

# Posters

## Monday 14 July

### Drug safety and toxicology

#### P001

##### **A comparative study on the cytotoxic effects of tanshinones isolated from *Salvia miltiorrhiza* on HepG2 cells**

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Tanshinones are diterpene quinones isolated from the roots of *Radix Salvia miltiorrhiza* (Danshen), a traditional Chinese medicine used in the treatment of cardiovascular diseases and recently reported to exhibit anti-tumour effects (Wang *et al.*, 2007). The anti-tumour potential of Danshen was investigated by studying the cytotoxicity of the major diterpenes, including tanshinone IIA, cryptotanshinone, tanshinone I and dihydrotanshinone on a human hepato-carcinoma HepG2 cell line in this study. *In vitro* cytotoxicity of tanshinones on HepG2 cells was determined by MTT assay and LDH leakage assay. The roles of caspases in tanshinones-mediated apoptosis were evaluated by Western blot and flow cytometric analysis. Generation of reactive oxygen species (ROS) was monitored by flow cytometry and enzymatic assay. Results from MTT assay showed that the tanshinones inhibited HepG2 cell growth in a concentration-dependent manner, with IC<sub>50</sub> at 25.1, 10.4, 3.7 and 1.3 µM for cryptotanshinone, tanshinone IIA, tanshinones I and dihydrotanshinone, respectively. LDH leakage assay showed that dihydrotanshinone caused necrosis in a wider concentration range (3.13–100 µM), while the others were effective at 25 µM or above. Flow cytometric analysis showed that all tanshinones caused significant apoptotic cell death, which involved the activation of caspase 3, caspase 8, and caspase 9, and cleavage of PARP as revealed by Western blot. The PARP cleavage was inhibited by caspase inhibitors such as Z-VAD-FMK, Z-IETD-FMK and Z-LEHD-FMK. Among the tanshinones studied, only dihydrotanshinone and tanshinone I caused oxidative stress (decrease in GSH/GSSG ratio) in the HepG2 cells. Cytometric analysis indicated the incidence of intracellular ROS generation occurred at 15 and 60 min. The involvement of ROS was confirmed when N-acetyl cysteine (10 mM) pre-treatment suppressed dihydrotanshinone-induced apoptosis. This study showed that the tanshinones induced apoptosis of HepG2 cells with different effectiveness and possibly through slightly different mechanism(s). The mechanisms and anti-tumour potential of these tanshinones thus merit further investigations.

##### **Reference:**

Wang X *et al.* Med Res Rev. 2007; 27: 133–148.

#### P002

##### **Influence of chronic exercise on the rewarding effect of amphetamine**

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The dopaminergic mesocorticolimbic and nigrostriatal systems are considered to be involved in reward-related associative learning, reinforcement, incentive salience and habit-like drug seeking, contributing to the development of drug dependence. The conditioned place preference experimental procedure is based on theoretical formulations of Pavlovian conditioning and despite some limitations provides unique information about the rewarding effect of contextual cues associated with a drug stimulus, being the most popular model to study motivational effects of drug and non-drug treatments in experimental animals. Using this procedure the aim of the present study is to verify if chronic pre-exposure to treadmill running modulates the mechanism of amphetamine addiction. Adult male Sprague-Dawley rats (200–300 g) were randomly separated in two groups: with and without chronic exercise (6–10 per group). Chronic exercise consisted in a 8 week treadmill running program, with increasing intensity. The conditioned place preference test was performed in both groups using the procedure and apparatus previously established by others, consisting of three phases: pre-conditioning, conditioning (8 consecutive days with 4 i.p. injections of 2 mg/kg amphetamine alternated with saline solution injections; each of the conditions associated with one of the compartments) and test phase (same conditions as the pre-conditioning). In pre-conditioning phase none of the animals presented a compartment preference, allowing us to use the totality of animals in the study. The group without exercise demonstrated reliable amphetamine-conditioned place preference ( $P < 0.01$ ). In the group with exercise the opposite was observed, a decreased amphetamine seeking behaviour, spending significantly more time in the compartment associated with the saline injection ( $P < 0.05$ ). These results are in accordance to recent studies with MDMA where a pre-exposure to a treadmill exercise decreased the later MDMA-associated compartment in this CPP paradigm (Chen *et al.*, 2008).

Supported by POCl/DES/60227/2004, FCT, Portugal

##### **Reference:**

Chen HI *et al.* Behav Brain Res. 2008; 187: 185–189.

#### P003

##### **Influence of d-methamphetamine and d-amphetamine on striatal dopamine in rats: a microdialysis study**

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d-Methamphetamine (METH) and d-amphetamine (AMPH) are psychostimulants with great abuse potential. These sympathomimetic drugs are both phenylethamines, METH being the N-methylated analogue of AMPH. Although METH is generally accepted to be more addictive and a more potent central stimulant than its analogues, there is scarce information on neurochemical differences in action between the two

drugs that could account for such differences. The aim of this work was to compare the acute dopamine dynamics induced by both drugs in the striatum, a major site for action for these stimulants. *In vivo* brain microdialysis coupled to high performance liquid chromatography with electrochemical detection (HPLC-ED) was used to measure dopamine (DA) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) release in the caudate-putamen (CPu) after intraperitoneal administration of 5 mg/kg AMPH or METH in freely-moving Sprague-Dawley rats ( $n = 5-7$ ), weighing 250–380 g. AMPH and METH raised CPu DA levels to a similar degree following an analogous time course. The peak effect induced by both was attained at *circa* 30 minutes and was not significantly different ( $C_{max-AMPH}$ :  $386 \pm 78$  vs.  $C_{max-METH}$ :  $250 \pm 42$  pg/30 µl;  $P = 0.12$ ). In addition the area under curve evoked by both stimulants was similar ( $AUC_{AMPH}$ :  $419 \pm 97$  vs.  $AUC_{METH}$ :  $311 \pm 62$  pg/30 µl/h;  $P = 0.35$ ). Consistently with the DA signal, both AMPH and METH decreased DOPAC levels in CPu to about 20% of the basal concentration, exhibiting a similar time course. This study clearly demonstrates that the two drugs show no differences in terms of changes in dopamine release and its pre-synaptic metabolism in the striatum. However, one cannot rule out possible neurochemical differences between AMPH and METH in other neurotransmitter pathways and/or in other brain regions.

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#### P004

##### **Pharmacodynamic study of lamotrigine in patients submitted to video electroencephalographic monitoring**

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Video-electroencephalographic (VEEG) monitoring is a crucial tool for presurgical evaluation. Drug reduction is often used as a seizure precipitation technique. The goal was to perform a pharmacodynamic evaluation of lamotrigine (LTG) based on correlations between seizures and LTG plasma levels, considering: (i) seizure type, (ii) co-medication profile and (iii) localization of epileptogenic focus (temporal vs. extratemporal). The study was conducted in 142 consecutive adult epileptic patients submitted to VEEG monitoring at the Coimbra University Hospital (CUH) (approved by Ethics Committee). LTG quantified by HPLC. PKS program (Abbott Diagnostics) was used to estimate ictal LTG serum levels. Four groups were established as follows: Group 1 (LTG + inducer agents); Group 2 (LTG + VPA); Group 3 (LTG + inducers and VPA); and Group 4 (LTG alone) 76 patients with partial epilepsy ( $n = 315$  seizures) were included in this analysis. Ictal LTG serum levels associated to extra-temporal epilepsy ( $n = 132$ ) were higher than those associated to temporal epilepsy ( $n = 179$ ) ( $P \leq 0.05$ ). Also, simple partial seizures seem to be associated to higher LTG levels, whereas lower LTG concentrations were mainly related to complex and generalized seizures. Furthermore, occurrences were associated to the lowest LTG serum concentrations in Group 1 ( $1.94 \pm 1.44$  mg/L). Secondary generalizations were often associated to the presence of an inducer such CBZ and related to patients who had extratemporal epilepsy. Although no clear relationship between secondary generalization and LTG plasma levels could be observed, significant differences related to simple and complex seizures with or without generalization were drawn.

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#### P005

##### **Lamotrigine in patients submitted to video electroencephalographic monitoring: effect of co-medication on seizure occurrence**

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More than 30% of epileptic people remain without seizure remission. These refractory patients are often considered to be submitted to video electroencephalographic (VEEG) monitoring. The aim of the present study was to perform a pharmacodynamic evaluation in 142 consecutive adult epileptic patients receiving lamotrigine (LTG) as add-on therapy and submitted to VEEG in the Coimbra University Hospital (CUH) [the local Ethics Committee of CUH approved this study]. Drug reduction was the methodology used to precipitate seizures in this setting. Correlations between seizures and serum levels was evaluated considering the: (i) co-medication profile and (ii) localization of epileptogenic focus (temporal vs. extratemporal). LTG was quantified by HPLC and CBZ and VPA were analysed by fluorescence polarization immunoassay. PKS program (Abbott Diagnostics) was used to estimate LTG, CBZ and VPA ictal serum levels. Only 76 patients presented partial epilepsy. Two groups were established according to the presence of CBZ ( $n = 33$ ) and VPA ( $n = 18$ ). Temporal epilepsy lobe was present in 21 and 13 patients in CBZ and VPA group, respectively. Neurological events seem to be mostly related to CBZ/VPA levels, since no significant changes were observed in LTG serum levels, before/after CBZ/VPA discontinuation. In addition, no differences were found in LTG levels between temporal and extratemporal epilepsy in both groups. It was clear that time to reach seizure was longer in LTG+VPA Group ( $76.4 \pm 49.3$  h) than that of LTG+CBZ ( $48.6 \pm 27.8$  h) ( $P \leq 0.05$ ). Therefore, the results suggest that a more favourable pharmacodynamic interaction could be attributable to the LTG+VPA association.

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## P006

**Evaluation of the effect of carbamazepine or valproic acid withdrawal on lamotrigine concentrations during video-electroencephalographic monitoring**

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Antiepileptic drug (AED) reduction may be used to precipitate seizures in medically refractory patients during video-electroencephalographic (VEEG) monitoring. Lamotrigine (LTG) was one of the first new generation AEDs to be introduced in Portugal (1994) for add-on therapy. However, inducing agents, as carbamazepine (CBZ), are known to increase the LTG clearance, while valproic acid (VPA) reduces the elimination of LTG. Furthermore, some authors suggested that a rapid tapering of concomitant CBZ may cause a compensatory effect on LTG half-life (Wang-Tilz *et al.*, 2005). The aim of the present study was to evaluate the effect of CBZ or VPA withdrawal in the LTG serum levels of those patients receiving LTG combined to CBZ or VPA therapy. The study has been performed in 80 adult epileptic patients submitted to VEEG monitoring in the Epilepsy Monitoring Unit of Coimbra University Hospital (CUH) (the local Ethics Committee of CUH approved this study and written informed consent was obtained from all patients). Patients were excluded if LTG dosage regimen suffered from any change during the study period. Accordingly, two groups were established: CBZ Group ( $n = 27$ ) and VPA Group ( $n = 22$ ). The analysis was accomplished by comparing dose normalized LTG trough serum levels during daily monitoring, by calculating the concentration-to-dose (C/D) ratio, in a 4-day period, after CBZ/VPA discontinuation. LTG was quantified by high-performance liquid chromatography (Castel-Branco *et al.*, 2001) and CBZ and VPA were analysed by fluorescence polarization immunoassay (Abbott Diagnostics Division). The C/D ratio obtained in Day 0 was compared to the values obtained in the three following days after drug withdrawal (Day 1, Day 2 and Day 3). No statistical differences were found during the study period in both treatment groups (Table 1). In conclusion, our results suggest no significant change in LTG serum levels during the first 3 days after inducer or inhibitor withdrawal.

Table 1. Comparison of LTG concentration (mg/L) normalized by dose (mg/kg/day) during the first 3 days after CBZ or VPA withdrawal

Day	LTG C/D ratio	
	CBZ withdrawal ( $n = 27$ )	VPA withdrawal ( $n = 22$ )
Day 0	0.54 ± 0.24	3.24 ± 0.98
Day 1	0.46 ± 0.22	3.01 ± 0.93
Day 2	0.48 ± 0.28	2.74 ± 0.99
Day 3	0.43 ± 0.23	2.66 ± 0.89
ANOVA	NS	NS

Results expressed as mean ± SD. NS, No statistical significant result.

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**References:**

Castel-Branco *et al.* J. Chromatogr. 2001; B 755: 119–127.  
Wang-Tilz *et al.* Eur. J. Neurol. 2005; 12: 280–288.

## P007

**In vitro iron chelation activity of selected polyphenolic compounds**

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Iron overload either caused by treatment of thalassemia or accompanying hereditary hemochromatosis represents a serious medical problem. Iron chelators used for the treatment have generally low clinical effectiveness due to poor compliance or low efficacy. The research of new iron chelators is therefore highly desired. In this pilot study, two flavonoids, two 4-methylcoumarins and two synthetic iron chelators from aroylhydrazone group were compared with effect of clinically used deferoxamine (DEF). Ferrous and total iron chelation activity was assessed by a simple spectrophotometric assay. In brief, potential iron chelators were mixed with ferrous or ferric ions in various ratios. The concentration of unbound iron was established by ferrozine at 562 nm. Because ferrozine colours specifically ferrous ions, in case of ferric ions, hydroxylamine 10 mM was used for their reduction. None of tested chelators was similarly effective ferrous chelator as compared with DEF. Interestingly, flavonoid apigenin was more active than synthetic chelators PIH and SIH. Both coumarins showed only negligible activity. On the contrary, ferric ions seemed to be chelated absolutely by synthetic iron chelators PIH and SIH in ratio 10:1, chelator : iron, respectively. DEF was more effective since its activity reaches 100% in ratio 1:1. The flavonoids rutin and apigenin together with 6,7-dihydroxy-4-methylcoumarin brought about significant ferric chelation while the acetic acid ethylester of 7,8-diacetoxy-4-methylcoumarin was again practically inactive. Iron overload is accompanied by oxidative stress which is exaggerated probably by excessive free iron. This study demonstrated that some flavonoids and 6,7-dihydroxy-4-methylcoumarin act as iron chelators in clinically relevant concentrations. Though their effects did not approach those of DEF and synthetic chelators, it should be taken in the consideration that additionally to their antioxidant properties they may be useful in pathological conditions associated with oxidative stress due to catalytic role of iron.

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## P008

**Anticancer effect of horse chestnut extract and escin**

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Natural products isolated from medicinal herbs have been the potential sources of novel anticancer drugs over the last few decades. Extracts of horse chestnut (*Aesculus hippocastanum*) seed (HCE) have been used in the treatment of chronic venous insufficiency, edema, and hemorrhoids. Recent studies suggest that horse chestnut and escin, a principal component of horse chestnut, may possess antiinflammatory, antihyaluronidase, and antihistamine properties. On the other hand, antiproliferative effect of horse chestnut have been studied only marginally. In the present work, we tested HCE and escin for their antiproliferative and proapoptotic effects on cancer cell lines. HCE and escin were obtained from CALENDULA (Slovakia). The cell proliferation was evaluated by MTT assay and anchorage-independent growth by colony forming assay (CFA). To understand the growth inhibitory effects, carcinoma cell lines (Jurkat, CEM, HeLa and MCF-7) were treated with various concentrations of HCE and escin. Cell cycle and apoptosis were analyzed by flow cytometry. Apoptosis was analyzed also by DNA fragmentation. Incubation of Jurkat, CEM, HeLa and MCF-7 cancer cells with HCE at 0.125 mg/mL for 72 h caused 38.8, 86.2, 79.6 and 59.6% reduction in cell survival. Cytotoxicity of escin was dose-dependent and incubation of Jurkat, CEM, HeLa and MCF-7 cancer cells at 0.031 mg/ml for 72 h caused 23.6, 55.3, 77.5 and 58.3% reduction in cell survival. CFA also confirmed growth-inhibitory effects of compounds studied. In HCE- and escin-treated cells we found significant increase in the fraction of cells with a sub-G<sub>0</sub>/G<sub>1</sub> DNA content, which is considered to be a marker of cell death by apoptosis. Apoptosis was also confirmed by the annexin V staining and DNA fragmentation. Our results suggest that HCE and escin suppresses cell proliferation and induces apoptosis in a dose-dependent manner in cancer cell lines. Our results suggest that both horse chestnut and escin may be useful candidate agents for cancer chemoprevention and treatment but further studies are warranted.

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## P009

**Effects of nickel on the adult rat brain Na<sup>+</sup>,K<sup>+</sup>-ATPase and Mg<sup>2+</sup>-ATPase activities: L-cysteine as a modulator**

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Nickel (Ni) is a heavy metal pollutant that is industrially applied in many forms and exerts multiple toxic effects. The aim of this study was to investigate the effect of L-cysteine (Cys, 7 mg/kg, a well-known heavy-metal-chelating-agent and antioxidant) on the alterations caused by Ni-administration (as NiCl<sub>2</sub>, 30 mg/kg) on the adult rat brain Na<sup>+</sup>,K<sup>+</sup>-ATPase and Mg<sup>2+</sup>-ATPase activities. Twenty-eight male Wistar rats were divided into four groups: A (saline-treated control), B (Ni), C (Cys), D (Ni and Cys). All rats were treated once daily with intraperitoneal injections of the tested compounds, for 1-week. The pre-mentioned biochemical parameters were measured spectrophotometrically in whole brain homogenates. Rats treated with Ni exhibited a significant reduction in brain Na<sup>+</sup>,K<sup>+</sup>-ATPase activity (−49%, B vs. A) that was not reversed by Cys-administration (−44%, D vs. A), while Cys (group C) also caused a significant inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase (−39%, C vs. A). Mg<sup>2+</sup>-ATPase activity was not affected by Ni, while Cys caused a significant decrease in the enzyme's activity (−17%, C vs. A; −17%, D vs. A). The observed inhibition of brain Na<sup>+</sup>,K<sup>+</sup>-ATPase due to high-dose exposure to Ni reflects a neurotoxic effect of this metal that might affect neuronal excitability and other crucial functions implicated in neurotransmission. This inhibition could be due to direct interaction of Ni with the enzyme or due to indirect inhibition of its activity via modulation of the ionic gradient maintenance. The co-administration of Cys could not (at least under the examined experimental conditions) reverse the Ni-induced inhibition of Na<sup>+</sup>,K<sup>+</sup>-ATPase, while it also inhibited Mg<sup>2+</sup>-ATPase (an enzyme functioning in order to maintain high intracellular Mg levels and participating in many important neuronal functions).

## P010

**Clinical and medication data collection for pregnancy risk assessment after inadvertent exposure**

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Limitations of the several teratogenic risk classification systems and bias problems in epidemiological teratogenic studies can be sources of errors and a possibility of confounding, causing mismanagement of pregnancies. Lack of vital clinical and medication data to guide decision making is an obstacle, and also limits case reports value. Reports properly described can provide useful signal of possible medication risks which might warrant further exploration. To support clinical decision, procedures must use methodological approaches to clinical questions assuming evidence based practice. The aim was to develop a standard operating procedures (SOP) for clinical data collection after inadvertent maternal medication exposure. SOP was developed using PICO model (Huang *et al.*, 2006) (Patient, Intervention, Comparison, Outcomes) and considering: 'Guideline on exposure to medicinal products during pregnancy: need for post-authorization data' (EMEA/CHMP/313666/2005); 'Reviewer guidance evaluating risk of drug exposure in human pregnancies' (FDA); 'Guidelines for Submitting Adverse Event Reports for Publica-

tion' (Kelly *et al.*, 2007). It was also based on physicians and pharmacists clinical experience. Data to be collected is integrated in categories related to the population, the mother or the foetus, and to the medication or other exposition factors, according to PICO model. The elements relevant to pregnancy risk assessment comprise for example in the case of the mother: the actual health problem, obstetric situation, medical and obstetric history, social habits, environmental or occupational exposures to toxics, elements related to the family and to the partner. On what concerns the medication, therapeutic or diagnostic, or exposure to other factors, is important to consider information like the moment of pregnancy, dose, and duration, as well as, if it can be stopped or if it is meant to continue during pregnancy. The information on these elements is graded in 'required', 'highly desirable' and 'if relevant' (2). SOP was projected to overcome the need for more accurate data on adverse event case reports and to better evaluate risk assessment.

#### References:

Huang X *et al.* AMIA Annu Symp Proc. 2006; 359–363.  
Kelly WN *et al.* Drug Saf. 2007; 30: 367–373.

#### P011

##### ***In vitro* pharmacological profiles of kinase inhibitors: comparison with other pharmaceuticals**

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Pharmacological profiling is a method for assessing the selectivity of compounds using *in vitro* assays. It is desirable in drug discovery to minimise the incidence of unwanted toxicities due to off-target activities, to reduce the risk of attrition. Kinase inhibitors as a class suffer from negative perceptions with respect to their safety profiles. In part this is due to the potential for kinase inhibitors to inhibit multiple kinases in the human genome, not all of which are fully characterised or understood. There is also a perception that kinase inhibitors are generally promiscuous compounds (inhibiting multiple other non-kinase targets). The objective of this study was to investigate whether kinase inhibitors are more likely than other classes of pharmaceuticals, to interact with non-kinase targets. The dataset consisted of 758 AstraZeneca compounds tested in a set of 591 *in vitro* radioligand binding and enzyme assays, giving 60129 datapoints in total. The assays were selected to give broad coverage of target biological space. Compounds were classified as kinase inhibitors based on the criteria of having an  $IC_{50} < 100 \mu M$  in a kinase assay. Overall promiscuity and hit rates (defined as  $\geq 50\%$  inhibition) in individual assays were compared between kinase inhibitors and other compounds. 199 compounds were classified as kinase inhibitors. Overall hit rates over all compound concentrations were similar between kinase inhibitors (10%) and non-kinase-targeted compounds (13%); this result was not markedly changed if results were limited to compounds tested at  $10 \mu M$ , the most common test concentration. Hit rate comparisons for individual assays identified 3 assays where kinase inhibitors showed significantly higher hit rates than non-kinase compounds at one or more concentrations: adenosine transporter, adenosine  $A_{2A}$  and phosphodiesterase 4 (PDE4). Data from this analysis demonstrates that compounds that inhibit the activity of kinases are not inherently more promiscuous than other classes of compound. A small set of non-kinase targets was identified where kinase inhibitors have an increased risk of interaction. To avoid toxicities associated with activity at these targets, pharmacological profiling in early drug discovery should be used to identify and screen out these liabilities.

#### P012

##### **Value of pharmacological profiles in interpretation of assay outputs**

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A key component of assay validation and interpretation is the use of 'selective' pharmacological reference compounds. The compounds are often chosen based on previously characterised activity at the molecular target or mechanism of interest. Pharmacological profiling is a method adopted to assess the *in vitro* selectivity of compounds. A common method used is to screen compounds in a panel of *in vitro*

assays covering a diverse range of molecular targets (enzymes, receptors, ion channels and transporters). This study explored the value of pharmacological profiling data in the interpretation of effects in 3 different *in vitro* assays and one example is illustrated here. Compound AZ1 was profiled in a panel of 141 *in vitro* radioligand binding and enzyme assays at a single concentration of  $10 \mu M$ . Noteworthy activity of AZ1 was detected at three targets when screened at  $10 \mu M$  and concentration-response curves were constructed in order to determine  $IC_{50}$ ,  $K_i$  and  $nH$  values. AZ1 was then tested in an *in vitro* functional 5-HT<sub>1B</sub> assay that measured forskolin-stimulated cAMP accumulation (Table 1).

Molecular target	$IC_{50}$ ( $\mu M$ )	$K_i$ ( $\mu M$ )	Mode of action
5-HT <sub>1B</sub> receptor	1.05	1.00	Antagonist
Serotonin transporter	0.45	0.07	Inhibitor
Phosphodiesterase 4	3.00	Not applicable	Inhibitor

In the 5-HT<sub>1B</sub> functional assay, no agonist activity (inhibition of forskolin-stimulated cAMP accumulation) was detected up to  $100 \mu M$ . However, AZ1 inhibited the response to the reference agonist, 5-HT, in a concentration-dependent manner. At concentrations  $>10 \mu M$  AZ1 caused an increase in the level of cAMP generated that was greater than that of forskolin alone. AZ1 behaved as an antagonist at the 5-HT<sub>1B</sub> receptor and an inhibitor of PDE4. Taken together these data provide a possible explanation of the 'super-antagonism' seen in the 5-HT<sub>1B</sub> functional assay. Understanding the pharmacological profile of compounds screened in *in vitro* functional assays, and in *in vivo* models, has value in the interpretation of data. Two more examples will be given on the poster.

#### P013

##### **Detecting drug-induced visual dysfunction preclinically: Comparison of the OptoMotry system in rats with previous data obtained in a zebrafish optomotor assay**

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Numerous marketed drugs cause either overt retinal toxicity, or affect functioning of the retina or visual pathways (Constable and Pirmohamed, 2004). Preclinical screens to detect this liability would be desirable. A zebrafish (Zf) optomotor assay correctly classified 19/27 (i.e. 70%) coded compounds (Richards *et al.*, 2008). We present data obtained in the rat OptoMotry system with the 8 misclassified compounds. In the Zf study, Zf larvae had been exposed to the test compound for 5 days, then tested in a multi-lane acrylic block placed on a screen displaying a moving black-and-white grating. After 45 s, the number of larvae that had swum to the end of the lane was counted, taking the average value over three runs in each direction. In the present study, rats were tested in an OptoMotry apparatus (Douglas *et al.*, 2005). This is a chamber formed by four screens displaying a vertical sine wave grating. The grating was rotated to elicit head-tracking movements. Grating frequency was increased until there were no discernable head-tracking movements. Rats received a single dose of compound and were retested 2 h post-dose. Of the eight compounds tested, six were associated with retinal toxicity/dysfunction in humans and/or mammalian species, and two were non-retinotoxic control compounds. The rat OptoMotry system correctly classified 4/6 of the 'retinotoxic' compounds and both of the non-retinotoxic control compounds. In conclusion, both methods are useful for assessing visual function. The Zf optomotor assay is a semi-automated, medium-throughput screen requiring small quantities of compound, that can be placed very early in the preclinical drug discovery phase. The rat OptoMotry method is more predictive, but not as high-throughput or automated as the Zf optomotor assay, so would be best positioned later in the preclinical phase.

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## P014

### Differential regulation of P2Y<sub>1</sub> and P2Y<sub>12</sub> receptor trafficking by sorting proteins

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ADP plays a key role in regulating platelet function by activation of P2Y<sub>1</sub> and P2Y<sub>12</sub> GPCRs. Previous work has shown that P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors desensitise via distinct mechanisms (Hardy *et al.*, 2005) and enter different sub-populations of clathrin-coated pits upon internalisation (Mundell *et al.*, 2006). We investigated whether post-endocytic sorting of these receptors is also regulated by divergent mechanisms. Specifically, we examined the role of proteins regulating receptor recycling (Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factors (NHERF)) and receptor down-regulation (Sorting Nexin 1 (SNX1); Marchese *et al.*, 2008) in P2Y receptor traffic. HA-tagged P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors were expressed in human 1321N1 astrocytoma cells and trafficking was studied using a combination of ELISA and confocal microscopy, to quantify and visualise receptor trafficking, respectively. Both P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors entered SNX1-positive endosomes following agonist-induced internalisation. siRNA-mediated depletion of SNX1 levels increased the rate of P2Y<sub>1</sub> receptor recycling, with ~30% more receptor returning to the cell surface compared to control cells. However, SNX1 did not appear to affect P2Y<sub>12</sub> receptor recycling. We subsequently generated GST-fusion proteins of the P2Y<sub>1</sub> and P2Y<sub>12</sub> receptor COOH-terminus tails. Although neither C-tail bound endogenous SNX1, both bound NHERF proteins from 1321N1 and platelet cell lysates. Interestingly, the P2Y<sub>1</sub> and P2Y<sub>12</sub> COOH-tails bound NHERF1 and 2 with varying affinity. P2Y<sub>1</sub> bound preferentially with NHERF2, whereas P2Y<sub>12</sub> showed a greater affinity for NHERF1. We are currently investigating the role of NHERF1 in P2Y receptor recycling. In conclusion, we have provided preliminary evidence that suggests sorting of P2Y<sub>1</sub> and P2Y<sub>12</sub> GPCRs is regulated by distinct molecular mechanisms. SNX1 appears to mediate slow recycling of the P2Y<sub>1</sub> receptor but not P2Y<sub>12</sub>. We have also shown, for the first time, that the P2Y<sub>12</sub> receptor binds preferentially to NHERF1 - an interaction which may be responsible for the rapid recycling of this GPCR.

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## P015

### μ-opioid receptor internalisation: studies using a pFluorin tag

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The extent of agonist-induced μ-opioid receptor (MOPr) internalisation is dependent upon the agonist. DAMGO induces greater internalisation than morphine (Johnson *et al.*, 2006). Here we report the use of a MOPr construct N-terminally tagged with a pH-sensitive mutant of green fluorescent protein (pFluorin) (Lober *et al.*, 2006). This emits fluorescence at physiological extracellular pH (7.4) but not in acidic environments such as that occurring in intracellular organelles (Lober *et al.*, 2006; Miesenbock *et al.*, 1998). The pFluorin-tagged MOPr was expressed in HEK 293 cells and imaged by confocal microscopy. For studies of agonist-induced internalisation we used receptor saturating concentrations of each drug to ensure comparable levels of receptor occupancy. In intact cells pFluorin fluorescence was only observed at the plasma membrane and this was abolished by lowering the extracellular pH to 6. Raising the intracellular pH with NH<sub>4</sub>Cl (50 mM) revealed fluorescence in intracellular compartments. The pFluorin-tagged MOPr was functional in that on exposure to agonist the pFluorin-tagged receptor coupled to activation of GIRK channels co-expressed in the same cells. Prolonged exposure of cells to the full agonist DAMGO (10 μM) resulted in a time-dependent decrease in cell surface fluorescence indicating receptor internalisation. The maximal decrease in fluorescence (24 ± 2%; n = 6) was achieved after 6 min of drug exposure. Over the same time period, the decrease in fluorescence induced by the partial agonist morphine (30 μM), was less (7 ± 1%; n = 4) than that induced by DAMGO. This decrease in fluorescence was not due to photobleaching since saline-treated cells imaged under the same conditions, showed no decrease in fluorescence over the same time interval. We are currently using the pFluorin-tagged receptor to examine agonist-induced MOPr clustering on the plasma membrane prior to internalisation. In conclusion, the pFluorin is a useful tool for studying the trafficking of MOPr in real time.

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## P016

### The second extracellular loop plays an important role in GPCR activation – the adenosine A<sub>2B</sub> receptor as a point in case

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Next to the transmembrane domains, the highly variable extracellular loops in G protein-coupled receptors (GPCRs) may be involved in their activation. However, the molecular mechanisms of this activation principle are still poorly understood, which is not surprising given the huge diversity of these extracellular regions among GPCR family members. In this study, the activating role of the second extracellular loop in the adenosine A<sub>2B</sub> receptor was investigated through random mutagenesis followed by a selection procedure using a yeast read-out system. Thus, a library of randomly mutated adenosine A<sub>2B</sub> receptors was screened for constitutive activity in yeast, as a typical readout of receptor activation. The yeast system used was the MMY24 *S. Cerevisiae* strain which has a chimeric G<sub>βi</sub> protein incorporated into its genome that enables mammalian GPCRs to couple to the yeast pheromone signaling pathway. Activation of this signaling pathway leads to transcription of the reporter genes HIS3 and LacZ, which were used for mutant

selection. From the random mutagenesis screen a number of highly constitutive active mutants were discovered. All of these mutations appeared to be clustered together, indicating that a defined region of the second extracellular loop is involved in adenosine A<sub>2B</sub> receptor activity. In the wild-type receptor, this region is highly cysteine-rich and most likely involved in the formation of one or more disulfide bridges, conferring structure and rigidity to the receptor structure. Therefore it is speculated that the second extracellular loop of the wild-type adenosine A<sub>2B</sub> receptor has an inactivating role. Disturbance in this region may lead to a change in receptor structure by which the loop is no longer able to silence the receptor.

## P017

### Characterisation of the effects of the phospholipase C inhibitor U73122 on P2Y receptor-mediated contractions of the rat isolated pulmonary artery

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P2Y receptor agonists induce vasodilation via endothelial P2Y receptors and vasoconstriction via smooth muscle P2Y receptors (Chootip *et al.*, 2002). However, the intracellular signalling pathways by which vasoconstriction is induced are not well characterised. The aim of this study was to determine the role of phospholipase C (PLC) in nucleotide-evoked vasoconstriction of rat small intrapulmonary artery (SPA) using the PI-PLC inhibitor U73122. 5 mm rings of endothelium-denuded rat SPA were mounted under isometric conditions in 1 mL organ baths at 37°C and a resting tension of 0.5 g. Contractions were elicited by addition of the P2Y receptor agonists UTP and UDP (300 μM), the FP receptor agonist PGF<sub>2α</sub> (10 μM) or KCl (40 mM) to the bath. UTP, UDP and PGF<sub>2α</sub> each evoked slowly developing contractions, which reached a peak within 5 min. Pre-incubation with U-73122 (1 μM and 3 μM) for 15 min had no effect on the resting tone of the arteries nor on the amplitude of the contractions evoked by UTP (n = 5), UDP (n = 5), PGF<sub>2α</sub> (n = 5) or KCl (n = 5). Increasing the concentration of U73122 to 10 μM and the preincubation period to 30 min, also left the responses to UTP, UDP and KCl unchanged (n = 6). In contrast, the contractions to PGF<sub>2α</sub> were inhibited significantly under these conditions (77 ± 6% of control, n = 6, P < 0.05). These results show that the PI-PLC inhibitor U73122 had no effect on P2Y receptor-mediated contractions of rat pulmonary artery under conditions that inhibited the responses induced by PGF<sub>2α</sub>. This calls into question the involvement of PLC in the P2Y receptor-mediated contraction of rat pulmonary artery and further studies are ongoing to determine the involvement of other intracellular signalling components.

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## P018

### Antagonist-mediated down-regulation of 5-HT<sub>7</sub> receptors by the atypical antipsychotics clozapine and olanzapine: evidence for functional selectivity

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Classically, ligands of G protein-coupled receptors have been classified primarily upon their affinity and efficacy to activate a signal transduction pathway. More recent reports indicate that the efficacy of a particular ligand can vary depending on the receptor mediated response measured (e.g. activating G protein(s), other downstream responses, inducing internalization). At the Gs-coupled 5-HT<sub>7</sub> serotonin receptor, we have previously demonstrated that one out of three inverse agonists (SB269970) induced both homo- and heterologous desensitization, similar to agonist stimulation (Krobert *et al.*, 2006). The primary objective of this study was to determine whether different antagonists/inverse agonists at the 5-HT<sub>7</sub> receptor also induced receptor internalization and/or degradation of 5-HT<sub>7</sub> receptors. The agonist 5-HT and three out of four inverse agonists tested induced internalization, but only the atypical antipsychotics clozapine and olanzapine (inverse agonists) induced degradation of 5-HT<sub>7</sub> receptors (~60% reduction within 24 h). Incubation with only clozapine or olanzapine targeted 5-HT<sub>7</sub> receptors to lysosomes and inhibiting lysosomal degradation with chloroquine blocked the down regulation of 5-HT<sub>7</sub> receptor density. Incubation with SB269970 decreased both 5-HT<sub>7</sub>(b) internalization and receptor density but increased 5-HT<sub>7</sub>(d) receptor density, indicating differential regulation among the 5-HT<sub>7</sub> splice variants. Taken together, the results show that various ligands differentially activate regulatory processes governing receptor internalization and degradation in addition to signal transduction. Thus, these data provide support for functional selectivity at the 5-HT<sub>7</sub> receptor.

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- Krobert KA *et al.* *Eur J Pharmacol*. 2006; 532: 1–10.

## P019

### N49 Phospholipase A<sub>2</sub>, a potent stimulus of mast cell related inflammation

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It has been recognized that phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is a crucial component of snake venom, which contributes greatly to snake venom induced inflammation in man. However, the mechanisms through which N49 PLA<sub>2</sub> provoke life threatening inflammation remain unclear. Recently, a N49 PLA<sub>2</sub>, TM-N49 from *Protobothrops mucrosquamatus* crude venom was characterized in our laboratory. Since the purification procedure developed is able to supply us with relatively large quantity of highly purified TM-N49, we investigated the ability of TM-N49 in induction of inflammation. The results showed that TM-N49 provoked a dose dependent increase in microvascular leakage in the skin of rats. The potency of TM-N49 in induction of skin edema appeared greater than the potency of bradykinin and histamine. Pretreatment of rats with compound 48/80 diminished TM-N49

induced skin reaction and reduced mast cell numbers in rats. Ginkgolide B and cyproheptadine, but not terfenadine and quinacrine inhibited TM-N49 elicited microvascular leakage when they were co-injected with the stimulus to rat skin. Moreover, TM-N49 was found to induce histamine release from human colon, lung and tonsil mast cells, and both metabolic inhibitors and Pertussis toxin were capable of inhibiting TM-N49 elicited histamine release. TM-N49 induced mast cell accumulation in the peritoneum of mice, which was inhibited by co-injection of ginkgolide B, cyproheptadine and terfenadine. Intravenous injection of monoclonal antibodies against CD18, ICAM-1 and CD11a also blocked TM-N49 induced mast cell accumulation. TM-N49 induced also serotonin release in the peritoneal lavage of mice. In conclusion, TM-N49 induced inflammation may be through activation of mast cells.

#### P020

##### A promising imidazole-free histamine H<sub>3</sub> receptor antagonist with good brain penetration and anticholinesterase activity

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Histamine H<sub>3</sub> antagonists, lacking the imidazole ring that is associated with the drawbacks of CYP450 inhibition and low CNS access, have been recently proposed as a new therapeutic approach to several cognitive disorders characterized by deficit of neurotransmitter release such as Alzheimer disease, a pathology currently treated with cholinesterase (ChE) inhibitors. In the search for an improved treatment, a new biphenylic derivative five has been developed (Morini *et al.*, 2006) as agent combining anticholinesterase activity with H<sub>3</sub> histamine receptor inhibition. The selectivity, affinity and potency at H<sub>3</sub> receptors and the inhibition of ChE were studied *in vitro*. The ability to enter the CNS (*ex vivo* binding) and to exert anti-amnesic activity (passive avoidance test) were investigated in 200–250 g female Wistar rats, applying experimental procedures supervised and approved by the Ministero della Salute (DL116/92). The *in vitro* results are summarized in Table 1.

	Binding test (pK <sub>i</sub> )			Functional test (pK <sub>o</sub> )					ChE pIC <sub>50</sub>
	Rat		Human	Human	Guinea pig			Rat	
	H <sub>3</sub>	H <sub>4</sub>	H <sub>3</sub>	H <sub>3</sub>	H <sub>3</sub>	H <sub>2</sub>	H <sub>1</sub> pD <sub>2</sub>		
Compound 5	8.9	4.6	9.5	8.8	8.6	inactive	4.8	5.2	
Thiopramide	8.6	7.3	7.3	7.2	9.0	inactive	inactive	not calc.	

In *ex vivo* study compound 5 was 3 fold more potent than the conventional H<sub>3</sub>-blocker thiopramide in inhibiting [<sup>3</sup>H](R)- $\alpha$ -methylhistamine cortical binding (ED<sub>50</sub> = 0.63 vs. 2.04 mg/kg i.p.). At 1.25 mg/kg i.p. it was as effective as the anti-Alzheimer drug donepezil and 7 fold more potent than thiopramide in reverting scopolamine-induced amnesia. These results suggest that the good CNS penetration

and the dual inhibition of cholinesterase and histamine H<sub>3</sub>-receptors could account for the anti-amnesic effect of compound 5, a potential benchmark for the development of non-imidazole H<sub>3</sub>-antagonists with therapeutic potential in cognitive disorders.

#### Reference:

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#### P021

##### Investigating activation of human G protein-coupled receptor kinase 2 by c-Src

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Arrestin binding to G protein-coupled receptors requires phosphorylation of the agonist-activated receptor by G protein-coupled receptor kinase (GRKs). Once arrestin is bound to the receptor, signaling via G proteins is arrested and the receptor is desensitized. The receptor can then be targeted to clathrin-coated pits to be internalized and alternative signaling pathways may be activated. Using a previously established FRET-based assay to monitor arrestin3 binding to the  $\beta_2$ -adrenergic receptor ( $\beta_2$ AR) in single living cells, we show that GRK2 phosphorylation by c-Src accelerates arrestin3 binding to the  $\beta_2$ AR, presumably by increasing GRK2 activity. HEK-293 cells were transfected with  $\beta_2$ AR tagged with YFP, arrestin3 tagged with CFP and GRK2 with or without c-Src. A slow (phosphorylation-dependent) and fast phase of arrestin3 binding to  $\beta_2$ AR was observed in agreement with previous work (Krasel *et al.*, 2005). Cells that were not transfected with c-Src displayed a slow phase with a half-life of about 24 seconds, whereas for cells co-transfected with c-Src the half-life was reduced by approximately 50% to about 13 seconds. PP2, a src-family kinase inhibitor, was used to show that the observed acceleration of arrestin3 binding to the receptor was caused by the catalytic activity of c-Src. Treatment with 10  $\mu$ M PP2 for 1 h had no effect on arrestin3 binding to the receptor in cells that had not been co-transfected with c-Src, but increased the half-life of arrestin3 binding to the receptor in cells co-transfected with c-Src to about 25 s, no different to that of cells without c-Src. It has previously been reported that c-Src-mediated tyrosine phosphorylation of GRK2 occurs at residues 13, 86 and 92 (Mariggio *et al.*, 2006). In order to show that phosphorylation of GRK2 by c-Src was required for the acceleration in arrestin3 binding to  $\beta_2$ AR we generated a series of phosphorylation impaired GRK2 mutants (Y13, 86, 92F, Y13F, Y86, 92F, Y86F & Y92F). There was no significant difference between the half-life of the slow phase of arrestin3 binding to  $\beta_2$ AR in cells transfected with Y13, 86, 92F, Y86, 92F, Y86F or Y92F GRK2 with or without c-Src. In the case of Y13, 86, 93F GRK2, the rate of arrestin3 binding was significantly slower than wild-type, suggesting that this mutant is in some way catalytically impaired. With Y86, 92F, Y86F and Y92F GRK2 the rate of arrestin3 was similar to wild-type. Co-transfection with c-Src resulted in a two-fold increase in the activity of Y13F GRK2 although both these rates were significantly slower than wild-type. Tyrosine residue 13 is probably not involved in the activation of GRK2 but maybe involved in receptor recognition. Other members of the GRK family were tested and it appears that the effect of c-Src on activity is unique to GRK2.

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Mariggio, S *et al.* Cell Signal. 2006; 18: 2004–2012.

## P022

### **Ionizing irradiation inhibits BKCa current in rat aorta smooth muscle cells via protein kinase C: implication to arterial hypertension development**

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It is known that ionizing irradiation increases vascular responsiveness and leads to arterial hypertension but mechanisms of its development still remain unclear (Soloviev *et al.*, 2005). In this study we tested the hypothesis that changes in the large conductance  $Ca^{2+}$ -activated  $K^+$  channels ( $BK_{Ca}$ ) activity in vascular smooth muscle cells (VSMCs) may contribute to irradiation-evoked arterial hypertension development.  $BK_{Ca}$  play an essential role in vascular tone regulation via its involvement to the changes of VSMCs membrane potential with following changes in intracellular  $Ca^{2+}$  concentration  $[Ca^{2+}]_i$  (Ledoux *et al.*, 2006). We also supposed that changes in  $BK_{Ca}$  activity under irradiation impact can be mediated by protein kinase C (PKC) (Barman *et al.*, 2004), a one of key regulatory enzyme involved in the signal transduction in VSMCs, and which activity can be increased under action of ionizing irradiation (Baskar *et al.*, 2007). The study was performed on isolated VSMCs from male Wistar rat aorta using standard patch-clamp techniques in whole-cell configuration. Animals (weight 180–210 g) were exposed to single whole-body ionizing irradiation (source  $Co^{60}$ , dose 6 Gy) and taken on 9<sup>th</sup> and 30<sup>th</sup> days postirradiation. Whole-cell outward  $K^+$  current separation in control VSMCs with selective inhibitors of different components of  $Ca^{2+}$ -activated  $K^+$  channels, apamin (1  $\mu M$ ), charybdotoxin (1  $\mu M$ ), paxilline (500 nM), showed that in rat aortic VSMCs the main component of whole-cell outward  $K^+$  current is  $BK_{Ca}$  current. Whole-cell outward  $K^+$  current in VSMCs from animals taken on 9<sup>th</sup> and 30<sup>th</sup> days postirradiation was significantly inhibited and consisted  $33.21 \pm 1.67$  pA/pF,  $n = 11$  and  $18.22 \pm 2.51$  pA/pF,  $n = 12$ , respectively vs. to control ( $63.21 \pm 4.7$  pA/pF,  $n = 10$ ). Paxilline (500 nM), selective  $BK_{Ca}$  current inhibitor, slightly inhibits reduced on 9<sup>th</sup> postirradiation whole-cell outward  $K^+$  current to  $17.19 \pm 1.1$  pA/pF ( $n = 9$ ) and had not effect on the  $K^+$  current in tissue taken on 30<sup>th</sup> day postirradiation ( $15.67 \pm 2.58$  pA/pF,  $n = 7$ ) showing that on this term postirradiation  $BK_{Ca}$  current eliminates completely. Application of chelerythrine (100 nM), led to significant restoration of  $K^+$  current in the tissue taken on 9<sup>th</sup> and 30<sup>th</sup> days postirradiation up to  $89.4 \pm 3.78$  pA/pF ( $n = 9$ ) and  $74.4 \pm 5.98$  pA/pF ( $n = 6$ ) respectively, suggesting that irradiation-evoked inhibition of  $BK_{Ca}$  current in aortic VSMCs is mediated by PKC. Taken together, these data indicate that one of mechanisms leading to elevation of vascular tone and subsequent arterial hypertension under ionising irradiation impact can be PKC-mediated inhibition of  $BK_{Ca}$  channels in VSMCs.

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## P023

### **The $M_2$ -negative feedback mechanism of motor nerve terminal mediates the worsening by apamin of fade induced by tetanizing stimulation of the phrenic nerve diaphragm muscle preparations of rats**

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Significant binding of radiolabeled apamin has been found in innervated muscles of myotonic dystrophy patients. It has been hypothesized that the beneficial effects of apamin applied to muscles of patients suffering from myotonic dystrophy could be due to a presynaptic action of toxin (Roncarati *et al.*, 2001), it has not been clarified as such presynaptic action of apamin could reduce the muscular excitability in such patients. To verify if the beneficial effects of apamin could be dependent on activation of the inhibitory presynaptic muscarinic receptors, the effects of apamin alone, or in combination with atropine, pirenzepine or methoctramine, were investigated in the neuromuscular preparations of healthy rats stimulated at 100 Hz. The effects of apamin were investigated in the phrenic nerve diaphragm muscle preparation of male Wistar rats (220–250 g) stimulated at 100 Hz. Apamin enhances the fade induced by indirectly stimulation of muscle. The effect of the toxin was not followed by any variation on maximal tetanic tension, and it did not appear when the muscle were directly stimulated. Apamin decreases the amplitude of muscular contraction when the preparations were indirectly stimulated at 0.2 Hz. In contrast, 4-aminopyridine facilitated the neuromuscular transmission in such experimental condition. The inhibitory effects of toxin were impaired by treatment of preparation with methoctramine, but they were not modified by pirenzepine. In contrast, methoctramine antagonized the increment by acetylcholine of fade induced at 100 Hz, but pirenzepine potentiated the inhibitory effect of cholinomimetic in such experimental condition. As whole, data show that the inhibitory effect produced by apamin has origin on motor nerve terminal, and it is not determined by blockage of potassium channels inducing an increment on acetylcholine release, thereby activating the inhibitory presynaptic muscarinic  $M_2$ -receptors. The inhibitory effect induced by apamin on neuromuscular transmission would be determined by a direct activation by toxin of the  $M_2$ -negative feedback control of the terminal. Such mechanism of action of toxin could be the origin of beneficial effects of such agent in patients suffering from myotonic diseases.

## P024

### **Modulation of $Ca^{2+}$ -activated $Cl^-$ current by large-conductance $Ca^{2+}$ -activated $K^+$ channel inhibitors in murine portal vein myocytes**

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Our laboratory recently reported large-conductance  $Ca^{2+}$ -activated  $K^+$  channel ( $BK_{Ca}$ ) activators augment macroscopic  $Ca^{2+}$ -activated  $Cl^-$  current ( $I_{ClCa}$ ) evoked with a fixed  $[Ca^{2+}]_i$ . This study extends this work by examining whether selective inhibitors of the  $BK_{Ca}$  modulate  $I_{ClCa}$  activity. *Balb/c* mice (6–8 week old) were killed

by cervical dislocation and exsanguination. Tissues removed immediately and portal vein myocytes obtained by enzymatic dispersion. Recordings were made using the whole cell voltage-clamp techniques with an external solution of (mM): NaCl 126, MgCl<sub>2</sub> 1.2, CaCl<sub>2</sub> 1.5, glucose 11, HEPES 10, TEA-Cl 10 (pH 7.2 with NaOH). The pipette solution contained (mM): TEA-Cl 20, CsCl 106, MgCl<sub>2</sub> 1.1, HEPES 10, Na<sub>2</sub>GTP 0.2, Na<sub>2</sub>ATP 3, BAPTA 10, CaCl<sub>2</sub> 7.8 (500 nM free  $Ca^{2+}$ ; pH 7.2 with CsOH). As previously reported, an inward current of  $-1.0 \pm 0.1$  pA/pF was observed at a holding potential of  $-50$  mV ( $I_{S0mV}$ ). Depolarisation to  $+70$  mV evoked a voltage- and time-dependent outward current of  $2.8 \pm 0.4$  pA/pF ( $I_{+70mV Instant}$ ), which increased over 1 s to  $9.3 \pm 1.4$  pA/pF ( $I_{+70mV Late}$ ;  $n = 7$ ). Repolarisation to  $-80$  mV ( $I_{S0mV}$ ) evoked an immediate inward current of  $-10.2 \pm 1.4$  pA/pF. Iberotoxin (100 nM) did not change depolarisation evoked current, but augmented  $I_{S0mV}$  ( $-21.9 \pm 5.6$  pA/pF,  $n = 6$ ,  $P < 0.05$ ) and prolonged current decay (at  $-100$  mV  $\tau$  increased from  $62.3 \pm 6.2$  ms to  $84.6 \pm 8.1$  ms,  $P < 0.001$ ). Paxilline (10  $\mu M$ ) inhibited  $I_{S0mV}$  ( $-0.9 \pm 0.3$  pA/pF,  $n = 4$ ,  $P < 0.05$ ),  $I_{+70mV Instant}$  ( $2.0 \pm 0.4$  pA/pF,  $P < 0.05$ ),  $I_{+70mV Late}$  ( $4.1 \pm 0.7$  pA/pF,  $P < 0.05$ ), and  $I_{S0mV}$  ( $-4.8 \pm 0.8$  pA/pF,  $P < 0.05$ ). Penitrim A (10  $\mu M$ ) blocked  $I_{+70mV Late}$ , reducing outward current to  $5.7 \pm 0.5$  pA/pF ( $P < 0.01$ ,  $n = 4$ ). Tamoxifen (10  $\mu M$ ) inhibited  $I_{S0mV}$  ( $-0.3 \pm 0.1$  pA/pF,  $n = 7$ ,  $P < 0.01$ ),  $I_{+70mV Instant}$  ( $0.7 \pm 0.2$  pA/pF,  $P < 0.01$ ),  $I_{+70mV Late}$  ( $1.2 \pm 0.2$  pA/pF,  $P < 0.01$ ) and  $I_{S0mV}$  ( $-1.1 \pm 0.2$  pA/pF,  $P < 0.01$ ). The observation that a further four modulators of  $BK_{Ca}$  activity also modulate  $I_{ClCa}$  suggests either the  $Ca^{2+}$ -activated  $Cl^-$  channel bares remarkably close similarity to the  $BK_{Ca}$ , or that modulation of  $I_{ClCa}$  is due to an interaction between channels, allowing pharmacological modulation of the  $BK_{Ca}$  to affect the  $Ca^{2+}$ -activated  $Cl^-$  channel.

## P025

### **TRPV1 expression can be induced in human primary lung fibroblasts by the inflammatory mediators TNF- $\alpha$ , LPS and IL-1 $\alpha$**

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The transient receptor potential vanilloid 1 (TRPV1) channel is found mainly on primary sensory neurons. Recent findings suggest that TRPV1 may also be expressed on various non-neuronal cell types including keratinocytes, urothelial cells, dental pulp fibroblasts and airway smooth muscle cells. In addition, it is now emerging that TRPV1 may be upregulated following nerve injury. Having recently cloned human TRPV1 from a fibroblast cell line, we sought to determine whether (i) human primary lung fibroblasts (HPLF) express TRPV1 and (ii) whether inflammatory mediators can induce TRPV1 expression. HPLF were treated with either TNF- $\alpha$ , LPS or IL-1 $\alpha$  from 0–72 h. RT-PCR was employed to detect changes in TRPV1 mRNA, whilst intracellular calcium signalling in response to capsaicin and resiniferatoxin was employed to detect functional TRPV1. HPLF were found not to endogenously express TRPV1 mRNA, and were not sensitive to challenge with capsaicin or resiniferatoxin. However, the inflammatory mediators TNF- $\alpha$ , LPS and IL-1 $\alpha$  all stimulated TRPV1 mRNA expression with similar kinetics. TRPV1 mRNA was observed at 24 h and 48 h post treatment with all three inflammatory mediators. In stark contrast to the untreated HPLF, significant calcium signalling in response to capsaicin and resiniferatoxin in 24 h and 48 h TNF- $\alpha$ , LPS or IL-1 $\alpha$  treated HPLF was observed. The data imply that TRPV1 expression can be induced by TNF- $\alpha$ , LPS or IL-1 $\alpha$  in HPLF and TRPV1 may play an important role in airway inflammation.

## P026

### **Quercetin antagonism of Bay K 8644 effects on rat tail artery L-type $Ca^{2+}$ channels**

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The functional interaction between two L-type  $Ca^{2+}$  channel activators, quercetin (Saponara *et al.*, 2002) and (S)-(-)-methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (Bay K 8644), has been investigated in vascular smooth muscle cells. L-type  $Ca^{2+}$  currents  $[I_{Ca(L)}]$  were recorded in freshly isolated rat tail artery myocytes using the whole-cell patch-clamp method. Bay K 8644 increased  $I_{Ca(L)}$  in a concentration-dependent manner with a  $pEC_{50}$  value of 8.25. Pre-incubation of myocytes with concentrations of quercetin *per se* ineffective as an L-type  $Ca^{2+}$  channel activator (0.1 and 0.3  $\mu M$ ), significantly inhibited the maximal response evoked by Bay K 8644, but left unaltered its potency. Quercetin 0.1  $\mu M$  prevented also the hyperpolarizing shift of the steady-state inactivation curve induced by 0.1  $\mu M$  Bay K 8644 without modifying its effects on  $I_{Ca(L)}$  activation, inactivation and deactivation kinetics, as well as on use-dependence and recovery from inactivation. Quercetin, at high nanomolar concentrations, which are consistently detected in plasma of healthy volunteers, antagonizes some effects of Bay K 8644 on L-type  $Ca^{2+}$  channels of isolated vascular myocytes.

### **Reference:**

- Saponara *et al.* Br J Pharmacol. 2002; 135: 1819–27.

## P027

### **$Ca^{2+}$ /calmodulin-dependent facilitation of P2X<sub>7</sub> receptor currents**

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The ATP-gated P2X<sub>7</sub> receptors are electrophysiologically unique among the members of the P2X ionotropic receptors family by the fact that they exhibit cationic currents that facilitate when brief agonist applications are repeated. The aim of this study was to elucidate the molecular basis of this facilitation phenomenon recorded by the whole-cell patch clamp configuration in HEK293 cells transiently expressing rat P2X<sub>7</sub> receptors. Repeated application of maximum

ATP concentration (5 mM, 10 s duration applied at 40 s intervals) resulted in a 2.5-fold increase in current density over the first four applications and then remained at this facilitated level for all subsequent applications (up to 4 min). Facilitation was highly dependent on the intracellular calcium activity and was significantly decreased by the calmodulin-binding peptide (CBP) while co-expression of the dominant-negative calmodulin (CaM1234) was less effective in inhibiting facilitation. By searching in a calmodulin target database, we identified an IQ-independent putative calmodulin binding site, made by 17 consecutive amino acid residues from I541 to R557, in the P2X<sub>7</sub> cytosolic C-terminus. A double-mutant receptor (I541T, S552C) in this region, generated in order to remove the putative calmodulin binding site, showed no Ca<sup>2+</sup>/calmodulin dependent facilitation. We also demonstrated by co-immunoprecipitation experiments that calmodulin interacts with the wild-type receptor and that this interaction is increased when receptors are activated by ATP. Calmodulin did not co-immunoprecipitate with the I541T, S552C double-mutant receptor. These results suggest that calmodulin is not pre-bound to the receptor but associates rapidly as the Ca<sup>2+</sup> concentration increases in the cell. This study presents, for the first time, evidence that calmodulin associates with a purinergic receptor, the P2X<sub>7</sub> receptor, and is responsible for its facilitation.

## P028

### TREK-1 channels as a possible therapeutic target for the treatment of unstable urinary bladders

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Bladder detrusor instability leading to urinary incontinence is a growing problem. Since potassium (K<sup>+</sup>) channels are responsible for setting myocyte excitability in the urinary bladder, the development of K<sup>+</sup> channel opening molecules is one of the strategies under development by the pharmaceutical industry. Targets include the ATP-sensitive K<sup>+</sup> channel (GlaxoSmithKline, GSK366074), the large conductance, Ca<sup>2+</sup>-sensitive K<sup>+</sup> channel (Tanabe Seiyaku, TA-1702) and the two pore domain (2-PK) potassium channel designated TREK-1 (Bristol-Meyers Squibb, BL-1249). Because of our on-going interest in 2-PK channels (Gardener *et al.*, 2004; Gönçzi *et al.*, 2006), experiments were conducted using BL-1249, the putative TREK-1 opener (Tertyshnikova *et al.*, 2005). The effects of BL-1249 on whole-cell K<sup>+</sup> currents were investigated using HEK293 cells stably transfected with TREK-1 cDNA; channel expression was verified by molecular biological techniques, while their pharmacological profile was studied using the TREK-1 modulators, arachidonic acid and floxetine. Using stepping voltage protocols, activation of the transfected HEK293 generated a non-inactivating K<sup>+</sup> current with outward rectification. Application of the opener arachidonic acid (10 μM) produced a 130 ± 50% increase of the current at +50 mV (*n* = 8) while floxetine (33 μM) inhibited the original TREK-1 currents by 91 ± 2% (*n* = 13). TREK-1 currents at +50 mV were also increased by 145 ± 25% (*n* = 13) following intracellular acidosis induced by a HCO<sub>3</sub><sup>-</sup>-rich (80 mM) solution. Application of the BL-1249 caused reversible and dose-dependent activation of the TREK-1 current, with an EC<sub>50</sub> value of 1.5 μM. It is concluded that BL-1249 is a TREK-1 opener; a study of its opening selectivity is on-going.

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## P029

### Stimulation of P2X7 receptors activates multiple permeability pathways with different selectivities in HEK-293 and RAW 264.7 cells.

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It is well known that P2X<sub>7</sub> receptor stimulation activates a pathway on the cell membrane which is permeable to large molecules. Although the identity of this pathway is still unclear, generally it is viewed as a non-selective, pore like structure which permeates all molecules smaller than 1 kDa. We used different fluorescent molecules to investigate the selectivity of this P2X<sub>7</sub> induced dye uptake pathway. We used two cell lines: HEK-P2X<sub>7</sub>, HEK-293 cells which stably express rat P2X<sub>7</sub> receptors, and RAW 264.7 cells, a macrophage derived cell line. We used DNA binding fluorescent dyes ethidium, YoPro-1 and ToTo-1, also lucifer-yellow and calcein as tracers. Measurements of fluorescent dye accumulation in cells were done with a confocal microscope. Stimulation of P2X<sub>7</sub> receptors with 1 mM ATP in Ca<sup>2+</sup> containing bathing solutions induces uptake of YoPro-1, ToTo-1, ethidium and lucifer-yellow but not of calcein into HEK-P2X<sub>7</sub> cells. On the other hand in Ca<sup>2+</sup> free bathing solutions ATP stimulated lucifer-yellow uptake disappears. Increasing intracellular Ca<sup>2+</sup> by ionophore Br-A23187 activates uptake of lucifer-yellow but not of YoPro-1 or calcein. Buffering intra- and extra-cellular Ca<sup>2+</sup> does not abolish ATP induced YoPro-1 and lucifer-yellow uptake. In RAW 264.7 cells, in nominally Ca<sup>2+</sup> free bathing solutions ATP induces uptake of YoPro-1, ToTo-1, ethidium, lucifer-yellow and calcein. In Ca<sup>2+</sup> containing bathing, solution an additional uptake which is delayed (starting about 15–20 min after ATP application) and has a very fast kinetics is observed. A very similar delayed and fast dye uptake can also be activated by Br-A23187 (only this time with a 10–15 min delay). Ca<sup>2+</sup> buffering do not affect ATP induced YoPro-1 or lucifer-yellow uptake into RAW 264.7 cells. When ATP induced ethidium and lucifer-yellow uptake is measured simultaneously some RAW 264.7 cells observed to uptake only ethidium but not lucifer-yellow. These results may indicate that in HEK-P2X<sub>7</sub> cells there are two uptake pathways, one of them is permeable to YoPro-1, ToTo-1 and ethidium and is independent of Ca<sup>2+</sup>. Second one is permeable to lucifer-yellow and is Ca<sup>2+</sup> dependent. In RAW 264.7 cells there may be two Ca<sup>2+</sup> independent pathways; one of them is permeable

to ethidium and the other one is permeable to lucifer-yellow. Additionally there may be a third non-selective and Ca<sup>2+</sup> dependent permeability in RAW 264.7 cells.

## P030

### Investigating the role of calcium channel β subunits in membrane targeting and regulation of voltage-gated calcium channels

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Calcium entry through voltage-gated calcium (Ca<sub>v</sub>) channels is important in diverse cellular processes including neurotransmitter release, gene expression and cardiac pacemaker activity. Ca<sub>v</sub> channels are composed of a pore-forming α<sub>1</sub> subunit associated with a number of auxiliary subunits including Ca<sub>v</sub>β subunits. Ca<sub>v</sub>βs enhance plasma membrane expression and modify the biophysical properties of Ca<sub>v</sub>α<sub>1</sub> subunits. Recent work suggests that Ca<sub>v</sub>βs may also act as scaffold proteins. This hypothesis is being tested by investigating Ca<sub>v</sub>β interactions both within and outside Ca<sub>v</sub> channel complexes. Preliminary studies comparing the sub-cellular distributions of CFP-tagged neuronal Ca<sub>v</sub>β isoforms (β<sub>1b</sub>, β<sub>2a</sub>, β<sub>3</sub>, β<sub>4</sub>) using confocal microscopy have shown that when expressed alone, all CFP-tagged Ca<sub>v</sub>βs exhibit strong nuclear localisation. CFP-Ca<sub>v</sub>β<sub>1b</sub>, β<sub>2a</sub> and β<sub>3</sub> are also present throughout the cytoplasm. To determine the roles of the hypervariable N- and C-terminal regions of Ca<sub>v</sub>βs in membrane targeting of Ca<sub>v</sub>α<sub>1</sub> subunits, CFP-tagged N- and C-terminal deletion mutants of Ca<sub>v</sub>β<sub>1b</sub> have been constructed. Preliminary data suggest that removal of one or both of these hypervariable domains has no significant effect on sub-cellular localisation of CFP-Ca<sub>v</sub>β<sub>1b</sub> or its ability to target Ca<sub>v</sub>α<sub>1</sub> subunits to the membrane. Patch clamp electrophysiology is in progress to confirm that CFP-tagged constructs are fully functional, and to assess the impact of removing hypervariable domains on Ca<sub>v</sub> channel biophysical properties. Co-immunoprecipitation and pull-down assays are also being developed to investigate whether non-Ca<sub>v</sub> channel proteins also interact with CFP-Ca<sub>v</sub>βs.

## P031

### Involvement of Na<sup>+</sup>-H<sup>+</sup> exchanger in hypoxia-mediated inhibition of voltage-gated K<sup>+</sup> channels in rat small pulmonary arterial myocytes

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Hypoxia-mediated inhibition of voltage-gated K<sup>+</sup> (K<sub>v</sub>) channels plays an important role in hypoxic pulmonary vasoconstriction (Mauban *et al.*, 2005). The exact mechanisms of this effect remain not completely understood. Therefore the effect of hypoxia on K<sub>v</sub> channel currents (I<sub>Kv</sub>) in pulmonary arterial myocytes (PAMs) freshly isolated from male Wistar rats (275–300 g) as previously described (Smirnov *et al.*, 2002; Tammaro *et al.*, 2004) was investigated using the patch clamp technique. Inhibition of I<sub>Kv</sub> was calculated as a percentage of the block of the whole cell conductance derived from current-voltage relationships measured with a 2 s voltage ramp applied from the holding potential of -80 mV. When PAMs were bathed in a bicarbonate buffered physiological saline solution (PSS), hypoxia blocked I<sub>Kv</sub> by 51 ± 8% (*n* = 6). When PAMs were bathed in HEPES buffered PSS the effect of hypoxia was virtually abolished. This difference suggests the importance of the mechanisms which are activated in the physiological conditions in the regulation of I<sub>Kv</sub>. Since the Na<sup>+</sup>-H<sup>+</sup> exchanger (NHX) could be one of such mechanisms, the effect of a non-selective inhibitor amiloride (100 μM) and the NHX specific inhibitor MIA (25 μM) was studied. Both amiloride and MIA significantly attenuated hypoxia induced block of I<sub>Kv</sub> (19 ± 5%, *n* = 8 and 20 ± 6%, *n* = 7, respectively, *P* < 0.01). The role of the NHX in the regulation of I<sub>Kv</sub> was further assessed in HEPES buffered PSS using the NHX inhibitors, different pH gradients and intracellular pipette solutions, and removal of extracellular sodium. Our findings suggest that the NHX activity contribute to the regulation of K<sub>v</sub> channels in PAMs during hypoxia.

Supported by the British Heart Foundation (grants PG03/059 and PG04/069).

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## P032

### Extracellular calcium modulates the inhibitory effect of 4-aminopyridine on K<sub>v</sub> channels in vascular smooth muscle cells

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Potassium channels play a prominent role in the regulation of cell membrane potential. Among these channels, voltage-dependent K<sub>v</sub> channels form a large family of proteins in which several subtypes have been identified. The aim of this work is to characterize the properties of 4-aminopyridine (4-AP)-sensitive K<sub>v</sub> channels in freshly isolated smooth muscle cells from the superior mesenteric artery by using whole-cell patch-clamp technique. The functional role of the 4-AP sensitive K<sub>v</sub> currents has been tested by measuring the contraction of isolated mesenteric artery mounted in a myograph. We have recorded potassium currents (I<sub>K</sub>) activating around -40 mV and showing a slow inactivation (τ ≈ 3 s) in response to prolonged depolarising stimulations (10 s). Only the inactivating component of the current was sensitive to 4-AP (0.1 to 5 mM). Changing the Ca<sup>2+</sup> concentration in the extracellular solution from 2 mM to 0 mM did not modify the amplitude or the kinetic parameters of the inactivating component of I<sub>K</sub>. Surprisingly, lowering the extracellular Ca<sup>2+</sup> concentration significantly decreased the inhibitory potency of the lower concentrations of 4-AP (0.1 to 0.5 mM) on I<sub>K</sub>: 0.5 mM 4-AP inhibited the inactivating component of I<sub>K</sub> by 8 ± 4% (*n* = 7) in the absence of Ca<sup>2+</sup> compared to 35 ± 4% (*n* = 8, *P* < 0.05 vs. without Ca<sup>2+</sup>) in the presence of 2 mM Ca<sup>2+</sup>. The effect of higher concentrations of 4-AP was unaffected. The component of I<sub>K</sub> that was sensitive to submillimolar concentration of 4-AP was resistant to stromatocin, a selective inhibitor of Kv2.1, and activated at more

negative voltage. Functional studies showed that 0.5 mM 4-AP significantly enhanced the contraction evoked by the  $\text{Ca}^{2+}$  channel agonist BayK8644 in the presence of 6–8 mM/KCl ( $n = 4$ ), while stomatoxin increased mesenteric artery reactivity to 7–13 mM/KCl. These data show that extracellular  $\text{Ca}^{2+}$  modulates the inhibition by 4-AP of Kv channels that are distinct from Kv2.1 and that contribute to the regulation of the cell membrane potential.

### P033

#### On pharmaco-physiology of mechanosensitive ionic vesical channels (detrusor, trigone)

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Spontaneous vesical detrusor (D) phasic (SPC) and trigonal (T) tonic (STC) contractions (amplitudes=A, frequency=F; guinea pig) are influenced by stretch (Martin *et al.*, 2007; Michailov *et al.*, 2007): Pharmacological effects will be given. Recording of motor (SPC/STC: isometric) activity (Martin *et al.*, 2007; Michailov *et al.*, 2007; Welsher *et al.*, 2007; Neu *et al.*, 2008). Table 1 demonstrates strong decrease of D-A of SPC in relaxed (3 mN), but not in stretched (50 mN) prep. after quinidine: High sensitivity to verapamil (1  $\mu\text{M}$  changed STC) and other complex drug/ionic effects (nifedipine, TEA) were observed (total  $n = 119$ ). Large variation of drug effects on STC/SPC probably is caused by stretch-dependent action potentials of vesical myocytes (Martin *et al.*, 2007; Michailov *et al.*, 2007).

Table 1. Initial values at tension 3 mN = 100%.

	3 mN		50 mN	
	A	F	A	F
Detrusor: Quinidine 100 $\mu\text{M}$ , Verapamil 10 $\mu\text{M}$				
Q:	16.1 $\pm$ 13.6	107.8 $\pm$ 13.1	94.9 $\pm$ 25.0	22.1 $\pm$ 56.3
V:	95.4 $\pm$ 37.3	42.3 $\pm$ 8.1	140.7 $\pm$ 37.8	66.3 $\pm$ 23.7
K:	354.2 $\pm$ 93.0	162.1 $\pm$ 56.0	467.5 $\pm$ 124.2	163.3 $\pm$ 39.9
Ca:	101.2 $\pm$ 3.3	66.9 $\pm$ 12.4	111.2 $\pm$ 8.8	72.7 $\pm$ 10.3
Trigone: Quinidine 100 $\mu\text{M}$ , Verapamil 1 $\mu\text{M}$ + 100 $\mu\text{M}$				
Q:	43.1 $\pm$ 4.6	405.2 $\pm$ 99.8	50.1 $\pm$ 10.4	203.2 $\pm$ 30.1
V1:	102.3 $\pm$ 10.5	70.2 $\pm$ 6.3	98.7 $\pm$ 4.3	89.0 $\pm$ 12.1
V100:	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	94.4 $\pm$ 3.6	115.2 $\pm$ 17.9
K:	96.9 $\pm$ 20.3	198.8 $\pm$ 71.3	86.8 $\pm$ 38.2	764.2 $\pm$ 121.3
Ca:	82.5 $\pm$ 7.8	65.8 $\pm$ 20.0	133.7 $\pm$ 36.5	18.8 $\pm$ 10.8

D-A = 4.8  $\pm$  0.9 mN, F = 2.9  $\pm$  0.3/min; T-A = 18.6  $\pm$  3.2, F = 0.3  $\pm$  0.1  
KCl (3x 5.6 mM),  $\text{CaCl}_2$  (3x 2.1 mM) ( $n = 44$ ,  $P < 0.05$ ,  $P < 0.01$ )

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### P034

#### On vesical electro-pharmacology and pharmaco-toxicology

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Description of *fundamental patterns* of action potentials (AP) in detrusor (D) myocytes, i.e. spikes (S), bursts (B), burst-plateaus (BP) and their stretch dependence (transformation of S into BP) opens new dimensions in vesical pharmacophysiology (Martin *et al.*, 2007; Michailov *et al.*, 2007; Welsher *et al.*, 2007; Neu *et al.*, 2008). Recording of AP (intracellular) and contractions (isometric) to electrical neurogenic (CENS: 10 Hz, 0.3 ms, 3 s) and myogenic (CEMS: 10 Hz, 40 ms, 3 s) stimulation of guinea-pig D-preparations (myocytes) (Michailov *et al.*, 2007). Results: (i) stretch (3 to 50 mN) had an augmentory effect on CENS/CEMS, (ii) induced transformation of S/B into BP, whereby (iii) hypothermia (37 to 31 °C) re-transformed BP into S, (iv) [KCl]-increase (> 2x: 5.6 mM=1x) and [CaCl<sub>2</sub>]-reduction (0.5x: 2.1 mM=1x) transformed also S into BP, (v) MgCl<sub>2</sub> (> 1x=1.2 mM) had opposite effects, (vi) TEA (500  $\mu\text{M}$ ) augmentory effect on CENS/CEMS was stronger in relaxed (3 mN) than in stretched (50 mN) preparations; verapamil (0.1–10  $\mu\text{M}$ ): inhibitory effect and transformation of S into BP, (vii) fenoterol (1 nM–1  $\mu\text{M}$ ): decrease of BP-frequency; PCP, pyrethroids (1–100  $\mu\text{M}$ ) transformed S into BP (total  $n = 110$ ). *Mechanosensitive channels* (Ca-activated K-) participate essentially in generation of electrical D-patterns and electro-mechanical coupling, whereby drugs/toxicants modified this. Further pharmaco-physiological and clinical observations could clarify importance of these results for D-collection/expulsion phase, pathology (incontinence, radiocystitis, etc.), and therapy.

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**P035****Antioxidant effects of a polyphenolic compound quercetin in mouse corpus cavernosum**

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Flavonoids are plant based phenolic compounds, and quercetin is the most abundant dietary member of this family. Quercetin is known to have antioxidant effects (Heijnen *et al.*, 2001). The decrease in the bioavailability of nitric oxide is important in the pathogenesis of erectile dysfunction (Jones *et al.*, 2002). This study investigated the possible antioxidant effect of quercetin on mouse corpus cavernosum. Male, Swiss albino mice weighing approximately 30 g were used. Isolated strips were incubated for 1 h followed by phenylephrine (5  $\mu$ M) induced contraction. Relaxations were induced by acetylcholine (0.1–1  $\mu$ M), electrical field stimulation (1–16 Hz, 30 V, 0.5 ms), or acidified sodium nitrite (0.5 mM). Oxidant stress was produced by superoxide anion generators; pyrogallol (50  $\mu$ M), hydroquinone (100  $\mu$ M), 6-anilino-5,8-quinolinedione (10  $\mu$ M), diethyldithiocarbamic acid (superoxide dismutase inhibitor; 8 mM), or diethyldithiocarbamic acid plus 6-anilino-5,8-quinolinedione. The effect of quercetin (10  $\mu$ M) on the relaxations influenced by these agents were recorded with isotonic transducers. All of the agents studied significantly inhibited the acidified sodium nitrite induced responses. Quercetin significantly restored these inhibited responses except the 6-anilino-5,8-quinolinedione studied groups. Quercetin failed to restore the inhibitions on acetylcholine or electrically stimulated responses. This study suggests that quercetin protects mouse corpus cavernosum against oxidant stress by scavenging superoxide anions and increasing exogenous nitric oxide bioavailability.

**References:**

Heijnen *et al.* *Environ Toxicol Pharmacol.* 2001; 10: 199–206.  
Jones *et al.* *Expert Opin Pharmacother.* 2002; 3: 889–897.

**P036****Stretch mediated alteration of nitric oxide bioavailability does not involve extracellular superoxide anion**

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We recently demonstrated that increasing the transmural stretch of isolated rings of rat aorta from 1 g to 5 g abolished the tonic vasodepressor influence of basally released nitric oxide (NO) whilst, conversely, the relaxant action of agonist-stimulated activity of NO remained unaffected. Production of superoxide anion (SA), which destroys NO, is known to be sensitive to mechanical forces. The aim of this study, therefore, was to determine if SA played a role in the altered NO bioavailability observed at different levels of transmural stretch. Rings of thoracic aorta were mounted in organ baths under 1 or 5 g resting tension. Responses to phenylephrine, acetylcholine and the NO donor spermine-NO complex (SPER/NO) were recorded  $\pm$  superoxide dismutase. In endothelium containing rings under 1 or 5 g stretch PE-induced contraction was unaltered in the presence of SOD suggesting that endogenous SA does not affect basal NO and is not the cause for the absence of basal NO at 5 g stretch. ACh-induced relaxation was also unaltered by SOD suggesting that SA does not alter stimulated endothelial NO production. In contrast SPER/NO-induced relaxation was enhanced by SOD at the mid-range concentrations with maximum responses unchanged. This suggests that SPER/NO-mediated relaxation is sensitive to partial impairment by SA and this impairment is independent of the level of initial stretch.

**P037****Polypeptide from *Chlamys farreri* inhibits apoptosis in HaCaT cells induced by ultraviolet B via inducible NO synthase and HSP pathway**

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There is a common perception during these years that it is the solar ultraviolet radiation that increases the incidence of human skin cancer and other adverse effects, so we need an effective chemoprevention strategy to inhibit it. Polypeptide from *Chlamys farreri* (PCF) has been studied as an antioxidant and photoprotective agent. Our previous study has demonstrated that PCF could reduce the intracellular reactive oxygen species (ROS) production and inhibit apoptosis in HaCaT cells induced by ultraviolet B (UVB) -radiation. This study is aimed at determining the roles that inducible NO synthase (iNOS) and HSP play in the dose of 20 mJ/cm UVB-radiation-induced HaCaT cells and the protecting effect of PCF. UVB-induced apoptosis was proved by MTT method and DNA fragments respectively. RT-PCR and Western blot showed that the expression of iNOS was increased in HaCaT cells by UVB-radiation followed by upgrade of NO level which was detected by ESR; however, the expression of iNOS and NO level was decreased in HaCaT cells which had been pre-treated with PCF. Moreover, Western blot showed that, as anti-apoptosis protein, levels of heat shock protein (HSP) 70 and HSP 90 were up-regulated in HaCaT cells which had been pre-treated with PCF. Finally, PCF and the iNOS inhibitor observed SMT were shown to inhibit the apoptosis of HaCaT cells induced by UVB-radiation at the dose of 20 mJ/cm. The opposite changes in the pre-treated HaCaT cells were shown to be positively dose-dependent, from 1.42 M/L to 5.68 M/L. All the differences reached statistical significance ( $P < 0.01$ ). We might conclude that iNOS, NO, and HSP played important roles in UVB-radiation-induced apoptosis of HaCaT cells and PCF could inhibit the apoptosis of HaCaT cells by affecting those factors. This study shows that PCF can inhibit UVB-mediated damages by modulating iNOS and HSP, and it could be as valuable as an effective chemoprevention strategy to protect human from the solar ultraviolet radiation.

**P038****Comparative studies of serum Semicarbazide-sensitive amine oxidase (SSAO) activity and nitrite/nitrate (NOx) concentrations in type 2 diabetic patients**

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Semicarbazide-sensitive amine oxidases (SSAO), such as monoamine oxidases (MAO A and B) deaminate endogenous amines to produce aldehyde, hydrogen peroxide and ammonia. Increased circulating SSAO activity may contribute to protein deposition, inflammation and thus may be involved in the pathophysiology of chronic vascular and neurological disorders, such as diabetic complications, chronic heart failure, and multiple types of cerebral infarcts (Ramonet *et al.*, 2003). Nitric Oxide (NO) is a highly unstable free radical in the circulating blood, it is oxidized rapidly to nitrite and nitrate and could therefore be of high relevance as a biochemical parameter in experimental and clinical studies (Stamler *et al.*, 1992). The aim of this work was to evaluate the SSAO activity and nitrite/nitrate (NOx) concentrations in serum of healthy donors and diabetics type 2 patients and related with some biochemical parameters investigating whether they could be useful as markers for type 2 diabetes. A radiochemical procedure was adapted in order to determine human serum SSAO activity using [<sup>14</sup>C]-Benzylamine (54Cm/L) as a substrate, in the presence of monoamine oxidase inhibitors clorgyline 10<sup>-4</sup> M for MAO A and selegiline 10<sup>-4</sup> M for MAO B. Biochemical analyses of concentrations of glucose, HbA1c, total cholesterol and triglyceride was determined in each case by standard laboratory methods. The NOx concentrations, obtained by the sum of nitrites and nitrates, were measured by the Griess reaction. Standard curves were made with sodium nitrite and potassium nitrate in saline solution (linear range 0–100  $\mu$ M/L). Serum SSAO activity was significantly increased in patients with type 2 diabetes (616.2  $\pm$  119.8 mU/L, (mean  $\pm$  SD,  $n = 20$ ) compared to controls (408.4  $\pm$  103.5 mU/L,  $P = 0.0001$ ),  $n = 20$ . In diabetic patients, serum SSAO activity is correlated with the serum creatinine (Person  $r = 0.676$ ,  $P = 0.0011$ ) and triglycerides (Person  $r = 0.5422$ ,  $P = 0.0335$ ). The NOx levels in type 2 diabetic patients were significantly higher (85.06  $\pm$  17.07  $\mu$ M/l) than those in the healthy control subjects (67.9  $\pm$  16.44  $\mu$ M/l,  $P = 0.0015$ ). Serum NOx values in type 2 diabetic patients were negatively correlated with age, serum creatinine and microalbuminuria. Serum NOx levels in control subjects positively correlated with serum SSAO activity (Person  $r = 0.5461$ ,  $P = 0.0321$ ). In conclusion, our results show a significant increase in serum SSAO activity and NOx concentrations, as other studies described. Taken together with the correlation established our findings demonstrate that both methods are useful to the evaluation of type 2 diabetes and they could be used as markers of diabetes.

Supported by FCT - Portugal.

**References:**

Ramonet *et al.* *Inflammopharmacology.* 2003; 11: 111–117.  
Stamler *et al.* *Science.* 1992; 258: 1898–1902.

**P039****Astaxanthin treatment protects against cardiovascular alterations in angiotensin II-infused mice**

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Hypertension is associated with oxidative stress and several studies have shown the beneficial effects of antioxidants. Elevated levels of angiotensin II (Ang II) have been implicated in the pathophysiological processes that occur in hypertension. The aim of this study was to investigate if chronic infusion of Ang II leads to progressive hypertension over a 2 weeks period and how that is modified by concomitant treatment with astaxanthin. D-1 mice were prepared by the infusion of Ang II at a dose of 1.4 mg/kg/day via osmotic minipumps implanted subcutaneously for 14 days (ANG) and compared with Sham-operated mice (SHAM). A group infused with Ang II were treated with astaxanthin (100 mg/kg/day) in the diet (AXT). Systolic blood pressure (SBP) was measured by the tail-cuff method. Ventricles were weighted and body weight ratio was calculated as a cardiac hypertrophy index and fibrosis was assessed by hydroxyproline content, using a colorimetric method (Jamall *et al.*, 1981). NADPH-oxidase activity was measured in aorta rings by lucigenin-enhanced chemiluminescence detection of superoxide. BP was comparable in the three groups before implantation of osmotic minipumps but progressively increased in Ang II-infused rats (ANG, 148  $\pm$  1 vs. SHAM, 104  $\pm$  1 mmHg,  $P < 0.05$ ). astaxanthin administration prevented the Ang II-dependent increase in SBP (127  $\pm$  1 mmHg). Ang II infusion increased fibrosis (from 28.8  $\pm$  3.9 to 46.6  $\pm$  3.7  $\mu$ g hydroxyprolin/mg protein) and cardiac hypertrophy index (from 3.8  $\pm$  0.1 to 4.3  $\pm$  0.1  $\pm$  0.1) and superoxide anion (O<sub>2</sub><sup>-</sup>) production (from 2848  $\pm$  423 to 4622  $\pm$  599 RLU/mg protein/min,  $P < 0.05$ ). Astaxanthin was able to prevent from these alterations. Our results suggest that astaxanthin significantly attenuated the pathophysiological processes that exert Ang II in this experimental model of hypertension.

Astaxanthin was kindly supply by BASF.

**Reference:**

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**P040****Pravastatin improve nitric oxide bioavailability after chonic treatment in SHR**

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There is accumulating evidence that statins, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, exert numerous beneficial effects that are apparently independent of their action on blood lipids. Statins significantly reduce cardiovascular morbidity and mortality. The aim of this work was to investigate the cardiovascular benefits of pravastatin on adult SHR. That statin was administered (20 mg/kg/day) to 16-weeks-old rats in the drinking water during 4 weeks and

compared with a control group receiving tap water. Systolic blood pressure, plasma cholesterol and urinary nitrite excretion were determined. Aortic and mesenteric rings were used for studying the endothelial function and superoxide anion production. The vascular response to ACh and sodium nitroprusside (SNP) was determined and the superoxide accumulation was assessed with the lucigenin-enhanced chemiluminescence technique. Result showed that pravastatin reduced systolic blood pressure in 20 mmHg without changes in plasmatic cholesterol. Relaxation to ACh was improved only in mesenteric arteries and no differences were observed in the SNP response. We found a larger nitrite excretion in treated rats ( $22.3 \pm 0.8$  vs.  $19.5 \pm 0.6 \mu\text{M}$ ,  $P < 0.05$ ). The pravastatin group showed a decrease in superoxide basal anion production ( $66.3 \pm 3.0$  vs.  $53.3 \pm 1.7 \text{RLU}/\text{mg}/\text{min}$ ,  $P < 0.05$ ) when evoked by NADPH ( $2351 \pm 208$  vs.  $1177 \pm 121 \text{RLU}/\text{mg}/\text{min}$ , when  $P < 0.05$ ). In conclusion, the beneficial effect of pravastatin on blood pressure could be related to an increased NO bioavailability due to a lower production of superoxide anions.

#### P041

##### **Crataegus microphylla improves endothelial dysfunction in streptozotocin-induced diabetic rats**

E.Koç<sup>a</sup>, G.Topal<sup>a</sup>, BS Uydes Dogan<sup>a</sup>, G.Melikoglu<sup>b</sup>, AH Mericli<sup>b</sup>, C Karaca<sup>c</sup>, T Altug<sup>c</sup>, O Ozdemir<sup>d</sup> *Istanbul University, Faculty of Pharmacy, Department of Pharmacology, Istanbul, Turkey;* <sup>b</sup>*Istanbul University, Faculty of Pharmacy, Department of Pharmacognosy, Istanbul, Turkey;* <sup>c</sup>*Istanbul University, Animal Research Laboratory Cerrahpasa Medical Faculty, Istanbul, Turkey;* <sup>d</sup>*Sanovel Pharmaceutical Company, Istanbul, Turkey* Diabetes-induced endothelial dysfunction contributes to increased cardiovascular risks. In this study, we aimed to investigate the effect of *Crataegus microphylla* leaf extract, which has been used as a folk medicine for some cardiovascular diseases (Rigelsky *et al.*, 2002) on the endothelial dysfunction in diabetic rats. Male Sprague Dawley rats weighing  $250 \pm 50$  g were injected with streptozotocin (STZ, 60 mg/kg, i.p.) to induce diabetes. Treatment with *C. microphylla* (100 mg/kg, day) or vehicle was initiated 4 weeks after induction of diabetes and continued for 4 weeks. Thereafter, vascular function was characterized in isolated aortic segments in organ bath chambers. Direct effects of *C. microphylla* on endothelium-dependent vasorelaxation were also investigated *in vitro*. *C. microphylla* induced a concentration-dependent vasorelaxation that was inhibited by nitric oxide (NO) synthase inhibitor, L-NG-nitro arginine. Endothelium-dependent vasorelaxation induced by acetylcholine was significantly reduced in aortae from diabetic rats and improved by the treatment with *C. microphylla* (maximum relaxation, % of precontraction-control:  $82.54 \pm 5.88$ , STZ-vehicle:  $61.72 \pm 4.48$ , STZ-*C. microphylla*:  $83.45 \pm 2.69$ ;  $P < 0.01$ ). We demonstrate that *C. microphylla* acutely cause NO-mediated vasorelaxation and chronically normalizes endothelial function in diabetic rats. These results suggest that *C. microphylla* might have beneficial effects on diabetes-induced endothelial dysfunction.

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##### **Reference:**

Rigelsky *et al.* Am J Health Syst Pharm. 2002; 59(5): 417–422.

#### P043

##### **The role of the serotonergic system in the antidepressant-like effect of TRIM in the rat forced swimming test**

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It is widely known that serotonin plays an important role in the antidepressant-like effect of drugs (Harkin *et al.*, 2003). The aim of this study is to investigate the involvement of serotonergic system in the antidepressant-like effect of TRIM, a nNOS inhibitor TRIM in the rat forced swimming test (FST). 220–300 g adult Wistar albino male rats were used in this study. Two way ANOVA *post hoc* Dunnett test was used for the evaluation of the results. Pretreatment with parachlorophenylalanine methyl ester (pCPA; 3 x 150 mg/kg, intraperitoneally (i.p.), an inhibitor of serotonin synthesis) ( $214.63 \pm 4.12$ ,  $n = 16$ ), methiothepin (0.1 mg/kg, i.p. a non-selective 5-HT receptor antagonist) ( $231.71 \pm 4.57$ ,  $n = 7$ ), WAY 100635 (0.1 mg/kg i.p. a selective 5-HT<sub>1A</sub> receptor antagonist) ( $181.63 \pm 7.46$ ,  $n = 8$ ), GR 127935 (3 mg/kg i.p. a selective 5-HT<sub>1B/1D</sub> receptor antagonist) ( $185.14 \pm 7.98$ ,  $n = 7$ ), cyproheptadine (3 mg/kg i.p. a 5-HT<sub>2</sub> receptor antagonist) ( $209.77 \pm 6.57$ ,  $n = 7$ ) or ketanserin (5 mg/kg i.p. a 5HT<sub>2A/2C</sub> receptor antagonist) ( $207.29 \pm 6.57$ ,  $n = 7$ ) prevented the effect of TRIM (50 mg/kg, i.p.) in the FST. The results of this study demonstrates that antidepressant-like effect of TRIM in the FST, seem to be mediated, at least in part, by interaction with 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptors.

##### **Reference:**

Harkin A *et al.* Neuropharmacology. 2003; 44: 616–623.

#### P044

##### **5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptors are involved in the anxiolytic-like effects of TRIM in the rat**

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Several studies have demonstrated that nitric oxide synthase (NOS) inhibitors have anxiolytic like effects in the plus-maze test (Faria *et al.*, 1997). The aim of this study was to evaluate the involvement of the serotonergic system in the anxiolytic-like effect of TRIM, a selective neuronal NOS inhibitor, in the elevated plus-maze test. 220–300 g adult Wistar albino male rats were used in this study. Two way ANOVA *post hoc* Dunnett test was used for the evaluation of the results. The anxiolytic-like

effect of TRIM was antagonised by pretreatment with the 5-HT depleting agent parachlorophenylalanine methyl ester (pCPA; 3 x 150 mg/kg, intraperitoneally (i.p.), an inhibitor of serotonin synthesis) ( $26.16 \pm 1.99$ ,  $n = 20$ ), methiothepin (0.1 mg/kg, i.p. a non-selective 5-HT receptor antagonist) ( $10.11 \pm 3.69$ ,  $n = 7$ ), WAY 100635 (0.1 mg/kg i.p. a selective 5-HT<sub>1A</sub> receptor antagonist) ( $20.20 \pm 3.28$ ,  $n = 8$ ), GR 127935 (3 mg/kg i.p. a selective 5-HT<sub>1B/1D</sub> receptor antagonist) ( $19.55 \pm 3.51$ ,  $n = 7$ ), cyproheptadine (3 mg/kg i.p. a 5-HT<sub>2</sub> receptor antagonist) ( $21.39 \pm 3.87$ ,  $n = 7$ ) or ketanserin (5 mg/kg i.p. a 5HT<sub>2A/2C</sub> receptor antagonist) ( $20.59 \pm 3.87$ ,  $n = 7$ ) prevented the effect of TRIM (50 mg/kg, i.p.) in the FST. In conclusion it is suggested that anxiolytic-like effects of TRIM is mediated at least in part by 5-HT<sub>1</sub> and 5-HT<sub>2</sub> receptors in the elevated plus-maze test in rats.

##### **Reference:**

Faria MS *et al.* Eur J Pharmacol. 1997; 323: 37–43.

#### P045

##### **Reduction of tissue plasminogen activator-induced haemorrhage by PJ34, a poly(ADP-ribose) polymerase inhibitor, after permanent cerebral ischemia in mice**

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The risk of intracerebral haemorrhage after tissue plasminogen activator (tPA) treatment of acute ischemic stroke remains one of the main impediments to its widespread use. This side effect of tPA seems to be related to tPA-induced up-regulation of matrix metalloproteinase-9 known to degrade the basal membrane components of blood vessels (Tsuiji *et al.*, 2005). In a model of cerebral ischemia, the poly(ADP-ribose) polymerase (PARP) inhibitor 3-aminobenzamide was shown to reduce brain MMP-9 overexpression (Koh *et al.*, 2005). In this context, we investigated whether the potent PARP inhibitor, PJ34 [N-(6-oxo-5,6-dihydrophenanthridin-2-yl)-2-(N,N-dimethylamino-acetamide)], affected the rate of haemorrhage due to tPA therapy in a model of permanent cerebral ischemia. Ischemia consisted in an intravascular occlusion of the left middle cerebral artery in male Swiss mice (30 g) anaesthetized with i.p. ketamine (50 mg/kg) and xylazine hydrochloride (6 mg/kg). Mice received either recombinant tPA (rtPA, Actilyse®, 10 mg/kg, i.v. 6 h after the onset of ischemia as a 10% bolus followed by an infusion over 30 min) or a combination of rtPA with the same protocol and PJ34 (3–6 or 12 mg/kg, i.p. just after and 4 h after the onset of ischemia). Control ischemic animals received the vehicle of rtPA and PJ34 (saline) with the same protocol. The neurological deficit (evaluated by a grip and a string test), infarct volume and intracerebral haemorrhage (counted on twelve 20  $\mu\text{m}$ -thick coronal brain slices) were assessed 48 h after ischemia. The rt-PA-treated ischemic mice showed a significant increased score of intracerebral haemorrhage compared to saline-treated ischemic mice ( $83 \pm 10$  versus  $50 \pm 8$ ,  $P < 0.05$ ). PJ34 reduced rt-PA-induced aggravation of haemorrhagic score at 3 mg/kg ( $37 \pm 7$ ,  $P < 0.01$ ) and 6 mg/kg ( $52 \pm 11$ ,  $P < 0.05$ ). PJ34 at 3 mg/kg also significantly improved the score in the string test ( $P < 0.05$ ) and tended to reduce the deficit in the grip test ( $P = 0.07$ ). Whatever the dosage, PJ34 had no effect on the infarct volume. The present study demonstrates that PARP inhibition prevents rtPA-aggravation of both intracerebral haemorrhage and neurologic deficit. Thus PARP inhibition could be a valuable strategy to optimise tPA thrombolysis in stroke.

##### **References:**

Tsuiji *et al.* Stroke. 2005; 36: 1954–1959.

Koh *et al.* Toxicology. 2005; 214: 131–139.

#### P046

##### **The effect of long-term resveratrol treatment on endothelial function of aorta from diabetic rabbit**

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The polyphenolic compound resveratrol presented in red wine has beneficial cardiovascular effects. In this study, the effect of long-term resveratrol treatment on endothelium-dependent relaxations to acetylcholine was investigated on aortae from alloxan diabetic rabbits. Superoxide production was also evaluated to explain the mechanism of action of resveratrol. Diabetes was induced with a single dose of alloxan (100 mg/kg, i.v.) dissolved in physiological saline in male New Zealand white rabbits weighing 2.0 to 2.5 kg. Concentration-response curve to acetylcholine ( $10^{-8}$ – $10^{-4}\text{M}$ ) was obtained in aortic rings with endothelium from control, diabetic, long-term resveratrol-treated (5 and 50 mg/L in drinking water for 10 weeks) diabetic rabbits. Acetylcholine produced concentration-dependent relaxation with a higher potency in endothelium-intact aortic rings of control rabbits than those of diabetic ( $n = 7$ – $11$ ,  $P < 0.05$ ). Resveratrol treatment increased endothelium-dependent relaxations to acetylcholine in aortae from diabetic rabbits ( $n = 5$ – $7$ ,  $P < 0.05$ ). The basal and NAD(P)H-induced superoxide productions were more pronounced in the artery segments from diabetic rabbits. Resveratrol treatment decreased superoxide production in the aortae from control and diabetic rabbits ( $n = 6$ – $10$ ,  $P < 0.05$ ). The increased relaxations in resveratrol-treated diabetic rabbits could be related to increased endothelial reactivity. The favourable effects of resveratrol may have potential clinical value.

**P047****A microplate-based spectrophotometric method for the determination of total antioxidant capacity of human plasma: modified cupric reducing ability assay**

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Reactive Oxygen Species (ROS) are continuously generated and exert physiological action during metabolism. At 'oxidative stress' status, overproduction of ROS reduces the content of the natural antioxidant defence system, then the biological macromolecules become vulnerable. Quantitation of the total antioxidant content of the body is important for the accurate assessment of the oxidative stress. In this study, we described a simple spectrophotometric method for the measurement of the total antioxidants level, in human plasma, using 96-well microplate. The reaction based on the reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$  by the antioxidants of plasma, including vitamins, thiol groups, reduced glutathione, uric acid and bilirubin. We used Neocuproine (Nc) as a chromogenic agent, to detect the generated  $\text{Cu}^{1+}$ , in neutral medium (Besada *et al.*, 1989; Apak *et al.*, 2005). The maximum absorbance of the formed 2 : 1 colored complex was 455 nm. Two hundred microliter diluted plasma sample or Trolox (as a reference antioxidant), and 50  $\mu\text{l}$  'Copper reagent' [Cupric ammonium chloride + Nc] was added directly to the 96-well microplate and the absorbance was read by a microplate reader, at 455 nm. Trolox was used from 0.98  $\mu\text{M}$  to 1.0 mM. The data were analyzed by a '4-parameter logistic' method. The correlation coefficient of the fitted curve was 0.999. Standard addition of Trolox to the human plasma, from 0–250  $\mu\text{M}$ , resulted a linear curve ( $n = 10$ ), with the correlation coefficient of 0.997, slope 0.0044 and intercept 0.015. This data demonstrated that, there were no chemical interactions between the chemicals added synthetically and the real matrix of the human plasma, and also the antioxidant capacity was additive. Using a previously described method, we found the antioxidant capacity of 32 young (ages between 20–30) and healthy men, as  $757.6 \pm 31.9 \mu\text{M}$  Trolox equivalent. The method proved to be efficient, reasonably selective, sensitive and easily accessible for the measurement of the antioxidant capacity of human plasma.

**References:**

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Besada A *et al.* Microchimica Acta. 1989; 99: 143–146.

**P048****Reactive oxygen species inhibit calcium- and carbachol-induced contractile responses in  $\beta$ -escin permeabilized rat bladder detrusor**

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Reactive oxygen species (ROS) alter muscle contraction by affecting either intracellular contractile proteins or  $\text{Ca}^{2+}$  movements of sarcoplasmic reticulum (SR) and mitochondria (Darnley *et al.*, 2001). We studied how ROS affect  $\text{Ca}^{2+}$  and carbachol contractions in permeabilized rat (Wistar, male, 200–250 g) bladder detrusor. Strips of detrusor were mounted in a chamber filled with Krebs' solution under 100 mg tension. Isometric contractions were recorded and expressed as % of 80 mM  $\text{K}^+$  (130.0  $\pm$  10.7 mg,  $n = 52$ ). Data are mean  $\pm$  SEM. Statistics was done by ANOVA/Bonferroni.  $P < 0.05$  was accepted as significant. Strips were permeabilized with 40  $\mu\text{M}$   $\beta$ -escin. Cumulative  $\text{Ca}^{2+}$  contractions elicited (pCa 7.5–4.5,  $E_{\text{max}}=64.2 \pm 3.3\%$ ,  $n = 9$ ) were inhibited by hydrogen peroxide ( $E_{\text{max}}=38.5 \pm 4.5\%$ ,  $n = 5$ ) or hydroxyl radical ( $E_{\text{max}}=33.5 \pm 7.4\%$ ,  $n = 6$ ). Scavengers of these radicals prevented this inhibition. While  $\text{Ca}^{2+}$  contractions were decreased with 1  $\mu\text{M}$  SR  $\text{Ca}^{2+}$ -ATPase pump inhibitor cyclopiazonic acid (CPA) ( $E_{\text{max}}=37.2 \pm 5.6\%$ ,  $n = 7$ ) or 1  $\mu\text{M}$  mitochondrial blocker carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) ( $E_{\text{max}}=46.4 \pm 4.3\%$ ,  $n = 7$ ), hydrogen peroxide or hydroxyl radical did not further inhibited these responses. After filling the intracellular stores with  $\text{Ca}^{2+}$ , a further contraction was elicited by 50  $\mu\text{M}$  carbachol (28  $\pm$  4.2%,  $n = 10$ ) that was decreased by hydrogen peroxide (5.5  $\pm$  1.1%,  $n = 7$ ), hydroxyl radical (9.9  $\pm$  1.4%,  $n = 6$ ) or superoxide anion generator pyrogallol (8.1  $\pm$  1.4%,  $n = 5$ ). Scavengers of these radicals prevented this decrease. CPA decreased the carbachol contraction to 15.8  $\pm$  3.0% ( $n = 6$ ) which was not additionally changed by hydrogen peroxide or hydroxyl radical but inhibited by pyrogallol (7.2  $\pm$  1.9%,  $n = 4$ ). FCCP decreased the carbachol contraction to 16.2  $\pm$  1.3% ( $n = 4$ ) and this was further inhibited by hydrogen peroxide (7.3  $\pm$  0.8%,  $n = 4$ ) or hydroxyl radical (9.2  $\pm$  0.9%,  $n = 4$ ) but not by pyrogallol. Hydroxyl radical decreased 50  $\mu\text{M}$  inositol triphosphate contraction from 10.9  $\pm$  0.6% to 7.8  $\pm$  0.5% ( $n = 5-8$ ) whereas none of the radicals affect carbachol-induced  $\text{Ca}^{2+}$  sensitization. We may propose that ROS inhibit contractions by affecting different intracellular  $\text{Ca}^{2+}$  stores in rat detrusor, i.e. hydrogen peroxide and hydroxyl radical acts on SR where superoxide anion acts on mitochondria. Supported by NOVARTIS.

**Reference:**

- Darnley GM *et al.* Exp Physiol. 2001; 86.2: 161–168.

## P049

### Studying ion channel pharmacology using the Dynaflo patch clamp system

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The patch clamp technique is still the golden standard for studying the interaction of drug molecules and ion channel receptors. Fast and reliable drug application is crucial for such interaction studies. The Dynaflo system uses cells lifted up from the bottom of the recording chamber and moves them in front of the openings of parallel drug ejection channels by a computer controlled system. Here we compare the Dynaflo system with conventional manifold-based drug application in whole-cell patch clamp experiments. Dofetilide and six Richter compounds inhibited human recombinant Kv11.1 voltage gated potassium channels (hERG) in HEK cells. There were no significant differences between drug potencies whether determined by the Dynaflo or the conventional patch clamp method. Analysis of current traces indicated that the fluid exchange was much faster when the Dynaflo system was used, and drug application was also more reliable, resulting in an increased throughput of drug testing. The Dynaflo system can be equipped with a temperature controlling system. Experiments done at 37 °C degrees were also performed and showed no significant temperature dependence of potencies of two compounds. We also tested, whether the application of different concentrations of trypsin in the cell harvesting protocol has any effect in the results and found no significant effect in the currents and in the potency of haloperidol. Comparison of the physiology and pharmacology of different subtypes of recombinant sodium channels did not show significant differences when conventional and the Dynaflo systems were used. Besides testing voltage gated sodium and potassium channels; the advanced drug application system of Dynaflo proved to be suitable for studying ligand gated ion channels, like NMDA, P2X, TRPV, and glycine receptors. In addition to HEK and CHO cells expressing recombinant ion channels; we also run experiments with one-day-old dorsal root ganglion cells. In conclusion the Dynaflo system produced pharmacological results comparable to that of conventional patch system, and proved to be superior concerning reliability of drug application.

## P050

### Evaluation of beat-to-beat variability parameters in anaesthetised dogs and guinea pigs

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Following the implementation of ICH S7B guidelines, there is a need for good predictors of pro-arrhythmic potential other than QT prolongation, which has an imperfect correlation with arrhythmogenicity. The evaluation of beat-to-beat variability of repolarisation (BVR) has been shown to help in predicting the occurrence of cardiac arrhythmias in anaesthetised dog preparations. We investigated whether BVR parameters (short-term variability (STV) and short-term instability (STI)) could be used in our pre-clinical assays to discriminate between hERG blocking compounds associated with arrhythmias in humans (e.g. dofetilide) and those generally considered to be safe (e.g. moxifloxacin). Anaesthetised dogs ( $n = 4$ /group), instrumented to measure QT interval, were administered dofetilide (0.02–0.09 mg/kg i.v.) or moxifloxacin (1–30 mg/kg i.v.). Anaesthetised guinea pigs ( $n = 5$ –6/group), instrumented to measure the left ventricular monophasic action potential duration (MAPD), were administered dofetilide (0.002–0.07 mg/kg i.v.) or moxifloxacin (15–31 mg/kg i.v.). STV and STI were calculated from 30 consecutive beats at baseline and following test substance administration (at  $C_{max}$ ). Dofetilide and moxifloxacin caused statistically significant increases in dog QT interval and in guinea pig MAPD ( $P < 0.05$ – $0.01$ ). In dogs, dofetilide caused significant increases in STV and/or STI at free plasma concentrations 8–40 times the human therapeutic concentration (STV  $2.2 \pm 0.2$  ms vs.  $1.3 \pm 0.1$  ms in vehicle group; STI  $2.1 \pm 0.3$  ms vs.  $1.1 \pm 0.2$  ms in vehicle group;  $P < 0.05$ ). In guinea pigs, dofetilide caused significant increases in STV and STI at free plasma concentrations 30 times the human therapeutic concentration (STV  $1.7 \pm 0.5$  ms vs.  $0.6 \pm 0.2$  ms in vehicle group; STI  $1.5 \pm 0.4$  ms vs.  $0.4 \pm 0.2$  ms in vehicle group;  $P < 0.05$ ). In contrast, moxifloxacin had no significant effect on STV or STI in dogs or guinea pigs at free plasma concentrations 1–9 times the human therapeutic concentration. These preliminary data, in accord with previous publications, suggest that increases in variability/instability of ventricular repolarisation may be more predictive for assessing the risk of drug-induced arrhythmias than increases in QT interval/MAPD alone.

## P051

### Relationship between antimuscarinic activity and clinical QT effects for drugs approved for the treatment of overactive bladder

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Several antimuscarinic (AM) drugs are approved for the treatment of overactive bladder. Activity at M2 and M3 muscarinic receptor subtypes is considered relevant for the efficacy of AM drugs. Some AM drugs have balanced M2/M3 selectivity (tolterodine, fesoterodine, and oxybutynin) while others are M3-selective (darifenacin, solifenacin). The proarrhythmic safety profiles of AM drugs have been characterized through preclinical assays (hERG), thorough QT (TQT) trials, and reports of Torsade de Pointes (TdP) and ventricular tachycardia in clinical trials and post-marketing surveillance. A number of approved AM drugs were compared for their effects at muscarinic receptors and cardiac safety-related pharmacology and clinical experience.

Compound	M3 Ki (nM)	hERG Ki (nM)	hERG:M3 Selectivity	TQT Results	TdP Reports
Atropine	0.24	>1000	>4000	No data	No
Oxybutynin	2.2	>8000	>3600	No data	No
Fesoterodine <sup>1</sup>	0.75	918	1224	No effect	No
Darifenacin	7.1	1100	155	No effect	No
Tolterodine	1.3	34	26	Modest <sup>2</sup>	No
Solifenacin	18.4	190	10	Modest <sup>2</sup>	Yes <sup>3</sup>
Terodiline	246	2065	8	No data	Yes

<sup>1</sup>tested as its metabolite and principal active moiety, 5-hydroxymethyl tolterodine; <sup>2</sup>only at supratherapeutic doses; <sup>3</sup>post-marketing surveillance data in Vesicare US label, Feb 2008.

The divergence of hERG and M3 affinities indicates that their respective binding sites are distinct, with no apparent relationship between the clinical efficacy mechanism and proarrhythmic potential of a range of AM drugs. Although modest hERG blockade is seen for some AMs, no clinical signal for QT prolongation is seen (fesoterodine, darifenacin) or a modest QT prolongation is seen at supratherapeutic doses only (tolterodine, solifenacin). Furthermore, of the AM drugs reviewed, TdP has been definitively associated only with terodiline, resulting in its withdrawal in 1991.

## P052

### Thorough QT study to evaluate the effect of fesoterodine on cardiac repolarization

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The primary objective was to assess the effect of fesoterodine on the corrected QT (QTc) interval. Double-blind, parallel-group study in healthy volunteers ( $n = 256$ ): fesoterodine 4 mg, fesoterodine 28 mg (supratherapeutic dose), moxifloxacin 400 mg (positive control), or placebo once-daily for 3 days. Triplicate 12-lead ECGs were obtained at 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18, and 23.5 h post-dose on Days 1 and 3 and at matching time points for baseline (BL) on Day 0. QTc at BL was defined as the mean of the 36 ECGs on Day 0. For central tendency assessment, placebo-subtracted maximum and time-averaged QTc changes from BL were analyzed. For outlier analysis, subjects with QTc >450, >480 and  $\geq 500$  ms or QTc change from BL  $\geq 30$  or  $\geq 60$  ms were analyzed. Placebo-subtracted Fridericia-corrected QTc (QTcF) changes from BL were 0.5, 2.5, and 10.2 ms (maximum), and 0.8, 0.1, and 12.7 ms (time-averaged) for fesoterodine 4 mg, fesoterodine 28 mg, and moxifloxacin, respectively, on Day 3. No subject had QTcF >480 ms. QTcF >450 ms was noted in 4.6, 3.1, 0, and 10.9% of subjects, and 30.60 ms QTcF changes occurred in 15.4, 18.8, 17.6, and 50.0% of subjects receiving placebo, fesoterodine 4 mg, fesoterodine 28 mg, moxifloxacin, respectively. QTcF change  $\geq 60$  ms occurred in one subject receiving moxifloxacin. Moxifloxacin results confirmed the study sensitivity. The upper limit of 95% confidence interval for placebo-subtracted QTcF change from BL was <10 ms for both fesoterodine doses. Based on these results, fesoterodine does not have the potential to affect cardiac repolarization.