potential GAG-binding sites on helical cytokines. Specific amino acid residues forming potential heparin-binding sites were identified for several cytokines, including interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), granulocyte colony stimulating factor (G-CSF), prolactin (PRL), erythropoietin (EPO), thrombopoietin (TPO) and granulocyte colony stimulating factor (GM-CSF). Three basic amino acids (arginine (Arg), lysine (Lys) and, to a lesser extent histidine (His)) were found to constitute the GAG binding sites and mediate the interactions with small heparin fragments, with the predicted free energies of binding ranging from -1 to -10 kcal/mol.



Investigating the mechanism of RXFP1 activation

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Introduction. The cognate receptor for relaxin is RXFP1, a G protein coupled receptor (GPCR) containing a large extracellular domain composed of a leucine rich repeat (LRR) region and an N-terminal low density lipoprotein receptor type A (LDLrA) module. The current hypothesis of ligand mediated RXFP1 activation is that a receptor homodimer is formed, with one receptor binding relaxin and in turn activating the second receptor in the dimer in a *transactivation* manner, possibly via the LDLrA module.

Aims. To test this hypothesis, co-expression of two different inactive RXFP1 mutant receptors, binding deficient (BD) and signalling deficient (SD), was performed to test if such co-expression could reconstitute normal relaxin-stimulated cellular responses, hence indicating transactivation.

Methods. Flow-cytometry was used to measure cell surface expression of transiently transfected mutant receptors in HEK293T cells, and this technique was adapted for FACS selection of cell lines that highly express these receptors. Inactive BD1 receptor (RXFP1-E277Q/D279N) was transfected into the cell lines highly expressing the semi-active SD1 (RXFP1-D451N) or SD3 (RXFP1-Y511G/Y599A) receptors, and relaxin-stimulated cellular responses were measured using plate-based CRE reporter gene assays as well as direct cAMP measurements.

Results. As expected, stimulation of BD1 or SD1 receptors alone with relaxin did not result in cAMP accumulation. The co-expression of BD1 and SD receptor variants did not reconstitute normal relaxin-stimulated cellular responses, however there was a small *decrease* in the efficacy of relaxin-stimulated cellular responses for semi-active SD variants when co-expressed with BD1.

Discussion. No clear evidence of RXFP1 transactivation was observed in these studies, however it is possible that the decrease in efficacy of relaxin on partially active SD variants indicated a dominant-negative effect that would support the transactivation hypothesis. There are many alternative mechanisms by which transactivation may be occurring, and the SD/BD model may have been inadequate to show this.

Medication safety issues in older Australians: results from a national medicines census

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Introduction. The use of medicines, including complementary medicines (CMs), for the prevention/treatment of disease is common. Information is limited, however, on patterns of medicines use, including concurrent use of multiple medicines, prevalence and types of medication safety issues, and users' reported responses to such issues.

Aims. To examine: the prevalence of concurrent use of medicines; prevalence and types of medication safety issues; self-reported response to medicines-related problems in Australians aged ≥ 50 years.

Methods. Cross-sectional study involving a questionnaire posted to a random sample of 4500 Australians aged ≥ 50 years, selected from the Australian electoral roll, between June 2009 and February 2010. Data were collected, using a pre-piloted 24-hour medicines diary, on self-reported medicines' use and experiences of suspected ADRs (sADRs). A potential drug interactions (PDIs) list was developed from scientific literature and reviewed by pharmacists.

Results. Response rate: 37.3%. A majority of respondents (87.1%, 95%CI:85.3-88.6%) had taken one or more medicines in the previous 24 hours. Of these, 5.5% were exposed to a PDI involving conventional medicines only, and 12.3% a PDI involving a medicines combination (including CMs). Of all respondents, 43.3% (95%CI:41.0-45.8%) had used ≥ 5 medicines and 10.7% (95%CI:9.3-12.3%) ≥ 10 medicines in the previous 24 hours; of these, 8.3% and 20.1%, respectively, were exposed to a PDI involving conventional medicines only. Overall, 18.4% (95%CI:16.2%-20.8%) of medicines users experienced sADRs in the previous year. In response, 76.4% of these informed their doctor about their last sADR and 38.9% stopped taking medicines.

Discussion. The prevalence of use of medicines, including concurrent use of multiple medicines, among Australians aged ≥ 50 years is high. A substantial proportion experiences sADRs and/or is exposed to potentially interacting medicines. These findings emphasise the need for open communication between health professionals and patients about all medicines used, including CMs, and for vigilance regarding ADRs, including drug interactions.

Study of natural health product adverse reactions (SONAR): active surveillance of adverse events following concurrent natural health product and prescription medicine use in community pharmacies

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Introduction. Many consumers use natural health products (NHPs) concurrently with prescription medicines. As NHP-related harms are under-reported through passive surveillance, the safety of concurrent NHP-prescription medicine use remains unknown.

Aims. To conduct active surveillance in participating community pharmacies to identify adverse events following concurrent NHP-prescription medicine use.

Methods. Participating pharmacy staff from ten community pharmacies in the Greater Toronto Area, Ontario, Canada, asked individuals collecting prescription medicines about (i) concurrent NHP-prescription medicine use in the previous three months and (ii) experiences of adverse events. If an adverse event was identified and if the patient provided written consent, a research pharmacist conducted a guided telephone interview to gather further, detailed information after also obtaining (and documenting receipt of) verbal consent. A study committee, comprising an NHP expert, a clinical expert, and a clinician with expertise in pharmacology and NHPs, assessed AE reports using accepted algorithms and the World Health Organization (WHO) causality assessment criteria to determine: (i) the likelihood of a causal relationship; (ii) the likelihood of an NHP-drug interaction; (iii) the need for pharmaceutical analysis of NHP(s) concerned.

Results. Over a total of 112 pharmacy weeks, 2615 patients were screened, of whom 1037 (39.7%; 95%CI: 37.8-41.5%) reported concurrent NHP and prescription medicine use. A total of 77 patients reported a possible AE (2.94%; 95%CI: 2.4-3.7%), which represents 7.4% of those using NHPs and prescription medicines concurrently (95%CI: 6.0-9.2%). Of 15 patients available for an interview, 4 (26.7%; 95%CI: 4.3- 49.0%) reported an AE that was determined to be "probably" due to NHP use, including concurrent use with prescription medicines.

Discussion. Although not without challenges, active surveillance of NHPs, particularly where used concurrently with prescription medicines, in the community pharmacy setting is feasible and can generate adverse event data of sufficient quality to allow causality assessment of potential harms.

The impact of medications on older people with falls

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Introduction. Medication exposure, defined as polypharmacy (≥ 5 medications) or exposure to Falls Risk Increasing Drugs (FRIDs, cardiovascular or psychoactive medicines) has been associated with poor outcomes in older adults with falls. However, there is limited evidence in relation to medication use including drug-drug interactions (DDIs) and clinically important drug-disease interactions (DDSI), and adverse outcomes in frail adults with falls.

Aims. To investigate medication exposures (polypharmacy, FRIDs, DDIs and DDSI) and falls in robust and frail hospitalised patients.

Methods. Patients aged over 60 years admitted with a fall to Royal North Shore Hospital (RNSH) were recruited. Clinical and medication data was collected on admission, discharge and at 2 months. Adverse outcomes were defined as rehospitalisation, falls, institutionalisation, and death.

Results. 129 patients were recruited (mean age 81.5 ± 7.8) with 59 robust and 70 frail. On admission, frail patients had significantly higher rates of polypharmacy (frail 68%, $p=0.001$), DDSIs (54%, $p=0.002$), DDIs (36%, $p=0.001$) and mean (\pm SD) FRID number (4 ± 2 , $p=0.01$) compared to the robust. Between admission and discharge, no significant change was seen in medication exposure. At 2 months, FRIDs were significantly correlated with adverse outcomes (1.8; 1.2-2.6). Adjusted ORs revealed no significant relationship between outcomes and polypharmacy (1.5; 0.5-4.3), DDIs (1.0; 0.8-1.4) or DDSIs (2.9; 0.95-8.9).

Discussion: Frail patients had higher medication exposure compared to the robust. FRID use was significantly correlated with adverse outcomes.

An essential medicines list for children: Are they still therapeutic orphans?

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Introduction. The Essential Medicines List (EML) is produced biennially by the World Health Organisation (WHO). The minimum medicine needs for a basic health care system are listed, including the most efficacious, safe and cost effective medicines for priority conditions. From its inception in 1997, the list had a focus on Adult medicines although there were some paediatric medicines listed. Goal 4 of the millennium development goals aimed to reduce child mortality; hence, there was a need to focus on medicines for children.

Aims. The aims were to assess the current state of the EML in regards to children; implement a policy of increasing paediatric medicines on the list and then reassess whether the policy had been effective.

Results. After assessing the 2003 EML, the WHO requested that we assess the 2005 list in which we identified 77 medicines on the EML relevant to children but with no paediatric formulation (PF) listed. After a series of meetings, the WHO established a subcommittee of the EML specifically for children's medicines as well as a children's EML (EMLc) in 2007. The subcommittee recommended medicines for children be added to the EML; these needing to be ratified by the full EML committee before addition to the EMLc. After 3 iterations of the list by 2011 some progress had been made with 23 medicines for children being added since the original assessment and over 310 medicines listed on the new EMLc (see Table).

Discussion. While there has been a significant improvement with the availability of EMLc, more formulations development is needed for children's medicines.

Category (Use in Children)	2005 – Number Missing	2011 – Number Added
Drug approved, PF existed	29	20
Drug approved, PF not existing	31	1
Drug not approved, PF not existing *1 Drug not approved but PF existed	17*	2

EML. http://whqlibdoc.who.int/hq/2011/a95053_eng.pdf

The ABCD of Clinical Pharmacokinetics

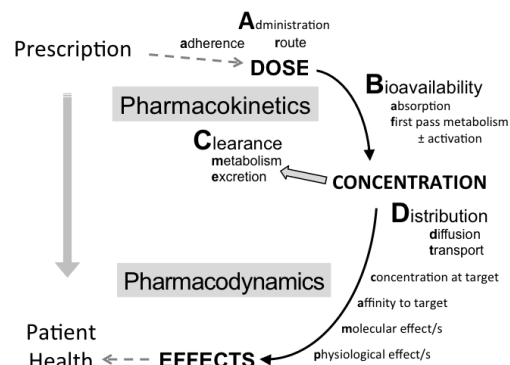
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Introduction. ADME is the acronym for *absorption, distribution, metabolism and excretion* that has described pharmacokinetics for 50 years.

Aims. To review the historical origin of ADME; to critically appraise current uses of ADME; and to present a new acronym for teaching clinical pharmacokinetics.

Discussion. ADME was first proposed by Nelson in 1961, rephrasing *resorption, distribution, consumption and elimination* used by Teorell in 1937. Drug: entering the body (A), moving about the body (D), changing within the body (M), and leaving the body (E). Over time the use of ADME has diversified according to the needs of the user, in particular for mechanisms: crossing the gut wall (A), movement between compartments (D), mechanisms of metabolism (M), excretion or elimination (E), and transport (T) is sometimes added. Variable use of ADME often causes confusion.

In teaching pharmacokinetic principles we follow the active drug moiety through the body in space and time using the schematic shown. We use the acronym ABCD, standing for *administration, bioavailability, clearance and distribution*. Administration is factors relating to dosing and adherence. Bioavailability is the active drug moiety arriving in the systemic circulation (Nelson's A). Clearance is the active drug leaving the systemic circulation (ME). Distribution is to the site/s of action (D). **Conclusion.** ADME is an old friend that has served pharmacokinetics well, but it has quirks that sometimes make teaching pharmacokinetic principles difficult. Seventy-five years after Teorell, ABCD is an alternative acronym for teaching clinical pharmacokinetics.



A population pharmacokinetic (popPK) model to describe the renal clearance and the effect of transporter genetic variants on the pharmacokinetics of metformin in patients with type 2 diabetes mellitus (T2DM).

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Introduction. Metformin is used in the treatment of T2DM despite large between-subject variability (BSV) in the PK. An effect of SNPs (OCT1, OCT2, OCT3, MATE1 and PMAT) on the apparent clearance (CL/F) of metformin has been reported, however, an effect on the renal clearance of metformin (CL_R) has not been investigated in patients with T2DM.

Aims. To investigate CL_R and effect of SNPs on CL_R in patients with T2DM.

Methods. Patients with T2DM (n=21) were recruited. Intensive blood sampling (8–10 samples) and urinary output was collected in 16 patients over a dosage interval (for immediate-release, IR, 12 h; extended-release, XR, 24 h). Sparse samples were collected from the remaining 5 patients (8 blood samples, 6 weeks) with the collection of timed (2–5 h) urine samples. A 2-compartment model was developed using NONMEM with parameters for apparent non-renal clearance (CL_{NR}/F), renal clearance (CL_R), oral availability (F), first-order absorption (k_a) for IR and zero-order clearance (CL_{CR}) and genotype (52 SNPs), were evaluated as covariates of the PK parameters.

Results. The mean and BSV of the PK parameters included: CL_{NR}/F, 24.6 L/h (57%); CL_R, 27.3 L/h (19%); V_C/F, 180 L (45%); F, 1 (57%). The fractional elimination via the renal route ranged from 11% to 80%. Only the measured urinary CL_{CR} was a significant covariate for CL_R. None of the SNPs was a significant covariate for CL_R.

Discussion. It would appear that the large variability in metformin PK is due to variable oral availability and variable excretion of metformin via the renal route. None of the SNPs were significant covariates for CL_R. More patient data are required to detect the covariate-effect of variant transporters.

Population pharmacokinetics of Factor VIII in paediatric patients.

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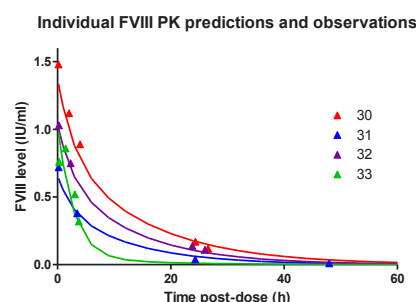
Introduction. The pharmacokinetics of Factor VIII (FVIII) in children with haemophilia A (HA) has been poorly characterised due to the requirement of intensive blood sampling in conventional studies. Despite this, knowledge of individual FVIII pharmacokinetics (PK) in prophylaxis is critical for maintaining a trough level of FVIII for prevention of haemarthrosis in haemophilic patients.

Aims. To produce a population pharmacokinetic model of FVIII in children based on a sparse sampling schedule, and to use this model to determine optimal doses in an individually-tailored fashion.

Methods. Sparse FVIII concentration data from four patients with HA at the Royal Children's Hospital was combined with rich data from 20 paediatric patients in a previous FVIII PK study, and analysed using the population PK software, NONMEM.

Results. Body weight, baseline von Willebrand's Factor (VWF), and baseline FVIII inhibitor level were all identified as patient characteristics significantly affecting FVIII CL or V. Doses calculated based on NONMEM estimates for the new RCH patients were able to be minimised, to a $43.75 \pm 15.73\%$ ($n=$, $p<??$) reduction in annual FVIII use.

Discussion. The identification of VWF as a significant covariate of FVIII clearance is a novel finding that may explain to a large degree variations in FVIII PK previously unexplained by other population PK studies, and will have implications in the treatment of patients with haemophilia A. The minimisation of FVIII dosage in these four patients is a promising starting point for increased cost-effectiveness in the use of an expensive treatment.



The pharmacokinetics of paracetamol and its metabolites: A population approach to investigating hepatic intrinsic clearance in old age.

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Introduction. Paracetamol can be used as a marker of intrinsic clearance by the liver. Investigating its pharmacokinetics in older people will provide important information about liver function in older age, which may be applied to other drugs metabolized in similar ways.

Aims. The aim of this study was to determine the impact of frailty on the pharmacokinetics of paracetamol and its metabolites in older inpatients.

Methods. Data from two studies were pooled; one conducted in healthy volunteers with intensive plasma sampling and the other an observational study of inpatients over 70 years old, who had sparse samples taken. Both the paracetamol glucuronide and sulfide metabolites were measured for each sample along with the parent drug concentration using HPLC. Population pharmacokinetic analysis was undertaken using NONMEM (version 7.2).

Results. The total study population was 219; 20 healthy volunteers and 199 inpatients. The average age of the volunteers was 35.7 years and the inpatients was 84.7 years. There were 139 frail patients and 61 non-frail. The best model was a one-compartment linear pharmacokinetic oral model with interindividual variability on all parameters. There was high variability in both populations.

Discussion. Frailty will be used to investigate the variability in the pharmacokinetics of paracetamol in the older patients. Decreasing variability in the model will allow for more predictable therapeutic outcomes in older people.

Baseline plasma urate: the only determinant for dosage selection of allopurinol.

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Introduction. Allopurinol is the most common treatment for gout and acts by lowering urate synthesis through xanthine-oxidoreductase inhibition. Many patients continue to experience acute gout attacks despite, apparently, adhering to allopurinol therapy. Lower doses are recommended in patients with impaired renal function but this approach often fails to lower plasma urate concentrations sufficiently.

Aim. To predict the optimum dosage of allopurinol in patients with gout.

Methods. Gouty patients (n=46) were treated with increasing doses of allopurinol ranging from 50-600 mg/day. Plasma urate and creatinine concentrations were measured at baseline and during treatment. The plasma concentrations of oxypurinol were measured in 26 of these patients. All urate concentrations were fitted to a dose-response model (Figure 1) by non-linear regression (R software version 2.15.0).

$$U_T = \left(1 - \frac{D}{ID_{50} + D}\right) * (U_P - U_R) + U_R$$

Results. Plasma concentrations of urate during treatment of allopurinol (U_T) were highly dependent on the dose of allopurinol (D) and the baseline plasma urate concentration (U_P). The best-fit estimates (mean, 95% CI) of the dose that inhibits 50% of urate concentrations (ID_{50}) and the apparent resistant plasma concentration of urate (U_R) were 226 mg (167-303) and 0.20 mmol/L (0.14-0.25), respectively. The addition of creatinine clearance (CL_{CR} , normalised to 120 mL/min) as a covariate did not improve the fit ($P=0.09$). In the subset of 26 patients, replacement of D with oxypurinol concentrations did not improve the fit (residual sum of squares = 0.18).

Discussion. Higher baseline plasma urate concentrations require higher doses in order to reach sufficiently low urate concentrations. The determinant of the final dosing rate of allopurinol is the baseline plasma urate concentration and is independent of renal function.

Optimising ribavirin therapy for hepatitis C patients.

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Introduction. Hepatitis C Virus (HCV) is treated with a combination therapy of ribavirin and pegylated-interferon. Increasing concentrations of ribavirin increase sustained virological response (SVR) rates while also increasing the risk of haemolytic anaemia. Hence, therapeutic drug monitoring has been recommended to individualise dosing regimens and achieve therapeutic drug concentrations.

Aim. To construct and validate a Bayesian model to predict day 28 plasma concentrations of ribavirin from either day 7 or day 14 plasma concentrations of ribavirin.

Methods. A population pharmacokinetic model was constructed (Kinetica version 5.0) using clinical data from Caucasian and Japanese populations. The model parameters were incorporated into the Bayesian forecasting software, Abbottbase. Plasma ribavirin concentrations were measured by HPLC in HCV patients (n=10) treated with ribavirin and pegylated-interferon at day 0, 7, 14 and 28 of therapy and were entered into Abbottbase. The day 28 AUC (observed) was then calculated. Trough and 2 h post-dose concentrations for day 7 or day 14 (n=10), were used to predict the AUC on day 28. Bias and precision were calculated for observed versus predicted AUC.

Results. A two-compartment population pharmacokinetic model provided the best fit to the ribavirin plasma concentrations. The mean (CV%) CL , V_1 and V_2 were 7.2 L/h (31%), 380 L (31%) 5917 L (40%), respectively. The observed versus predicted AUC at day 28 indicated a bias and precision of 3.3% and 15.7% from the data at day 7. Bias and precision were 2.2 % and 12.3% from the data at day 14.

Discussion. The bias and precision indicate that the constructed model is sufficiently accurate to predict dose regimens from plasma concentrations taken on day 7 or 14. Application of this approach should result in early dose adjustment which may improve SVR rates while minimizing toxicity.

High dose intravenous paracetamol in major surgical patients

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Introduction. Inadequate control of pain postoperatively leads to prolonged recovery time and increased complication risks. Safety of unlicensed supratherapeutic paracetamol has not been established although stat doses $\leq 90\text{mg/kg}$ following molar extraction¹ and 8g/day for 3 days have been shown to be safe in healthy adults^(2,3).

Aim. To examine the safety of unlicensed supratherapeutic doses of paracetamol in major surgical patients.

Methods. Irish Medicines Board granted ethical approval. Patients were recruited into one of four groups: 1.5gq4h open bowel resection or; 1g q6h open bowel resection, laparoscopic bowel resection or mastectomy. Daily blood samples were drawn for AST, ALT, αGST , INR, paracetamol and metabolites. Urinary metabolite ratio of paracetamol's main metabolites was assessed.

Results. 33 patients were recruited. There was no overt evidence of hepatotoxicity occurring in any patient during the study period, although towards the end of the period, there was evidence that liver enzymes in some patients were beginning to change slightly. This was not reflected in any changes of αGST , a sensitive marker of liver damage. Paracetamol disposition was shown to change, however half life remained relatively constant. There were a number of changes in paracetamol's metabolism, with increased glucuronide and sulphate metabolite production following surgery that maintained this rate of elimination.

Discussion. Doses $\leq 9\text{g/day}$ given to major surgical patients for ≤ 5 days postoperatively produced no evidence of hepatotoxicity. Changes to paracetamol metabolic ratios indicate underlying changes to the mechanism of metabolism. The clinical utility of higher doses of postoperatively needs to be established.

Zacharias M et al (2007) *Anaesth Intensive Care* Aug;35(4):544-9

GelotteCK et al. (2003) *Journal of ClinTox.* 41(5):726

Gelotte CK (2007) *ClinPharmacolTher*81(6):840-8.

Dosage adjustment of medications in patients with renal impairment: how consistent are drug information sources?

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Introduction. Renal insufficiency and advancing age decrease the glomerular filtration rate (GFR), which requires the dosage individualisation of many drugs to prevent adverse events. However, a significant percentage of patients with renal disease are administered inappropriately high doses of drugs (Markota et al, 2009). Lack of quantitative data in the available drug information sources and inconsistency in dosing information may augment the problem of dosing error.

Aims. To determine the consistency among five drug information sources regarding the dosing recommendations provided for drugs considered problematic in patients with renal impairment.

Methods. All five drug information sources viz. British National Formulary, American Hospital Formulary System Drug Information, Australian Medicines Handbook, Medical Information Management System, and Drug Prescribing in Renal Failure (published by American College of Physicians) were reviewed and information on recommendations for dosage adjustment in renal impairment was extracted and analysed for 61 drugs recommended as requiring caution (Veterans' MATES, 2012).

Results. Recommendations tended to vary in the information sources. Only moderate agreement between the sources was observed (inter-rater reliability: Fleiss Kappa: 0.3; average pairwise percent agreement: 67%). Qualitative data were not well defined and there was lack of consistency in quantitative values. Drugs including glibenclamide, metformin and codeine were marked as contraindicated in one source but not mentioned in others. Also, drugs considered as not requiring dosage adjustment in one source (e.g. teriparatide, candesartan and bupropion) had explicit recommendations in other sources.

Discussion. There should be an evidence-based approach on drug dosage adjustment in order to bring uniformity to the recommendations. Regular updating of the content of the drug information sources is also important.

Markota NP et al (2009) *Journal of Nephrology* 22(4):497

Veterans' MATES (2012) www.veteransmates.net.au/VeteransMATES_TherBrief.pdf

Development and validation of software to calculate the drug burden index: a pilot study

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Introduction. The Drug Burden Index (DBI), a novel pharmacologic risk assessment tool that measures an individual's total exposure to anticholinergic and sedative medicines, has been associated with impaired physical and cognitive function, frailty, falls and increased hospitalisation in older adults.

Aims. (1) To develop software which calculates and generates reports on DBI; (2) To use data from fictitious patients to test this DBI software for accuracy.

Methods. Based on previous work by Dr Sepehr Shakib, Microsoft Access 2010 was used to build and design The DBI Calculator©. Fictitious patient data was collected from the Scenarios of the Disease State Management Education Sections of the Australian Journal of Pharmacy (Aug 2010 to Aug 2012). The fictitious data was used to calculate DBI using the manual calculation and The DBI Calculator©. Cohen's Kappa statistics were used to calculate the degree of concordance between manual and automated DBI scores.

Results. The software has been designed with a front and a back end; the front end allows the user to enter data, the back end allows for data management and analysis. The software design allows for ease of uploading onto a secured, de-identified, password-protected website. The user enters patient data and clicks "Calculate DBI" which immediately reports the DBI with the significance of the calculation for the patient. Preliminary results indicate good agreement between the software and manual calculation (Cohen's Kappa 0.81) of the 16/25 fictitious patient scenarios used with DBI>0.

Discussion. We have developed a reliable calculator to report DBI in older patients taking multiple medications. Further studies will assess usability and application of The DBI Calculator© in clinical settings such as Home Medicine Reviews.

Hilmer SN et al (2007) Arch Intern Med 167:781-7.

Identifying effective strategies to prevent drug-drug interactions in hospital: a user-centered approach

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Introduction. Drug-drug interactions (DDIs) are an important, yet preventable cause of medication errors in hospitals. Research has shown that doctors and pharmacists are often unable to recognise potential DDIs. To prevent DDIs, numerous strategies, including alert systems, reference sources, and personalised prescriber feedback have been implemented worldwide. These strategies are rarely evaluated and are typically implemented without input from the individuals using those strategies.

Aims. To ascertain the most appropriate mechanism to prevent DDIs in a hospital, as viewed by users.

Methods. Eight drug safety experts and 18 doctors took part in semi-structured interviews. Participants were asked about their confidence in identifying DDIs, their opinion on currently used strategies, as well as their views on possible future strategies to prevent DDIs. Interviews continued until saturation of themes was achieved. Transcripts were analysed and coded to identify key themes and compare groups.

Results. Reference sources and ward-pharmacists were consistently consulted as current DDI prevention strategies, however users also identified multiple limitations with these strategies. No doctors reported being completely confident in identifying dangerous DDIs and junior doctors were less confident in their ability than senior doctors. Most doctors thought that alert systems would be an effective strategy to implement. This was because alerts would highlight potential problems in situations where individuals were too busy, or not cognisant of the possibility of DDIs.

Discussion. The lack of confidence by doctors regarding DDIs suggests that a strategy which doesn't rely on individuals seeking the information out for themselves would be most appropriate at this site. While the literature has identified numerous problems with the implementation of DDI alerts, this study showed that users were receptive to the idea. By involving users in DDI strategy design we expect greater policy adherence and satisfaction.

Ropivacaine concentrations (total and unbound) following transverse abdominis plane (TAP) block for analgesia following abdominal surgery – A pilot study.

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Introduction. The use of local anaesthetics for analgesia following a range of surgical procedures has been minimally investigated in patients where protein binding may have changed as a result of surgical stress.

Aim. To evaluate the safety of single bolus dosing of ropivacaine (ROP) followed by continuous infusion of ROP via TAP block catheter following abdominal surgery.

Methods. Towards the end of surgery, 12 patients received 20mL of 0.5% ROP as a single bolus on both sides followed by 10mL/h of 0.2% ROP infusion for 48h bilaterally, starting 30min post bolus. Serial blood samples were drawn pre-dose, post bolus at 15, 30, 45, 60min and 2, 4, 8, 12, 16, 24 and 48h; and analyzed for total and unbound plasma ROP concentrations by HPLC/UV. AAG concentrations were measured by ELISA in selected samples. Patients were monitored for any signs and symptoms of CNS or CVS toxicity (including serial ECG's).

Results. Following the bolus dose, mean (\pm SD) peak (C_{max}) total and unbound ROP concentrations were 2.04 (\pm 0.81) mg/L and 0.044 (\pm 0.016) mg/L, respectively. Mean time to peak (t_{max}) was 18.75 (15-30) min. During the ensuing infusion phase, total and unbound ROP concentrations rose to a mean (\pm SD) C_{max} of 3.3 (\pm 1.79) mg/L and 0.065 (\pm 0.041) mg/L, respectively.

Discussion. Although total ROP concentrations approached or exceeded reported neurotoxicity thresholds (Knudsen et al, 1997), no patients had unbound concentrations approaching the unbound toxicity threshold, nor showed any signs or symptoms of toxicity or ECG changes. This was consistent with increases in AAG concentrations in most patients. Hence there is scope to consider using higher ROP dosages with safety in patients to maximise therapeutic benefit, despite toxicity data derived from healthy volunteers with normal protein binding.

Knudsen K et al (1997) Br J Anaesth 78:507-14

Impact of pharmacist-led dosing of vancomycin and aminoglycosides in hospitalised patients

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Introduction. Aminoglycosides and vancomycin are commonly used antibiotics and display narrow therapeutic indices and inter-patient-variable pharmacokinetics. Therefore, hospital practice is to perform dose individualisation and therapeutic drug monitoring (TDM) with the aim of achieving and maintaining therapeutic concentrations. TDM and dosing is primarily the responsibility of the prescriber, with variable input from clinical pharmacists.

Aim. To determine the impact of pharmacist-led dosing for vancomycin and aminoglycosides across a multisite health service.

Methods. A pre- and post-intervention cohort study was conducted (150 patients pre and post). The intervention involves clinical pharmacists ordering drug-levels and independently adjusting doses once therapy has been initiated by the treating medical team. Clinical pharmacists were trained, assessed and accredited to provide the service 7-days per week. Endpoints included the time to first therapeutic level; proportion of patients reaching therapeutic level and the appropriateness of TDM (a composite measure of correct timing of levels and subsequent dosage adjustments).

Results. 300 patients were included (100 vancomycin and 50 aminoglycoside courses pre and post-intervention). Patient characteristics, duration of therapy, indication and number of levels were similar across the pre- and post-cohorts. For patients receiving vancomycin the time to first therapeutic level was significantly less for the pharmacist-led dosing group (1.9 vs 2.6 days, $p=0.008$); the proportion of patients ever reaching therapeutic levels was similar (77% for pharmacist-led vs 72% for physician-led, $p=0.41$); the proportion of dosing adjustments fully compliant with specific criteria in the guideline was significantly greater in the pharmacist-led dosing group (60% vs 50%, $p=0.004$).

Discussion. The pharmacist-led service has resulted in a significant improvement in the time to reach therapeutic level and correct dose adjustments. This is now an established service, provided as a routine component of clinical pharmacists' practice and is a model for further expansion.

Development of a population PKPD model to describe the effect of paracetamol on the International Normalised Ratio (INR).

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Introduction. Paracetamol is one the most common substances taken in overdose. (Reith et al, 2009) Paracetamol may increase International Normalised Ratio (INR) in paracetamol poisoning without hepatic injury by reducing functional factor VII. (Whyte et al, 2000)

Aims. The aim of this study was to develop a population model to describe the PKPD of paracetamol and its effect on INR.

Methods. A total of 167 patients were included in the dataset (31 paracetamol overdose patients, 9 control overdose patients, 20 cross-over clinical trial patients, 107 retrospective paracetamol overdose patients); 63 were men, the median age (range) was 22 years (13–71). A structural population PKPD model was developed in Phoenix® NLME™. 167 patients contributed a total of 907 paracetamol plasma and INR observations.

Results. The pharmacokinetics of paracetamol were best described by a 1-compartment model with first order input and linear disposition. A modified baseline E_{max} model described the effect of paracetamol on INR by inhibition of the activation of vitamin K-dependent coagulation factors. The population mean estimates ± SE (CV%) of the pharmacokinetic parameters volume of distribution, clearance, absorption rate constant (K_a), and lag time (t_{lag}) were 10.6 ± 1.8 (17) L, 2.3 ± 0.3 (13) L·h⁻¹, 3.1 ± 5.3 (172) h⁻¹, and 0.2 ± 0.1 (36) h, respectively. The population estimates ± SE (CV%) of the pharmacodynamic parameters E_{max} and EC₅₀ were 0.4 ± 0.1 (30) (increase in INR) and 512 ± 256 (50) μM, respectively. An additive residual error model was used. Covariates investigated in the final model included age, sex, and treatment with N-acetylcysteine (NAC).

Discussion. Preliminary findings demonstrated that the model adequately estimated population PKPD parameters for paracetamol and provided the basis for covariate analyses.

Reith D et al (2009) Clin Exp Pharmacol Physiol 36(1):35-42.

Whyte IM et al (2000) Ther Drug Monit 22(6):742-8.

Assessment of frailty and prescribing criteria in older people: A systematic review

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Introduction: Rational prescribing (RP) in frail older people is complex and difficult. In this group, there is limited evidence on effectiveness of medication, drug pharmacokinetics and pharmacodynamics differ from younger people and multiple co-morbidities with higher risk of adverse drug events are more likely (Couteur et al, 2004). Various criteria have been developed to measure inappropriate prescribing but their applicability to frail older population is uncertain (Gnjidic & Hilmer, 2010).

Aims: The primary aim of this systematic review was to identify studies describing the use of frailty measures for evaluating inappropriate prescribing in those aged 65 and older. The secondary goal was to address the missing parameters in the prescribing tools to increase their utility for frail individuals.

Methods: A search was conducted in PubMed and EMBASE (1990- 2011). Original studies written in English that utilized frailty assessment and criteria to evaluate inappropriate prescribing in frail individuals were included. Excluded are: studies of specific drugs or groups of drugs and of particular disease conditions.

Results: Ten of 573 studies met the inclusion criteria. All papers measured certain parameters of frailty, such as *performance based tests*, *measures of co-morbidity*, etc. Six studies used the Beers criteria to explicitly identify inappropriateness while Medication Appropriateness Index (MAI); an implicit criteria was used by two studies and combination of both in other two studies.

Discussion: Although some parameters of frailty measurement have been used, there appears a need of more user-friendly and detailed criteria for assessing frailty in older individuals. Prescribing tools should address both *medication* and *patient* related factors such as life expectancy and functional status to minimize inappropriate prescribing in frail individuals.

Couteur et al, (2004), Aust Fam Physician, 33(10): 777-81

Gnjidic D, Hilmer SN, (2010), Aging Health, 6(6):705-16

Allopurinol hypersensitivity: an examination of all published cases, 1950-2011

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Introduction. Allopurinol is the primary therapy for chronic gout. Utilization has increased with the growing prevalence of gout, thus exposing more patients to the risk of allopurinol hypersensitivity (AH), a rare, idiosyncratic hypersensitivity reaction that can be fatal.

Aims. To review all cases of AH in order to better understand the risk factors contributing to this syndrome.

Methods. A literature search (Medline and Embase; 1950-2011) was performed to identify all cases of AH which either fulfilled the diagnostic criteria of Singer & Wallace (S&W) or reported cutaneous involvement alone.

Results. A total of 653 patients with AH were identified; 575 fulfilled the S&W criteria while 78 had only mild cutaneous involvement. Data was often incomplete. Hence, the following results reflect the fractions of the subsets of the population for whom data were available. The majority (315/387; 81%) of patients were Asian. Allopurinol was prescribed for non-approved indications (asymptomatic hyperuricaemia and acute gout) in 211/317 (66%) patients. The most common (117/239; 49%) allopurinol dose was 300 mg/day (range 10-1000 mg/day). Approximately 90% (342/377) developed AH within 75 days of commencing allopurinol. The major S&W manifestations of cutaneous reaction (rash, Erythema Multiforme (EM), Stevens-Johnson Syndrome (SJS) or Toxic Epidermal Necrolysis (TEN)), worsening renal function and acute hepatocellular injury were present in 560/575 (97%), 258/575 (45%) and 238/575 (41%) patients, respectively. Minor S&W manifestations of fever, eosinophilia and leukocytosis occurred in 273/575 (47%), 218/575 (38%) and 142/575 (25%) patients, respectively. The majority (228/323; 70%) were treated with corticosteroids. Overall mortality rate was 15% (93/599). Patients with severe cutaneous manifestations (EM, SJS or TEN) had an increased mortality rate (45/139; 32%).

Discussion. Asian ethnicity appears to confer a greater risk of AH. Many cases of AH could have been avoided if allopurinol had been prescribed appropriately.

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Association between age, lean body weight, frailty and inducible SIRT1 expression response to sera: the CHAMP study

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Introduction. Tissue SIRT1 expression and activity increase with caloric restriction and often decrease in old age. Sera from either middle age rats or humans subjected to caloric restriction increase SIRT1 expression in cultured cells. This suggests that there are circulating factors that influence SIRT1 expression and potentially correlate with ageing and age related health.

Aims. To determine the effect of frailty in older people on such circulating factors.

Methods. We measured SIRT1 expression in cells induced by culture in presence of sera from nested group of subjects from CHAMP, a study of community dwelling males greater than 70 years of age.

Results. Inducible SIRT1 expression was not different between frail and robust subjects. However subjects with expression values in the lowest quartile of values were likely to be frail and had higher lean body weight.

Discussion. This suggest that low expression of SIRT1 induced by sera is paradoxically associated with some markers of better health in older men.