INTRINSIC ACTIVITY

VOL. 3 (SUPPL. 2)
9 SEPTEMBER 2015

WWW.INTRINSICACTIVITY.ORG
ISSN 2309-8503

PUBLISHED BY APHAR, THE AUSTRIAN PHARMACOLOGICAL SOCIETY

21st Scientific Symposium of the Austrian Pharmacological Society

Joint Meeting with the British Pharmacological Society and the Pharmacological Societies of Croatia, Serbia and Slovenia

Graz, 16–18 September 2015

MEETING ABSTRACTS

Intrinsic Activity is an online, open-access publication medium published by the Austrian Pharmacological Society (APHAR). The Journal welcomes contributions in the fields of Pharmacology, Pharmacotherapy and other fields in biomedicine.

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ISSN: 2309-8503

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Intrinsic Activity, 2015; **3**(Suppl.2)
http://www.intrinsicactivity.org/2015/3/S2
published online: 9 September 2015

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(available online at http://www.intrinsicactivity.org/2015/3/S2)

Meeting abstracts:

Cardiovascular Pharmacology and Endocrinology

A1.1

Potent irreversible $P2Y_{12}$ inhibition does not reduce LPS-induced coagulation activation in a randomized, double-blind, placebo-controlled trial

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.1
http://www.intrinsicactivity.org/2015/3/S2/A1.1

Background: Platelets play an important role in coagulation activation. $P2Y_{12}$ receptor inhibition may be beneficial in inflammatory states. Prasugrel, a potent, irreversible inhibitor of $P2Y_{12}$ receptor-induced platelet activation may reduce coagulation activation in a human LPS model.
Methods: A double-blind, randomized, crossover trial with a minimum washout period of 6 weeks was performed. Sixteen subjects were randomly assigned to a treatment group that received prasugrel or placebo two hours prior to infusion of a bolus of LPS (2 ng/kg body weight), while four subjects were assigned to a control group receiving prasugrel or placebo without LPS. Histone-complexed DNA (hcDNA), coagulation- and platelet-specific parameters were measured by enzyme immunoassay. Leukocyte aggregate formation was analyzed by flow cytometry, and thromboelastometry was performed.
Results: LPS infusion markedly activated coagulation. However, prasugrel did not reduce changes in prothrombin fragment F1+2, thrombin–antithrombin complexes, microparticle-associated tissue factor, CD40 ligand, P-selectin, platelet–leukocyte aggregation, hcDNA levels or the coagulation profile measured by thromboelastometry. hcDNA plasma levels increased approximately six-fold after LPS infusion in both treatment groups, but not in the control groups.
Discussion: Potent irreversible $P2Y_{12}$ inhibition by prasugrel does not affect LPS-induced coagulation activation. hcDNA plasma levels increased six-fold after infusion of LPS, indicating the formation of neutrophil extracellular traps during sterile inflammation.

A1.2

Hemodynamic effects of free fatty acids

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.2
http://www.intrinsicactivity.org/2015/3/S2/A1.2

Background: In healthy subjects infusion of free fatt acids (FFA) stimulates sympathetic nervous system activity, impairs endothelium-dependent vasodilation and increases limb blood flow. The net effects on the cardiovascular system are inconsistent, with some but not all studies reporting increased pressor responses. The underlying mechanism (cardial *vs.* vascular) is not well studied. Thus, the aim of the present study was to assess the combined effect of FFA infusion and stress on pressor responses imploying two stressors eliciting either a cardiac (Stroop test) or vascular (cold face test) dominated pressor response.
Methods: Twenty healthy non-smoking subjects (10 women, 10 men) participated in this randomized, double-blind, cross-over study, involving 2 study days with a washout period of at least 7 days

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between the study days. Each subject received an intravenous lipid emulsion with heparin or saline on alternate study days. After a 15-minute baseline period the two stress tasks were performed. Thereafter, the intralipid/saline infusion was started, lasting until the end of the experiment. Stress tasks were repeated after 180 minutes of infusion. Blood pressure, heart rate, stroke volume, cardiac output (CO) and total peripheral resistance (TPR) were measured. FFA and stress effects were tested by a series of 2 (placebo/FFA) × 2 (base *vs.* task) ANOVAs.

Results: The intralipid infusion had no influence on mean arterial pressure levels but significantly altered the underlying pattern. Compared to saline, absolute levels of cardiac output increased ($F = 9.98$; $p < 0.005$) and total peripheral resistance ($F = 4.46$; $p < 0.05$) decreased. Although the Stroop test and cold face test elicited the expected myocardial (significant increase in CO and decrease in TPR) and vascular (significant decrease in CO and increase in TPR) pattern of responses, respectively (all $F > 4.38$; $p < 0.05$), these responses were uninfluenced by the intralipid infusion.

Discussion: The results suggest that in young healthy subjects acute increases in FFA primarily influence the underlying mechanism of the pressor response by decreasing TPR and increasing CO but neither magnitude nor pattern of the stress response itself, irrespective of the type of stressor applied.

A1.3

Receptor characterization of serotonin and bradykinin actions on isolated rat peripheral arteries

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.3
http://www.intrinsicactivity.org/2015/3/S2/A1.3

Background: Serotonin, a monoamine neurotransmitter, induces vascular effects predominantly after binding to 5-HT_1 or/and 5-HT_2 receptors, while bradykinin, a pharmacologically active peptide, produces its effects through the selective activation of B_1 and B_2 kinin receptors. Accordingly, the aim of this study was to determine whether serotonin 5-HT_2 receptors and bradykinin B_2 receptors are involved in serotonin- and bradykinin-produced responses of the investigated blood vessels, respectively.

Methods: Femoral and common carotid arteries were isolated from male Wistar rats, cut into circular segments, and placed in an organ bath filled with Krebs-Ringer bicarbonate solution. Serotonin- and bradykinin-produced cumulative concentration-dependent contractile curves were obtained in vascular rings previously equilibrated at basal tone.

Results: Serotonin and bradykinin produced concentration-dependent contractions of carotid and femoral arteries, respectively. Ketanserin (a 5-HT_2 receptor antagonist) abolished serotonin-evoked contractions of examined blood vessels. On the other hand, HOE 140 (icatibant, a selective B_2 kinin receptor antagonist) significantly, but not completely, reduced the contraction induced by bradykinin in femoral arteries.

Discussion: 5-HT_2 and B_2 receptors have pivotal role in serotonin- and bradykinin-induced contractile actions in investigated blood vessels, respectively. Nevertheless, the importance of 5-HT_2 receptors was shown to be essential for the serotonin-induced effect on the common carotid artery, while we can presume that apart from B_2 receptors, bradykinin-induced contractile responses of the femoral artery probably includes parallel activation of B_1 receptors in a smaller extent.

Acknowledgements: This investigation was supported by the Ministry of Education and Science of the Republic of Serbia, grant no. 175023.

A1.4

Effect of the potassium channel opener pinacidil on isolated human internal mammary artery grafts from patients with type-2 diabetes mellitus

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.4
http://www.intrinsicactivity.org/2015/3/S2/A1.4

Background: Arterial grafts used for coronary artery bypass grafting (CABG) surgery have a tendency to develop vasospasm. Diabetes melllitus increases cardiovascular risk, induces endothelial dysfunction and alters basal vascular tone in blood vessels through different mechanisms. Potassium channel openers (PCOs) have the ability to inhibit vasospasm. Little is known about the effects of PCOs on human arteries that are used as CABGs obtained from patients with diabetes. Thus, the aim of this study was to investigate the involvement of smooth muscle K^+ channels in the effect of the PCO, pinacidil, on vasorelaxation of human internal mammary arteries (HIMA) obtained from patients with type-2 diabetes mellitus.

Methods: Segments of HIMA were obtained from patients undergoing coronary bypass surgery. Rings of HIMA without endothelium were mounted in an organ bath system and isometric tension was recorded. The experiments followed a multiple curve design. Pinacidil was used as PCO for vasorelaxation of HIMA precontracted with 5-hydroxytryptamine (100 μM).

Results: Pinacidil (0.01–100 μM) produced a concentration-dependent vasorelaxation of HIMA ($pD_2 = 5.9 \pm 0.3$, $n = 16$). Glibenclamide (GLB, 10 μM, $n = 6$), a highly selective blocker of ATP-sensitive K^+ (K_{ATP}) channels, 4-aminopyridine (4-AP, 1 mM, $n = 6$), a nonselective blocker of voltage-gated K^+ (K_V) channels, and tetraethylammonium (TEA, 1 mM, $n = 6$), a nonselective blocker of calcium-dependent K^+ (K_{Ca}) channels, induced a shift to the right of the concentration-response curves for pinacidil. There was no difference between the maximal vasorelaxation effects (E_{max}), produced by 0.1 mM of pinacidil in the absence and presence of K^+ channel blockers.

Discussion: Pinacidil induced endothelium-independent vasorelaxation of HIMA from diabetic patients. It seems that different potassium channels, located in the vascular smooth muscle, are partly involved in vasodilatation of HIMA induced by pinacidil. This study demonstrates that the mechanism of pinacidil includes potassium-channel-dependent and potassium-channel-independent effects.

Acknowledgements: This work was supported by scientific research grant no. TR31020 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

A1.5

Effects of wine polyphenol resveratrol on the renal artery of diabetic rats

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.5
http://www.intrinsicactivity.org/2015/3/S2/A1.5

Background: The anti-diabetic effects of resveratrol are well documented. It has been shown previously that vasorelaxation of rat renal artery (RA) induced by resveratrol is partly endothelium-dependent and involved nitric oxide production. The endothelium-independent relaxation of RA by resveratrol is mediated by activation of smooth muscle big calcium-sensitive potassium (BK_{Ca}) channels. However, the mechanisms by which resveratrol causes vaso-dilatation of RA from diabetic rats are not defined. Thus, the aim of this study was to investigate the mechanisms of resveratrol-induced vasorelaxation of RA from diabetic rats.
Methods: Insulin-dependent diabetes in male Wistar rats was induced by alloxan. Rings of RA were mounted in an organ bath for recording isometric tension. The experiments followed a multiple curve design. Contractions of RA were provoked by phenylephrine or by KCl (100 mM).
Results: Resveratrol relaxed RA of normal rats more potently than RA of rats with diabetes (EC_{50} were 8 and 40 μM, respectively). L-NAME and methylene blue partly antagonized the relaxation of RA of normal animals only. A selective blocker of ATP-sensitive potassium (K_{ATP}) channels, glibenclamide, and non-selective and highly selective blockers of BK_{Ca} channels, tetraethylamonium and iberiotoxin, did not affect the effects of resveratrol in both experimental models. High concentration of resveratrol (100 μM) completely inhibited KCl-induced contractions of RA in both experimental models.
Discussion: In conclusion, we have shown that resveratrol induces a strong endothelium-dependent relaxation of RA of normal rats. In diabetic rats, resveratrol induced NO-independent relaxation of RA. These observations indicate that the early stage of insulin-dependent diabetes mellitus is associated with a functional defect of the endothelium of RA. K_{ATP} and BK_{Ca} channels are not involved in resveratrol-induced relaxations of RA. It seems that the effects of resveratrol on RA of diabetic rats involves mechanisms independent of endothelium, K_{ATP} and BK_{Ca} channels. We need further investigations to evaluate this mechanism.
Acknowledgements: This work was supported by scientific research grant no. TR31020 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

A1.6

Resveratrol wine polyphenol relaxes rat renal artery in diabetic rats: the role of smooth muscle voltage-sensitive potassium channels

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.6
http://www.intrinsicactivity.org/2015/3/S2/A1.6

Background: A polyphenol present in red wine, resveratrol, is thought to be responsible for cardiovascular benefits associated with moderate wine consumption. Recently it has been documented that resveratrol reduced hyperglycemia and improved metabolic parameters in animal models of diabetes. Over the past 10 years, we have reported potent effects of resveratrol in preventing contractions of different rat and human blood vessels. However, the effect of resveratrol on vasculature of diabetic animal is not defined. Thus, the aim of this study was to investigate the mechanisms of resveratrol-induced vasorelaxation of the renal artery (RA) of diabetic rats.
Methods: Diabetes in male Wistar rats was induced by alloxan. Rings of RA were mounted in an organ bath for recording isometric tension. Contractions of RA were provoked by phenyephrine. The experiments followed a multiple curve design. Expression of different voltage-sensitive potassium (K_V1.1, 1.2., 2.1 and 4.2) channels in the vascular wall of RA was evaluated by immunohistochemistry.
Results: Resveratrol relaxed RA of normal rats more potently than RA of rats with diabetes (EC_{50} were 8 and 50 μM, respectively). A nonselective blocker of K_V channels, 4-aminopyridine, partly inhibited the relaxation of RA of normal as well as of diabetic rats. However, margatoxin, a selective antagonist of K_V1.x channels, completely antagonized the relaxation of RA of diabetic rats only. In contrast, a selective antagonist of K_V4.2 channels, phrixotoxin antagonized the effect of resveratrol on the RA of normal rats only. The vascular wall of RA of diabetic and non-diabetic rats showed variable positivity with applied antibodies. RA of normal rats expressed K_V1.2, K_V1.3 and K_V4.2 channels; the RA of diabetic rat expressed K_V1.1 and K_V1.2, but not K_V4.2 channels. The K_V2.1 channels are expressed neither in RA of diabetic nor in RA of normal rats.
Discussion: In conclusion, we have shown that resveratrol induces a stronger relaxation of RA of normal rats than diabetic rats. It seems that resveratrol induces relaxation of RA by activation of smooth muscle K_V4.2 channels in normal rats and K_V1.x channels in diabetic rats.
Acknowledgements: This work was supported by scientific research grants no. TP31020 and OI175064 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

A1.7

Investigation of the antifibrillatory drug interactions between valsartan and diltiazem in isolated perfused rabbit hearts

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.7
http://www.intrinsicactivity.org/2015/3/S2/A1.7

Background: In view of the reliability of the serial-shock method of measuring ventricular fibrillation threshold (VFT) in assessing the antifibrillatory potency of many antiarrhythmic drugs [1] and the alarming reports of the proarrhythmic effects of several anti-arrhythmic agents [2], we decided to use the above technique to study the antifibrillatory interactions that may occur when antiarrhythmic and antihypertensive drugs from different classes are combined. In several previous studies, we have investigated the antifibrillatory interactions between the antihypertensive drug valsartan and lidocaine (as class I antiarrhythmic agent), propranolol (as class II antiarrhythmic agent) and amiodarone (as class III antiarrhythmic agent). In this abstract, we report the antifibrillatory interactions between valsartan and the class IV antiarrhythmic agent diltiazem.

Methods: Studies were carried out on hearts isolated from New Zealand white rabbits of either sex weighing 1.5 to 2 kg. The details of the method and the stimulation connections have been given previously [1].

Results: In six hearts, measurement of VFT was made in the absence of any drug throughout the experiments. In this group, no significant change in the threshold was observed. Perfusion with diltiazem produced a significant, dose-dependent increase in VFT. On the other hand, perfusion with valsartan did not cause any significant change in the threshold. In addition, there was no significant difference between the increase in VFT produced by the infusion of 0.02 µmol of diltiazem and the effect when it was combined with 1 µmol of valsartan. This is in contrast to a synergistic antifibrillatory effect of the combined use of diltiazem and amiodarone which we reported recently [3].

Discussion: The lack of antifibrillatory interactions between valsartan and diltiazem may suggest its safety in combining with class IV antiarrhythmic agents in the treatment of hypertensive patients developing cardiac arrhythmias. However further studies are required to establish this in the clinical setup.

Acknowledgements: This work was supported by a grant from the College of Medicine Research Center (CMRC), King Saud University, Riyadh, Saudi Arabia.

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A1.8

Regional heterogeneity of vascular dysfunction in *db/db* mice

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.8
http://www.intrinsicactivity.org/2015/3/S2/A1.8

Background: It is well recognized that diabetes mellitus adversely affects the vasculature. However, whether various arteries exhibit differential vulnerability to the diabetic milieu remains to be explored. We compared the functional and molecular alterations in the aorta, carotid and femoral arteries in relation to the progression of the diabetic status in *db/db* mice and examined some plausible mechanisms underlying the differential adaptation of these arteries.

Methods: Using wiremyography, the vasodilatory and contractile responses of aortae, carotid and femoral arteries isolated from *db/db* and control mice at 6, 10 and 14 weeks of age were examined to assess the endothelial and vascular smooth muscles function. As well, protein expression of superoxide dismutase (SOD) isoforms were examined in the three arteries. In parallel, body weight, plasma glucose, C-reactive protein, 8-isoprostane, cholesterol and triglycerides were measured.

Results: There were age-related increases in body weight, plasma glucose, 8-isoprostane, C-reactive protein, and triglycerides in *db/db* mice. In comparison to the aorta and femoral artery, the carotid artery was the most resilient and maintained normal functional responses at the three age points examined. The aortae of *db/db* mice exhibited progressive loss of endothelium-dependent and -independent vasodilatation, while concurrently having enhanced vasoconstriction. The femoral arteries of *db/db* mice showed reduced endothelium-dependent, hyperpolarizing factor-mediated vasodilatation and attenuated contractile responses. The femoral arteries of control and *db/db* mice lacked the expression of SOD-3 in contrast to the aortae and carotid arteries.

Discussion: Substantial heterogeneity exists between the aorta, carotid and femoral arteries both at functional and molecular levels. The carotid artery maintained unaltered functional responses despite marked increases in systemic oxidative stress in *db/db* mice, likely because the carotid artery relaxed in response to superoxide anion or peroxynitrite; this response may reflect a physiological strategy to maintain blood supply to the brain under stressful conditions. Both the vasodilatory and contractile responses in the femoral arteries of *db/db* mice were attenuated, probably due to the lack of the expression of SOD-3 in the femoral arteries leading to marked oxidative damage. Understanding regional differences in vasomotor control, coupled with advanced drug delivery systems will help developing therapies that target specific vascular beds with reduced systemic side effects.

Acknowledgements: N.A.S. received a scholarship from the Egyptian Ministry of Higher Education.

A1.9

Anti-addiction drug ibogaine and the heart: a delicate relation

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.9
http://www.intrinsicactivity.org/2015/3/S2/A1.9

Background: Ibogaine is an indole alkaloid derived from the African shrub *Tabernanthe iboga*. Although never licensed as therapeutic drug, ibogaine is used as anti-addiction medication in dozens of alternative-medicine clinics worldwide. Recently, alarming reports of QT-interval prolongation in the electrocardiogram, life-threatening cardiac arrhythmias, and sudden death cases associated with the ingestion of ibogaine have been accumulating.

Methods: In order to estimate the cardiac risk connected with ibogaine intake, we assessed the effects of the drug and its long-lived active metabolite noribogaine on cardiac ionic currents and action potentials (APs). Therefore, by using the whole-cell patch-clamp technique, currents from tsA cells expressing human cardiac ion channels, and APs from human induced pluripotent stem cell-derived ventricular-like cardiomyocytes were recorded.

Results: We report that therapeutic concentrations of ibogaine significantly inhibit human ether-a-go-go-related gene (hERG, $hK_V11.1$) potassium channels, and retard action potential repolarization in human cardiomyocytes. The latter finding represents the first direct experimental proof that ibogaine application implies a cardiac arrhythmia risk for humans. In addition, we found that noribogiane also inhibits hERG channels and prolongs the human cardiac AP in similar concentrations as its parent drug ibogaine. These results explain the clinically observed delayed incidence of cardiac adverse events sometimes even several days after ibogaine intake.

Discussion: The use of ibogaine as anti-addiction drug is associated with a cardiac arrhythmia risk due to hERG channel block. Hereby, noribogaine may represent the main player responsible for long-term cardiac toxicity after ibogaine ingestion. If considered an indispensable drug for anti-addiction therapy, we urge the responsible medical regulatory authorities to specify adequate standards and exclusion criteria to pave the way for a safer ibogaine anti-addiction therapy in the future.

Acknowledgements: This work was supported by the Austrian Science Fund FWF (grant P23060 to K.H.). Ibogaine was kindly donated by Sacrament of Transition (Maribor, Slovenia).

A1.10

Privileged ER Ca^{2+} refilling in vascular endothelial cells: evidence for a role of the Na^+/Ca^{2+} exchanger (NCX)

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.10
http://www.intrinsicactivity.org/2015/3/S2/A1.10

Background: The endoplasmic reticulum (ER) is an organelle involved in the majority of cellular processes such as lipid synthesis, protein synthesis and folding, and post-translation modification. The ER is also the main intracellular Ca^{2+} store. Ample experimental evidence suggests that there is a relation between Ca^{2+} signals and the above-mentioned processes. Under ER stress conditions, misfolded proteins accumulate in the ER; this, in turn, leads to Ca^{2+} leakage from the ER and, in general, to an alteration in the healthy Ca^{2+} transport to and from the ER. Deterioration of the ER function, as happens during ER stress, appears linked to several diseases such as neurodegenerative disorders (Parkinson's, Alzheimer's), bipolar disorders and diabetes. Since changes in Ca^{2+} in the ER can affect the quantity and the efficiency of protein folding, it is important to understand the mechanism of ER Ca^{2+} refilling. Na^+/Ca^{2+} exchangers (NCX), Ca^{2+} ATPases (SERCA), inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR) regulate Ca^{2+} movement into and out of the ER, including to and from the extracellular space. We investigate the role of NCX in the transport of Ca^{2+} in endothelial cells under various conditions of cell stimulation and membrane polarization on the heels of previous findings showing that in vascular smooth muscle cells the NCX plays a critical role in the refilling of the SR with extracellular Ca^{2+}.

Methods: We employed Fura-2AM as a ratiometric cytoplasmic Ca^{2+} indicator and D1ER cameleons as luminal ER Ca^{2+} indicators to image Ca^{2+} signals in our cell system by standard fluorescence microscopy. We also measured the membrane potential by whole-cell patch and micro-electrode methods.

Results: Our findings point to an involvement of the NCX Ca^{2+} influx mode in the refilling of ER. The data suggest a significant contribution of NCX reverse-mode operation in addition to, or in conjunction with, store-operated Ca^{2+} entry via STIM-Orai in a process of privileged refilling of the ER at small or negligible changes in global cytosolic Ca^{2+}. We propose that this process occurs in plasma membrane (PM)–ER junctions. These results are corroborated by a comparison between our own measurements of the membrane potential and calculation of the NCX potential in the experimental condition used during the experiments.

Discussion: Our results provide further elucidation of the mechanism and function of a previously hypothesized subplasmalemmal Ca^{2+} control unit during the refilling of the ER under physiological conditions.

Acknowledgements: This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no. PIIF-GA-2012-330657 (QuMoCa project).

A1.11

Effect of plasma from high and low serum unconjugated bilirubin individuals on cholesterol efflux

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.11
http://www.intrinsicactivity.org/2015/3/S2/A1.11

Background: Mildly elevated bilirubin levels, a characteristic of Gilbert Syndrome (GS), have been inversely correlated with cardiovascular disease onset and mortality. Protection of lipids, proteins and other macromolecules from oxidation by bilirubin represents the most commonly accepted mechanism contributing to cardiovascular protection [1]. A critical step of atherosclerosis pathogenesis is the formation of cholesterol-enriched pro-atherogenic foam cells. Therefore, increased macrophage cholesterol efflux is expected to result in an overall anti-atherosclerotic effect. However, there are no data on how high serum levels of unconjugated bilirubin (UCB) might affect macrophage cholesterol efflux.

Methods: In this study, THP-1-derived macrophages were differentiated by PMA treatment and then labelled with [^{3}H]cholesterol. Cholesterol efflux was assessed in presence of human plasma from 120 individuals for 4 h. The subjects were divided into two age- and gender-matched groups, with high and low serum UCB with a cut-off point at 17.1 µM. A paired *t*-test was performed to analyse the data for statistical significance.

Results: The cholesterol efflux mediated by serum from high- and low-UCB individuals was 5.45% and 5.83%, respectively. Individuals with higher serum UCB showed significantly lower cholesterol efflux capacity, even after correction for Apo-A1 or HDL levels in plasma ($p < 0.001$).

Discussion: A number of studies have shown that the risk of mortality from cardiovascular disease is remarkably reduced in GS individuals. This protection may be explained by bilirubin's ability to protect blood lipids and LDL from oxidation [1]. Some studies showed that the individuals with GS had significantly reduced levels of total cholesterol, low-density lipoprotein cholestrol (LDL-C), triacylglycerol (TAG), oxidized low-density lipoprotein (oxLDL), very-low-density lipoprotein (VLDL), small dense low-density lipoprotein (sd-LDL), and elevated HDL / LDL ratios in plasma [2]. However, our results showed that plasma with higher bilirubin levels, as found in GS, does not contribute to higher cholesterol efflux from macrophages, and even had an inverse effect. This suggests that different pathways might be involved in the cardiovascular protection by increased plasma bilirubin.

Acknowledgements: This work was supported by the Austrian Science Fund (FWF; project P25971-B23) and by the Vienna Anniversary Foundation for Higher Education (Hochschuljubiläumsstiftung der Stadt Wien; project H-297332/2014).

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A1.12

Cardiac dysfunction in adipose triglyceride-deficient mice: role of the ubiquitin–proteasome system

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Intrinsic Activity, 2015; **3**(Suppl. 2):A1.12
http://www.intrinsicactivity.org/2015/3/S2/A1.12

Background: Adipose triglyceride lipase (ATGL) represents a key enzyme of the lipolytic cascade. Global ATGL deficiency in mice leads to massive accumulation of neutral lipids in adipose and multiple non-adipose tissues [1]. In hearts of ATGL knockout mice, ectopic storage of triglycerides results in progressive development of lethal cardiomyopathy [1]. Recently it was demonstrated that ATGL knockout mice suffer from pronounced cardiac oxidative inflammatory stress and defective PPARα signaling [2, 3]. Since dysfunction of the ubiquitin–proteasome system (UPS) has been closely linked to various cardiac pathologies, we investigated if disturbances in cellular protein degradation might contribute to the observed cardiac phenotype.

Methods: Western-blot analysis and quantitative PCR were used to compare protein ubiquitination and markers of inflammatory oxidative stress between cardiac tissue of wild-type and ATGL knockout mice. Furthermore, mice were treated with the PPARα agonist Wy14,643 to test for the role of defective PPARα signaling in this scenario.

Results: Western-blot analysis revealed significantly increased amounts of ubiquitinated cardiac proteins in ATGL-deficient hearts. In parallel, protein expression of the ubiquitin-activating enzyme E1a, which initiates protein ubiquitination, was significantly upregulated in cardiac ATGL deficiency. Both effects were reversed upon cardiomyocyte-directed overexpression of ATGL in ATGL knockout mice. In parallel, we observed activation of cardiac NF-κB signaling in these hearts. Chronic treatment of ATGL knockout mice with the PPARα agonist Wy14,643 (which substantially improves cardiac performance) reversed accumulation of ubiquitinated proteins, prevented activation of NF-κB, and decreased oxidative stress.

Discussion: In summary, our data suggest a hitherto unrecognized link between proteasomal function, PPARα signaling and cardiovascular disease.

Acknowledgements: This work was supported by the Austrian Science Fund (P24005, F3003 and W901 DK Molecular Enzymology to B. M.).

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A1.13

Role of aldehyde dehydrogenase 2-catalyzed nitric oxide formation in nitroglycerin-induced vasorelaxation

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Intrinsic Activity, 2015; **3**(Suppl. 2):A1.13
http://www.intrinsicactivity.org/2015/3/S2/A1.13

Background: The antianginal drug nitroglycerin (GTN) causes vasodilation through activation of soluble guanylate cyclase (sGC) by release of nitric oxide (NO) or a related species, resulting in accumulation of 3′,5′-cyclic guanosine monophosphate (cGMP) in vascular smooth muscle. In 2002, Stamler and coworkers showed that aldehyde dehydrogenase-2 (ALDH2) catalyzes bioconversion of GTN to 1,2-glyceryl dinitrate (1,2-GDN) and nitrite. Nitrite was proposed to be reduced to NO by components of the mitochondrial respiratory chain. However, we found that a minor pathway of ALDH2-catalyzed GTN bioconversion, accounting for about 5% of total turnover, results in direct formation of NO. Site-directed mutagenesis revealed that two vicinal cysteine residues adjacent to the catalytically active C302 are essential for the major nitrite pathway but not involved in GTN reduction to NO. Mutation of C301 and C303 to serine led to > 95% loss of 1,2-GDN formation but enhanced sGC activation and NO formation. It was the aim of the present study to test whether the direct NO formation that was observed with purified C301S/C303S ALDH2 explains GTN bioactivation in vascular smooth muscle.

Methods: Wild-type ALDH2 and the C301S/C303S mutant were overexpressed in murine ALDH2-deficient aortic smooth muscle cells by recombinant adenoviral vectors. Protein expression of wild-type and mutated ALDH2 was analyzed by western blot. Activation of purified sGC by transfected aortic smooth muscle cells was studied with increasing concentrations of GTN. GTN denitration was assayed as 1,2-GDN formation by thin-layer radio-chromatography.

Results: Adenoviral overexpression led to virtually identical protein expression levels of wild-type and mutated ALDH2. In the presence of wild-type and C301S/C303S-transfected cells, GTN activated purified sGC with EC_{50} values of 2.87 ± 0.68 and 0.11 ± 0.01 µM, respectively, demonstrating more than 10-fold higher GTN affinity of the mutant. Denitration activity of wild-type and C301S/C303S ALDH2 was 2.39 ± 0.33 and 0.62 ± 0.29 pmol/min/ng ALDH2 (determined in western blots using the purified protein as standard).

Discussion: Our results demonstrate that bioactivation of GTN in vascular smooth muscle involves direct reduction of GTN to NO. This reaction is catalyzed by ALDH2 in a minor pathway that is not linked to clearance-based GTN metabolism yielding 1,2-GDN and inorganic nitrite.

Acknowledgements: This work was supported by the Austrian Science Fund (grant P24946).

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A1.14

Adenosine kinase mediates adenosine attenuation of cardiomyocyte microtubule cytoskeletal densification

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.14
http://www.intrinsicactivity.org/2015/3/S2/A1.14

Background: Microtubules play essential roles in cell size, shape and intracellular trafficking. In the heart however, extensive densification of the cardiomyocyte microtubule cytoskeleton under hypertrophic stress conditions is associated with contractile dysfunction. Myocardial adenosine attenuates cardiomyocyte microtubule densification, hypertrophy and heart failure in the setting of pressure overload, but the mechanism(s) by which adenosine regulates microtubule dynamics is not clear. Here we investigated the role of adenosine receptors and intracellular metabolism by adenosine kinase (ADK) in adenosine regulation of cardiomyocyte microtubule dynamics.

Methods: Cultured neonatal rat ventricular cardiomyocytes (NRVMs) were stimulated with phenylephrine (50 μM) or constitutively activated Raf+Akt (10 MOI/cell) to induce hypertrophic growth and microtubule densification. To examine the impact of adenosine receptors or adenosine metabolism in adenosinergic effects, NRVMs were further treated with adenosine (10 μM; plus adenosine deaminase inhibitor pentostatin (1 μM)) or 2-chloroadenosine (CADO; 5 μM) in the presence of selective adenosine receptor antagonists (1–5 μM) or ADK inhibitors (iodotubercidin (0.2 μM) or ABT-702 (0.2 μM)). In addition, ADK or 5′-cytoplasmic nucleotidase (5′cNi) were over-expressed to examine the impact of adenosine conversion to AMP. Microtubule dynamics, cell signaling, and cell morphology were analyzed by subcellular fractionation, western blot and immunofluorescence.

Results: Phenylephrine or Raf/Akt caused cardiomyocyte microtubule stabilization and hypertrophy. Both of these processes were attenuated by adenosine or CADO. While adenosine receptor antagonists only modestly blocked adenosine effects on microtubules, adenosine kinase inhibitors or expression of 5′cNi potently reversed microtubule destabilization by adenosine and restored cardiomyocyte hypertrophy. Conversely, ADK over-expression potentiated adenosine destabilization of microtubules. Remarkably, adenosine attenuated microtubule stabilization and cardiomyocyte hypertrophy in Raf/Akt-transfected cells despite unmitigated mTORC1 and ERK pathway signaling. The ADK dependent destabilization of microtubules by adenosine was not associated with increased activation of AMPK.

Discussion: Intracellular conversion of adenosine to AMP attenuates microtubule stabilization and cardiomyocyte hypertrophy independent of AMPK, even in the setting of constitutive hypertrophic signaling. ADK attenuation of microtubule cytoskeletal network expansion may serve to limit cardiomyocyte growth during metabolic stress conditions.

A1.15

Effects of risk-factor clustering on baroreflex sensitivity and blood pressure variability in borderline hypertensive rats

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Intrinsic Activity, 2015; **3**(Suppl.2):A1.15
http://www.intrinsicactivity.org/2015/3/S2/A1.15

Background: Primary hypertension is a common disease of unknown etiology, which strikes over a billion people worldwide. Numerous factors have been established to increase the risk of developing this condition. The aim of this study was to investigate the effects of stress, salt loading and genetic predisposition on neurogenic cardiovascular control, implicated in the pathogenesis of hypertension. For this purpose, experiments were conducted in borderline hypertensive rats (BHR) with family history of hypertension.

Methods: Experiments were performed in twelve-weeks old BHRs equipped with a radiotelemetry device for direct blood pressure (BP) recording. Animals were randomized in three experimental groups and monitored for 15 weeks: the first group (control) included BHRs recorded under baseline physiological conditions (genetic predisposition; risk factor 1); the second group of animals was salt-loaded with 0.9% saline solution (risk factors 1+2); the third group of BHRs was salt-loaded and exposed to combined environmental stress models in two time blocks: shaker stress plus crowding stress, then isolation stress plus air-jet stress plus tilt stress (risk factors 1+2+3). Arterial BP was digitalized at 1000 Hz and analyzed in Dataquest A.R.T. 4.0 software (Transoma Medical, Data Science International, USA). Autonomic cardiovascular control was assessed by spectral analysis of systolic BP (SBP), diastolic BP (DBP) and heart rate (HR). Evaluation of spontaneous baroreflex sensitivity (BRS) was done by the sequence method.

Results: Control BHRs displayed higher values of BP, but still in the normotensive range, and no change in BP variability. In these rats HR was lower than in other groups, no alternation in HR variability was noted, while BRS sporadically increased. Salt-loaded BHRs exhibited BP levels comparable to the control BHRs, and lower SBP variability. HR also decreased over time and maintained low for 15 weeks. No change in HR variability was found. BRS was increased and the increase persisted during the follow-up period. Salt-loaded plus stressed BHRs exhibited overt hypertension. However, changes in BP variability were inconsistent. Both HR (decrease) and BRS (increase) were altered, with no change in HR variability.

Discussion: The present results show that rats genetically predisposed to hypertension exhibit periodical increases in BRS suggesting that the baroreflex responds to slight elevation of BP, through the engagement of the vagus nerve, which leads to lower HR values. Addition of salt loading to BRS unveiled that it is stimulus enough to trigger long-term changes of BRS in genetically predisposed animals. At this point, the baroreflex can still maintain BP in the normal range. Decrease in BP variability in these rats confirms a

buffering effect of the baroreflex on BP. Adding environmental stress to salt loading in BHRs leads to overload of autonomic mechanisms which results in a notable BP increase. Occasional changes in BRS and of the HR observed in these rats suggest that the baroreflex is still trying to overcome the disruption of homeostasis.

Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant no. III 41013).

A1.16

Doxorubicin nanoparticles conjugated with *N*-(2-hydroxypropyl) methacrylamide exhibit low cardiotoxicity in rats

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Intrinsic Activity, 2015; **3**(Suppl. 2):A1.16
http://www.intrinsicactivity.org/2015/3/S2/A1.16

Background: The clinical use of the highly effective antineoplastic agent doxorubicin is limited by late and irreversible life-threatening cardiotoxicity. Recently engineered doxorubicin-conjugated nanoparticles improved therapeutic index and tolerability. The aim of this study was to investigate cardiotoxicity induced by doxorubicin nanoparticles conjugated with *N*-(2-hydroxypropyl)methacrylamide.

Methods: Twenty-two male Wistar rats equipped with a radiotelemetric device (TA11PA-C40) were randomized into four experimental groups: Group 1 (HPMA; $n = 5$) was treated with *N*-(2-hydroxypropyl) methacrylamide (5 mg/kg; i.v.); group 2 (saline; $n = 5$) was treated with 0.9% NaCl (0.5 ml; i.v.); group 3 (HPMA-DOX; $n = 5$) was treated with *N*-(2-hydroxypropyl)methacrylamide conjugated with doxorubicin (5 mg/kg; i.v.), and group 4 (DOX; $n = 7$) was treated with doxorubicin (5 mg/kg, i.v.). Body weight (BW), blood pressure (BP), heart rate (HR), HR short-term variability (HRV), left ventricular ejection fraction (EF_{LV}) and left ventricular end-diastolic volume (EDV) were monitored for 140 days. Kaplan-Meier survial curves were calculated for all groups. At the end of the experiment, rats were euthanized and the harvested hearts were used for pathohistology.

Results: In the HPMA and saline groups, BW of rats increased over time and median survival was 140 days. BP, HR and HRV were comparable in both groups. However, EDV was increased in 3 HPMA-treated rats in respect to saline-treated rats. There were no pathohistological signs of cardiotoxicity in either the HPMA or saline group of rats. In HPMA-DOX rats BW increased over time and median survival was 140 days. BP, HR and HRV of these rats were comparable to controls while EDV was increased and EF_{LV} was decreased in 3 rats. Pathohistology revealed fibrosis in 3 rats. DOX rats exhibited a significant decline in BW and low median survival (16 days). In all DOX rats BP and HR were normal while EDV was increased and EF_{LV} and HRV were decreased. Pathohistological examination uncovered typical signs of cardiotoxicity in all DOX rats including severe fibrosis, vacuolization, necrosis and inflitration.

Discussion: Our results indicate that HPMA-DOX-treated rats have a better survival and lower cardiotoxicity than DOX-treated rats. These findings are in agreement with previous reports on doxorubicin survival in rats. EDV is the earliest indicator of heart failure in conventional echocardiography. Increase in EDV and decrease of EF_{LV} indicate left ventricular dilatation associated with heart failure in all rats treated with doxorubicin but only in 3 rats treated with HPMA-DOX. The increase of EDV in HPMA rats may reflect the increase of circulating volume due to the plasma-expander properties of HPMA as there was no pathohistological confirmation of cardiotoxicity in this group of rats. HRV was depressed only in DOX rats, which is an expected finding since reduction of HRV has been reported to predict poor survival in clinical settings.

Acknowledgements: This work was supported by (1) the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant no. III 41013), and (2) High Impact Research HIR of the Ministry of Higher Education, Malaysia (grants UM.C/625/1/HIR/MOHE/MED/17 and UM.C/625/1/HIR/MOHE/MED/33).

A1.17

Ets-2, a possible marker of early instability in coronary artery bypass grafting patients: modulation by drugs

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Intrinsic Activity, 2015; **3**(Suppl. 2):A1.17
http://www.intrinsicactivity.org/2015/3/S2/A1.17

Background: Endothelial progenitor cells (EPCs) play a key role in endothelial repair processes. It is well known that the functionality of the EPCs is poor in patients with diabetes mellitus type 2 (DM2) and cardiovascular disease (CVD), although the exact mechanism of dysfunction is still uncertain. Several studies have pointed out the importance of adequate therapy (*e.g.* therapy with EPCs) for endothelial repair, helping to reduce the alterations in the processes of re-endothelization in patients with DM2 and CVD and therefore decrease the occurrence of CVD. Recently, the SDF-1 axis and the CXCR4 co-receptor have become a key element in the study of CVD. Moreover, it has been hypothesized that members of the E26 family of transcription factors are involved in the development of CVD, and in patients with DM2, the specific alteration of the transcription factor Ets-2 could contribute to the dysfunction of the EPCs. Our main objective was (i) to determine whether the degree of expression of Ets-2–SDF-1α/CXCR4 is capable of predicting the release of circulating EPCs, (ii) to relate the data found with clinical and laboratory parameters of patients undergoing coronary artery bypass grafting (CABG), and (iii) to study their modulation by DPP4 inhibitor drugs (sitagliptin).

Methods: Ninety CABG patients were divided into diabetic and non-diabetic patients (NDM). Peripheral mononuclear cells were obtained by Ficoll-Hypaque density gradient centrifugation. Expression of Ets-2, CXCR4, and SDF-1 were measured by western blotting. The effects of sitagliptin on EPCs in culture were measured by western blotting, ELISA and immunofluorescence.

Results: In patients with DM2, release of EPCs, determined by levels of SDF-1α, is a late effect due to the high levels of glucose and low levels of HDL, but this effect decreases in patients treated with insulin. In DM2 patients a low expression in the SDF-1/CXCR4 axis was observed in comparison to NDM patients (NDM: 3.20 ± 2.3 *vs.* DM2: 1.41 ± 1.4; NDM: 1.32 ± 1.0 *vs.* DM2: 1.08 ± 0.9), which was associated with low levels of expression of Ets-2 (NDM: 1.60 ± 1.5 *vs.* DM2: 1.17 ± 1.0). However, an increase in expression of Ets-2 was observed in patients without cardiovascular risk factor (2.12 ± 1.5), associated with early stages of cardiovascular instability, while the expression was decreased in patients with longer evolution of CVD. In EPCs in culture, sitagliptin improved cell morphology and

increased the expression of SDF-1α and CXCR4 at 24 hours; this effect did not depend on stimulus by apoptotic bodies.

Discussion: In patients with DM2, release of EPCs is a result of the SDF-1α axis and the CXCR4 co-receptor. Poor functionality of circulating EPCs is associated with decreased expression of the transcription factor Ets-2 in advanced stages of CVD in patients with DM2. Ets-2 could be an early marker of cardiovascular instability associated with states of hyperlipidemia. Therapy with sitagliptin in EPCs in culture could help to reverse the poor functionality of the EPCs, especially in patients with DM2.

Acknowledgements: This work was supported by FIS PI12/00590 (plan estatal I+D+I 2013–2016).

A1.18

Neurocardiogenic remodelling in normotensive and spontaneously hypertensive pregnant rats

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Intrinsic Activity, 2015; **3**(Suppl. 2):A1.18
http://www.intrinsicactivity.org/2015/3/S2/A1.18

Background: Normal pregnancy is associated with maternal cardiovascular adaptations in order to provide foetal positive outcome. This circulatory adjustment may affect the health of pregnant women with preexisting hypertension. Similar findings were observed in other species, including rats. The focus of this research was to investigate the influence of pregnancy-induced adaptations on blood pressure and heart rate variability in pregnant Wistar (WR) and spontaneously hypertensive rats (SHR).

Methods: All experiments were performed in conscious female WR ($n = 8$) and SHR ($n = 6$) equipped with a radiotelemetry device. Systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) were derived from the arterial pulse wave as maximum, minimum and inverse inter-beat interval. Spectral analysis of BP and HR was performed on 7-minutes-long recordings in total (0–3 Hz), very low frequency (VLF: 0–0.2 Hz), low frequency (LF: 0.2–0.8 Hz) and high frequency (HF: 0.8–3 Hz) range. Spontaneous baroreceptor reflex sensitivity (sBRS) was evaluated using the sequence method.

Results: Pregnancy significantly reduced SBP and increased HR in both strains, without affecting sBRS. By the end of pregnancy, total SBP variability in WR significantly increased, while a significant decrease was observed in SHR. In non-pregnant state there was a significant difference in VLF and LF bands between the two strains, which was not observed in mid and late pregnancy.

Discussion: Our results show for the first time differences in neurogenic control of the cardiovascular system during normotensive and hypertensive pregnancies. As expected, accentuated activity of the renin–angiotensin system along with an increased sympathetic drive during hypertension was noticed in non-pregnant SHRs. These phenomena, seen as increased VLF and LF SBP bands, could not be noticed in pregnant SHR dams, probably due to prevailance of mechanisms inducing vasodilation. Normalization of SBP and BP variability in SHRs reveal potential adaptational mechanisms that can be beneficial for maternal health during pregnancy in the hypertensive state.

A1.19

Rice bran enzymatic extract reduces hyperlipidemia and related hepatic steatosis in ApoE$^{-/-}$ mice

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Intrinsic Activity, 2015; **3**(Suppl. 2):A1.19
http://www.intrinsicactivity.org/2015/3/S2/A1.19

Background: ApoE$^{-/-}$ mice spontaneously develop nonalcoholic fatty liver disease secondary to hyperlipidemia. Rice bran has been associated with lipid-lowering and anti-inflammatory properties in several rodent, primate and human models. We aimed to evaluate the impact of a rice bran enzymatic extract (RBEE) diet supplementation on hepatic steatosis.

Methods: Seven-week-old ApoE$^{-/-}$ mice were fed a high fat diet (HF) supplemented or not with 1% or 5% RBEE (w/w) for 23 weeks. Wild-type C57BL/6J mice were kept under standard diet for the same period as the healthy controls. Serum total cholesterol, HDL-C, triglycerides, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with commercial kits. Extraction of lipids from liver and feces was performed following the Folch method. HMG-CoA / mevalonate ratio, determined spectrophotometrically, served as estimation of HMG-CoA reductase activity. Lipid droplets in the liver were visualized by Oil Red O staining. PPARα protein expression was measured by western blot from liver homogenates.

Results: ApoE$^{-/-}$ mice were characterized by increased total cholesterol ($p < 0.001$) and triglycerides ($p < 0.001$), and reduced HDL-C ($p < 0.001$). 5% RBEE diet supplementation reduced total cholesterol ($p < 0.01$) and triglycerides ($p < 0.05$) while both, 1% and 5%, supplements augmented HDL-C ($p < 0.01$ and $p < 0.001$ for 1% and 5%, respectively). Increased ALT ($p < 0.01$) and AST ($p < 0.05$) were induced by HF diet. RBEE supplementation was able to reduce AST increase regardless of the dose ($p < 0.001$) but had no effect on ALT levels. HMG-CoA reductase activity was downregulated by 1% ($p < 0.01$) and 5% ($p < 0.05$) RBEE supplements. Finally, 5% RBEE diet supplementation increased cholesterol excretion in feces ($p < 0.01$) and elevated levels of PPARα protein expression in the liver. As a result of all above, liver steatosis observed in HF-fed mice ($p < 0.001$) was sharply reduced by 1% RBEE diet supplementation as shown by Folch extraction ($p < 0.001$) and Oil Red O staining ($p < 0.001$). Oil Red O staining was also lower for the 5% RBEE group.

Discussion: Among the bioactive compounds present in RBEE are phytosterols, γ-oryzanol and tocols. The serum lipid pattern may be improved due to greater fecal exception induced by phytosterols and γ-oryzanol, coupled with the inhibition of cholesterol synthesis through reduction of HMG-CoA reductase activity. This fact, combined with the induction of liver PPARα expression resulted in the improvement of steatosis induced by the high fat diet. These results suggest that RBEE supplementation might be beneficial for the prevention of hyperlipidemia and related hepatic steatosis.

Acknowledgements: C.P.T. is a recipient of a FPU fellowship from the Spanish Government.

A1.20

Chloroquine protects injured rat kidney in an experimental model of ischemia–reperfusion (I/R) injury

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.20
http://www.intrinsicactivity.org/2015/3/S2/A1.20

Background: Acute kidney injury (AKI) still remains an unresolved problem in pharmacotherapy and renal inflammation is a major factor in its development. Chloroquine, a well-known antimalarial drug, possesses pleitropic effects as well as antiinflammatory, anticoagulant and antioxidative actions. The effects of chloroquine on renal function may involve significant increases in urine flow rate, glomerular filtration rate and sodium excretion, as well as stimulation of nitric oxide synthase. However, its role in experimental models of renal ischemia–reperfusion (I/R) injury is unknown. We aimed to analyze the acute effects of a single dose of chloroquine administered intravenously at three different times in the experimental model of I/R injury in rat.

Methods: Male adult Wistar rats ($n = 57$, body weight 250–300 g) were subjected to bilateral renal ischemia (45 min) followed by reperfusion with saline lasting for 4 hours. Chloroquine was administered i. v. at doses of 0.3 mg/kg and 3 mg/kg 30 min before ischemia, 30 min before reperfusion and 5 min before reperfusion. Selected parameters of glomerular and tubular function, histological score and kidney injury molecule-1 (KIM-1) staining score were followed in sham-operated animals and in rats subjected to I/R injury, pretreated with either saline or chloroquine. These markers were obtained from the appropriate serum, urine or tissue samples at the end of the reperfusion period.

Results: Chloroquine (0.3 and 3 mg/kg, i. v.) protected the I/R injured kidney in a U-shaped manner. Both doses were protective regarding biochemical and histological markers of I/R injury (serum urea, creatinine and fractional excretion of sodium, as well as total histological score, tubular necrosis score and KIM-1 staining score) ($p < 0.05$ *vs.* corresponding controls, *i. e.* rats subjected to I/R injury and treated with saline only). The protective effects of the lower dose of chloroquine were more profound. Time-related differences between pretreatments were not observed ($p < 0.05$, all).

Discussion: Our study shows for the first time that a single dose of chloroquine (0.3 mg/kg i. v.) could attenuate the injured rat kidney in a non-time-dependant manner. It is also important to point out that beneficial effects of acute pretreatment with chloroquine in this experimental model could be confirmed by KIM-1 staining scores.

Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project no. 175023).

A1.21

Over-expression of V_{1A} receptors in the hypothalamic paraventricular nucleus induces baroreflex desensitization and increases cardiovascular variability during stress

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.21
http://www.intrinsicactivity.org/2015/3/S2/A1.21

Background: The hypothalamic paraventricular nucleus (PVN) is a key integrative site of neuroendocrine control of the circulation and of the stress response. It is also the major source of the neuropeptide vasopressin (VP), and co-expresses V_{1A} receptors ($V_{1A}R$). Therefore we sought to investigate the role of $V_{1A}R$ in PVN in cardiovascular control. We hypothesized that, by increasing the number of vasopressin $V_{1A}R$ in PVN and by selectively blocking their activity, we can modulate PVN neuronal activity involved in autonomic cardiovascular control.

Methods: Experiments were performed in conscious male Wistar rats equipped with a radiotelemetric device implanted into the abdominal aorta for registration of cardiovascular parameters. The experimental group of animals was subjected to unilateral *in vivo* gene transfer into the right PVN of adenoviral vectors (Ads) containing information necessary to induce expression of enhanced green fluorescent protein (eGFP), used as a marker, and over-expression of $V_{1A}Rs$. Control animals were either subjected to gene transfer of Ads containing information for eGFP or were sham-operated. Rats were recorded with and without selective blockade of vasopressin V_{1A} receptors ($V_{1A}RX$) in the PVN, both under baseline conditions and during exposure to acute air-jet stress. Blood pressure (BP), heart rate (HR) and their short-term variability as well as spontaneous baroreflex sensitivity (BRS) were evaluated using spectral analysis and the sequence method, respectively.

Results: Under baseline conditions, $V_{1A}R$ over-expressing rats exhibited reduced BRS and this was antagonized by $V_{1A}RX$. Exposure to stress increased BP, HR, BP variability, and decreased BRS in all rats. In $V_{1A}R$ rats, stress induced a marked increase of BP variability and HR variability, all of which were prevented by $V_{1A}RX$ pre-treatment. In wild-type rats, $V_{1A}RX$ did not modify cardiovascular parameters under baseline conditions but prevented stress-induced BP variability increase.

Discussion: The present findings show for the first time that $V_{1A}Rs$ in the PVN are involved in local (autocrine/paracrine) regulation of neurons involved in the control of the baroreflex function and cardiovascular short-term variability. During exposure of wild-type rats to stress, $V_{1A}Rs$ in the PVN were responsible for an increase of BP variability. In rats over-expressing $V_{1A}R$ in the PVN, baroreflex was desensitized both under baseline conditions and stress, while cardiovascular variability was markedly increased by stress. These findings indicate that the level of expression (*i. e.* density) of $V_{1A}R$ in the PVN influences cardiovascular vulnerability to stress. The findings also implicate a possible role of somato-dendritic release of VP and of $V_{1A}Rs$ in the PVN in cardiovascular pathology, especially hypertension and heart failure, whose poor prognosis is associated with baroreflex desensitization and enhancement of cardiovascular short-term variability.

Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant no. III 41013) and BBSRC (BB/G006156/1 and BB/J01515/1).

A1.22

Effects of a water extract of *Ocimum basilicum* on glycemia in normoglycemic and alloxan-induced diabetic rats

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Intrinsic Activity, 2015; **3**(Suppl. 2): A1.22
http://www.intrinsicactivity.org/2015/3/S2/A1.22

Background: Basil (*Ocimum basilicum*) is herbaceous perennial plant of the family Lamiaceae (mints). The whole plant has been used as traditional medicine for household remedy against various human ailments from antiquity, and nowadays it is used as aroma additive in food, pharmaceuticals and cosmetics. Basil extracts affect glycemia primarily by preventing the occurrence of postprandial hyperglycemia and increasing the usability of glucose in peripheral tissues.
Methods: Experiments were carried out on laboratory Wistar rats. Animals were treated with a water extract of *O. basilicum* for seven days. Alloxan was used to induce hyperglycemia. The effects of the water extract of *O. basilicum* on glycemia were evaluated using the oral glucose tolerance test and by measuring blood glucose levels in alloxan-induced diabetic rats. In addition, the effect of the treatment on the body weight of the rats was recorded.
Results: The body weight of the diabetic animals treated with a water extract of *O. basilicum* was significantly decreased compared to the body weight in the control group ($p < 0.05$) and the experimental group that was treated only with basil extract ($p < 0.01$). In the group of the diabetic animals treated with the water extract of *O. basilicum*, there was a significantly lower increase of the body weight compared to the control group ($p < 0.05$) and the experimental group that was treated only with basil extract ($p < 0.01$). After the induction of hyperglycemia with alloxan, the water extract of *O. basilicum* significantly lowered glycemia ($p < 0.01$).
Discussion: The aqueous extract of basil did not lead to significant decreases in blood glucose in normoglycemic animals during the seven-day treatment. In contrast, in diabetic animals there was a statistically significant reduction of serum glucose levels. The treatment with a water extract of *O. basilicum* prevents disorders in glucose homeostasis induced by pro-oxidant effects of alloxan. The water extract of *O. basilicum* has no significant influence on the change in body mass in animals with alloxan- induced hyperglycemia.
Acknowledgements: This work was supported by the Ministry for Education, Science and Technological Development of the Republic of Serbia (project no. 41012) and by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina (project no. 114-451-3551/2015-03).

Neuropharmacology

A2.1

Unnatural amino acids as a novel tool to study the folding of the serotonin transporter

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.1
http://www.intrinsicactivity.org/2015/3/S2/A2.1

Background: The serotonin transporter (SERT) is a membrane protein, comprising cytosolic N- and C-termini, 12 transmembrane domains (TMDs) and a large extracellular loop between TMDs 3 and 4. SERT is responsible for the rapid reuptake of serotonin from the synaptic cleft, thus terminating neurotransmission. Mutations in the first cytoplasmic loop and the C-tail region of SERT lead to misfolding and ER retention of the transporter. The folding-defective mutants can be rescued by treatment with noribogaine and heat shock protein (HSP) inhibitors [1,2].
Methods: Amber codons (TAG) were introduced into 23 locations of human SERT. HEK 293 T cells were co-transfected with plasmids encoding these constructs, the orthogonal (suppressor) tRNA and the evolved amino acyl-tRNA synthase pair. This allows for incorporation of *p*-benzoyl-L-phenylalanine at the amber codon. This specific unnatural amino acid (UAA) is also suitable for UV-induced photo-crosslinking, performed by irradiating the cells (365-nm UV light source) and identifying the cross-linked products by gel electrophoresis using a SERT-specific antibody.
Results: A series of residues located in the N- and C-termini of SERT, as well as within cytoplasmic loop and TMD regions, were replaced by the amber codon. All 23 mutants were functionally screened by measuring specific [^{3}H]5-HT uptake. Upon incorporation of the UAA, the functional activity of the mutants ranged from 10 to 80 % of the wild-type uptake levels. However, some mutants were not recovered by adding UAA to the culturing media, even though they could be functionally rescued by the pharmacochaperone noribogaine. Moreover, UV-induced cross-linking experiments produced high molecular weight species, indicating an association of the mutants with partner proteins. Interestingly, the detected cross-linked species were not identical among the mutants we examined. This suggests that specific partners are coupled to SERT proteins trapped at distinct stages along the folding trajectory. On the other hand, no cross-linked products were found for amber codons introduced at locations known to face the lipid bilayer, although the same mutations exhibited specific [^{3}H]5-HT uptake comparable to wild-type SERT.
Discussion: Understanding the folding trajectory of SERT and other solute carrier 6 family members is of key physiological relevance, since point mutations in these proteins result in misfolding and cause clinically relevant phenotypes in people. Pharmacochaperoning may become a useful therapeutic option in the treatment of these diseases.
Acknowledgements: This work was supported by FWF projects SFB35-10 (to M.F.) and P27518-B27 (to S.S.). We are grateful to Thomas Sakmar (Rockefeller University, USA) for the generous gift of the UAA expression system and Jacob Andersen (University of Copenhagen, Denmark) for providing several SERT mutants.

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A2.2

Exocyst-dependent trafficking of the wild-type dopamine transporter (DAT) and folding-deficient DAT mutants

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.2
http://www.intrinsicactivity.org/2015/3/S2/A2.2

Background: Uptake through the dopamine transporter (DAT) represents the primary mechanism used to terminate dopaminergic transmission in the brain. Synaptic function depends on the targeting and delivery of a large number of different proteins at the presynaptic membrane, including neurotransmitter transporters, ion channels, anchoring and cell adhesion molecules, and a variety of signal transduction modulators. DAT requires an intact C-terminal PDZ-binding motif to reach the cell surface; the closely related serotonin transporter SERT does not. Previous experiments showed that the PDZ-binding motif of GAT1 engaged the exocyst. The exocyst is a multiprotein complex required by many membrane proteins for delivery to and insertion into the plasma membrane. Here, we tested the hypothesis that DAT requires the exocyst for reaching the cell surface.
Methods: Briefly, the cells were transiently transfected with plasmids encoding DAT, SERT or NET, along with different amounts of the plasmid encoding Exo70; 48 h after transfection, uptake of radio-labelled substrate was determined to quantify surface expression of transporters.
Results: DAT relied on the exocyst to reach the cell surface. Surprisingly, SERT did not require the exocyst complex to reach the cell surface, regardless of whether the experiments were performed in HEK 293 cells (a cell line of fibroblast origin) or in CAD cells. We examined the effects of exocyst components on transporter expression by performing radiolabelled substrate uptake assays in HEK 293 and CAD cells.
Discussion: Exo70 mediates DAT targeting to presynaptic membranes. Identification of proteins as DAT interactors along with the molecular bases and physiological significance of such interactions will result in a better understanding of the role that DAT plays in regulating DA homeostasis in the brain.

A2.3

Mechanism of low-efficacy substrate efflux at the human serotonin transporter

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.3
http://www.intrinsicactivity.org/2015/3/S2/A2.3

Background: The dopamine transporter (DAT) and the serotonin transporter (SERT) terminate dopaminergic and serotoninergic synaptic transmission by reuptake of their cognate neurotransmitters from the synaptic cleft. Mutations in SERT and DAT lead to their misfolding and ER retention. This is of clinical relevance: several mutations have been identified in DAT, which give rise to a syndrome of infantile/juvenile dystonia and Parkinsonism. Misfolding of proteins can be rescued by their cognate ligands, provided that they act as scaffolding molecules and assist in proper protein folding by lowering the energy barrier between folding intermediates. Compounds, which exert this action, are referred to as pharmacochaperones. For SERT, the folding trajectory is thought to proceed through the inward-facing conformation. SERT and DAT have a rich pharmacology, because they are also important targets for illicit substances derived from amphetamine and cathinone. We tapped into a phenethylamine library of compounds (PAL) to search for low efficacy in inducing neurotransmitter efflux through SERT and DAT when compared to amphetamines. This indicates that the compounds trap SERT and DAT in a conformational state in the transport cycle. Thus, these compounds are of interest as candidate pharmacochaperones: they are predicted to rescue folding-deficient SERT and DAT mutants, if this state is visited during the folding trajectory. Thus, the aim of our study was to identify the conformational state, to which PAL compounds bind, by analysing their effects on the transport cycle.
Methods: Substrate translocation through neurotransmitter transporters require a series of conformational changes which can be inferred from electrophysiological analysis of substrate-induced currents that are carried through the transporter: the peak current reflects substrate-induced charge movement; the steady-state current indicates inward-facing conformation visited by the transporter during the conformational cycle. These currents were measured by whole-cell patch clamping of HEK 293 cells stably expressing hSERT. The compound PAL-1045 was studied as an example of a partial releaser for SERT (and DAT) and currents induced by this compound were compared to 5-hydroxytryptamine-induced currents.
Results: Steady-state amplitudes of currents though SERT decreased with increasing concentrations of PAL-1045. This suggests that PAL-1045 readily diffuses through the cell plasma membrane and displays high affinity for the inward-facing conformation of SERT. This was confirmed by increased steady-state amplitudes with increasing concentrations of PAL-1045 when pH of the external solution was lowered from 7.4 to 5.5 decreasing its membrane diffusibility. Slow recovery of 5-HT-induced peak currents on PAL-1045 application and subsequent washout also argues for a longer dwell time of PAL-1045 in its binding site, which precludes intracellular serotonin binding and efflux. Thus, PAL-1045 may be a potential pharmacochaperone for rescue of folding mutants of SERT.
Discussion: Taken together, our observations provide evidence for a mechanism resulting in low-efficacy substrate efflux through SERT in the presence of PAL-1045. The results have implications for the development of low-efficacy releasers as therapeutic agents for addiction therapy and as pharmacochaperones for the treatment of folding mutants in SERT and DAT.
Acknowledgements: This work was supported by the FWF-funded SFB 35; S.B. is supported by the FWF-funded doctoral programme CCHD.

A2.4

Transient receptor potential ankyrin 1 channels participate in somatic pain hypersensitivity in experimental colitis

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.4
http://www.intrinsicactivity.org/2015/3/S2/A2.4

Background: Gastrointestinal disorders such as inflammatory bowel disease (IBD) are associated with pain symptoms also described in rodent models of IBD such as that induced by dextran sulfate sodium (DSS). Central sensitization has been proposed to contribute to the somatic pain symptoms in IBD and related rodent models. The transient receptor potential ankyrin 1 (TRPA1) channel expressed by a subpopulation of primary sensory neurons of the dorsal root ganglion (DRG) and trigeminal ganglion (TG) is a major transducer of nociceptive signals produced by inflammation and tissue injury and is involved in hypersensitivity conditions. There is indication that TRPA1 contributes to visceral pain-like behavior in DSS-evoked colitis. The present study was designed to investigate the role of TRPA1 channels in the colitis-evoked mechanical and thermal hypersensitivity at the somatic level.
Methods: Colitis was induced in C57BL/6 male mice by adding 2% DSS to the drinking water for 7 days. Following this treatment, on day 8, control and DSS-treated mice were tested for various parameters of colitis as well as mechanical sensitivity in the abdominal and facial skin and thermal sensitivity in the plantar skin. Pharmacological blockade of TRPA1 by the selective antagonist HC-030031 (100 mg/kg, i.p.) and genetic deletion of TRPA1 were used to investigate the role of TRPA1 in DSS-induced colitis. The pain sensitivity to mechanical stimuli was evaluated with von Frey hairs (facial and abdominal region) and to thermal stimuli with the hot- and cold-plate method (plantar skin). Colitis-associated parameters, such as body weight, disease activity score, colon length, colon weight and colonic myeloperoxidase (MPO) activity, were measured. The expression of mRNA of various TRP channels (TRPA1, TRPV1 and TRPV4) was quantified in isolated DRGs and TGs of control and DSS-treated mice. On day 8, control and DSS-treated mice were also tested for behavioural (freezing, locomotion, rearing) and molecular changes (c-Fos in spinal cord) in response to a chemical pain stimulus (intrarectal instillation of 2% allylisothiocyanate; AITC) in the presence or absence of HC-030031 (100 mg/kg, i.p.).
Results: Induction of colitis was confirmed by a decrease in body weight and colon length and an increase in colon weight, disease activity score and MPO activity. DSS increased the mechanical (abdominal and facial) and thermal (hot) sensitivity in mice. The TRPA1 antagonist reduced mechanical sensitivity of both the abdominal and facial region. DSS treatment caused an increase in TRPA1 mRNA expression in the DRG. Intrarectal AITC evoked freezing behaviour which was reduced in the presence of the TRPA1 antagonist.
Discussion: Taken together, the current findings indicate that the TRPA1 channel participates in colitis-associated pain hypersensitivity at the somatic level.
Acknowledgements: P.J. is a recipient of a Marietta Blau Fellowship, Federal Ministry of Science, Research and Economy, Austria.

A2.5

K_V7 channels: potential targets for antinociceptive action of paracetamol

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.5
http://www.intrinsicactivity.org/2015/3/S2/A2.5

Background: Paracetamol (acetaminophen; APAP) is a widely used analgesic and is well understood in the context of its benefits and side effects. Multiple pathways have been proposed to explain the mechanism underlying its analgesic action, yet a clear explanation remains elusive. The postulated mechanisms include inhibition of cyclooxygenase enzymes, effects on the descending serotonergic inhibitory pathways, and interactions with opioidergic systems and nitric oxide pathways. Paracetamol is mainly eliminated by glucuronidation and sulfation, while some of it is converted into a reactive intermediate, NAPQI (*N*-acetyl-*p*-benzoquinone imine) by cytochrome P450 enzymes. The M current is characteristic of the neuronal subtypes of voltage-gated potassium channels (K_V7 family). Inhibition of M currents is linked to enhanced neuronal excitability while their augmentation causes neuronal silencing, with established translational use in pain management and epilepsy. Moreover, oxidative modification of K_V7 channels has been shown to enhance M currents with a triplet of cysteines in the channel S2–S3 linker as the mediator of oxidative sensitivity. The project aims at understanding whether NAPQI is involved in the antinociceptive action of paracetamol.
Methods: tsA201 cells were transfected using the Turbofect kit (Fermentas). DRG neurons were dissected from 10 day old rats and cultured at 37°C/5% CO_2 for 2 days. Electrophysiological recordings were made using the perforated patch-clamp technique. NAPQI was perfused for 3 minutes at each concentration followed by washout for 5 minutes.
Results: Our preliminary data show a progressive enhancement of M current in heterologously expressed $K_V7.2$ homomers starting at 0.3 µM NAPQI with a threefold rise at 10 µM, which is maintained during a control washout for 5 minutes. The $K_V7.2/7.3$ heteromers and capsaicin-positive DRG neurons showed a similar profile with a maximal response at 0.3 µM and 1 µM NAPQI, respectively, and a sustained decrease in the amplitude of current during washout.
Discussion: These results indicate that the mechanism of action of paracetamol could be explained by the enhancement of M current and consequently a decrease in excitability of DRG neurons by its metabolite NAPQI.
Acknowledgements: This project is funded by the doctoral program "Cell Communication in Health and Disease" (CCHD; co-financed by FWF and the Medical University of Vienna; W1205).

A2.6

Neuropeptide Y knockout alters behavioural effects of environmental enrichment

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.6
http://www.intrinsicactivity.org/2015/3/S2/A2.6

Background: Environmental enrichment (EE), an improved laboratory housing condition to enhance rodent welfare, reduces anxiety and facilitates stress coping of mice. Neuropeptide Y (NPY), a key peptide for the processing of stress, has similar behavioural effects. Given these resemblances, the current work investigated the role of NPY in the behavioural effects of EE.
Methods: The behavioural phenotype of wild-type (WT) and NPY knockout (NPY KO) mice housed either under standard environment (SE) or EE was assessed in various behavioural tasks. After a 9-week differential housing period anxiety was evaluated with the elevated plus maze (EPM) and the open field test (OF), while stress coping and depression-like behaviour was measured with the stress-induced hyperthermia test (SIH) and the forced swim test (FST), respectively.

One day after the last behavioural test NPY levels in the amygdala and hippocampus were measured by PCR and ELISA.
Results: NPY KO abolished the EE-induced anxiolytic effect in the EPM. In particular, EE-housed WT mice made significantly more entries to the open arms of the EPM compared to SE-housed WT mice, an effect not seen in NPY KO mice. In contrast, anxiety, locomotor and depression-like behaviour in the OF and the FST were influenced by genotype, but not housing condition. NPY KOs showed increased anxiety, reduced locomotor activity and enhanced depression-like behaviour independent of housing conditions. Housing itself did however affect climbing behaviour during the FST as both EE-housed WTs and NPY KOs spent more time climbing. The SIH suggested a negative effect of EE for NPY KOs as EE-housed NPY KOs had higher stress-induced rectal temperatures compared to SE-housed NPY KOs. Increased EE-induced amygdalar and hippocampal NPY gene expression in WT mice also suggests an interaction between NPY and EE. The corresponding NPY peptide levels did not differ between the groups indicating enhanced NPY turnover in EE-housed mice.
Discussion: The current molecular and behavioural data favour the contention that NPY contributes to the anxiolytic effects of EE. The absence of NPY abolishes this beneficial effect and even induces negative effects in response to environmental stimulation.
Acknowledgements: Supported by the Austrian Science Fund (FWF grant P25912-B23).

A2.7

The role of hydrogen sulfide in the autonomic nervous system

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.7
http://www.intrinsicactivity.org/2015/3/S2/A2.7

Background: Hydrogen sulfide (H_2S) is a toxic gas also produced in mammalian tissues where it can exert various functions as gasotransmitter, such as opening of smooth muscle K_{ATP} channels and resulting in vasorelaxation. A recent study showed that H_2S is endogenously generated and released in sympathetic ganglia and potentiates ganglionic transmission [1].
Methods: Experiments were performed on primary cultures of rat superior cervical ganglion (SCG) or on transfected tsA cells. Neurotransmitter release was determined by measuring the outflow of radioactivity from cultures labelled with [^{3}H]noradrenaline. Electrophysiological recordings were performed by using the perforated patch-clamp technique.
Results: In SCG primary cultures, we found that in radiotracer release experiments, basal tritium overflow as well as outflow triggered by either electrical fields or depolarizing K^+ concentrations were enhanced by 0.1 to 1 mM of the H_2S donor NaHS in a concentration-dependent manner. In electrophysiological experiments, H_2S hyperpolarized the SCG membrane potential and reduced action potential firing. In SCG neurons, hyperpolarisation of membrane potential can be caused by an enhancement of currents through K_V7 channels [2]. Unexpectedly, NaHS inhibited currents through K_V7 channels in a concentration-dependent manner, whether endogenously expressed in SCG neurons or heterologously expressed in tsA cells.
Discussion: These results show that H_2S regulates various functions of ganglionic neurons. Nevertheless, diazoxide, a well-known K_{ATP} channel opener, also hyperpolarized the SCG membrane potential leading to the hypothesis that the membrane hyperpolarization caused by H_2S could be an effect mediated by K_{ATP} channels.
Acknowledgements: M.D.R. is member of the doctoral programme "Cell Communication in Health and Disease" (CCHD; co-financed by the Austrian Science Fund FWF and the Medical University of Vienna; W1205).

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A2.8

Mechanisms underlying the excitation of rat sensory neurons via metabotropic 5-HT receptors

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.8
http://www.intrinsicactivity.org/2015/3/S2/A2.8

Background: Serotonin (5-HT) is an inflammatory mediator and involved in pain sensation. Ionotropic 5-HT_3 receptors of dorsal root ganglion (DRG) neurons are thought to mediate this effect. However, the role of metabotropic 5-HT receptors is still unknown. Here, the contribution of metabotropic 5-HT receptors and their functional interactions with K_V7, TRPV1 and Ca^{2+}-activated Cl^- channels (CaCCs) were investigated.
Methods: Using the perforated patch-clamp technique in voltage- and current-clamp mode on primary cultures of rat DRG neurons, effects of 5-HT receptor ligands on membrane potential and currents through K_V7, TRPV1 and Ca^{2+}-activated Cl^- channels were investigated.
Results: 5-HT increased the excitability of DRG neurons and caused depolarizations. This effect was not altered by the 5-HT_3 receptor antagonist tropisetron, but reduced by the 5-HT_2 receptor antagonist ritanserin. Moreover, this excitation of DRG neurons by 5-HT was inhibited by the TRPV1 antagonist iodoresiniferatoxin (I-RTX) and the CaCC (TMEM16) blocker $CaCC_{inh}$-A01, but not by the TMEM16A-specific blocker $T16A_{inh}$-A01. Furthermore, this 5-HT-induced excitation was inhibited by the 5-HT_{2A} receptor-specific antagonist 4F 4PP oxalate rather than by the 5-HT_{2C} receptor-specific antagonist RS-102221 hydrochloride. Currents through K_V7 channels of DRG neurons were not inhibited by 5-HT. By contrast, 5-HT enhanced currents through TRPV1 channels in DRG neurons. This increase of the TRPV1 current was inhibited by the 5-HT_2 receptor antagonists ritanserin and ketanserin. Moreover, the enhancement was also inhibited by blocking both 5-HT_{2A} and 5-HT_{2C} receptors. As expected, this enhancement of currents through TRPV1 channels by 5-HT was inhibited by the PLC-blocker U73122, the PKC blocker GF109203X, the Ca^{2+}-ATPase blocker thapsigargin and the Ca^{2+} chelator BAPTA-AM, respectively. Additionally, 5-HT also enhanced currents through CaCCs. The involvements of 5-HT_2 receptors in the potentiation of CaCC currents via 5-HT and related signaling mechanisms will be investigated further.

Discussion: These results indicate that the 5-HT_2 receptor-induced increase in excitability is not mediated by K_V7 channel inhibition, but rather by sensitization of TRPV1 channels and activation of CaCCs. Additionally, this effect involves activation of both PLC and PKC.

A2.9

In vitro effects of ethanol and gabapentin treatment

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.9
http://www.intrinsicactivity.org/2015/3/S2/A2.9

Background: The anticonvulsant drug gabapentin, a structural analogue of GABA, showed beneficial effects in the treatment of alcoholism and its consequences. Although the mechanisms of action of gabapentin are not fully understood, studies suggested that gabapentin's neuroprotective effects could be achieved via $GABA_A$ receptors. The aim of this study was to investigate the potential protective action of gabapentin on the well-known neurotoxic effects of chronic alcohol consumption and withdrawal.
Methods: The dose–response relationship and the time course of ethanol and gabapentin treatment were established on human embryonic kidney (HEK) 293 cells, either non-transfected or stably expressing α1β2γ2S $GABA_A$ receptors. A trypan blue exclusion assay and MTT test were performed to assess cell viability. Membrane preparations of stably transfected HEK 293 cells treated with 100 mM ethanol in combination with 1 μM gabapentin for 96 h were used in [^{3}H]flunitrazepam and [^{3}H]TBOB binding studies to determine the number and affinity of benzodiazepine and convulsant binding sites, and their allosteric interactions with GABA binding sites. 100 μM bicuculline, a $GABA_A$ receptor antagonist, was used in other to counteract the effects of gabapentin. The levels of mRNA encoding the α1, β2 and γ2S receptor subunits in stably transfected HEK 293 cells following ethanol and gabapentin treatment were determined by semiquantitative RT-PCR analysis.
Results: Treatment with ethanol at concentrations higher than 100 mM for 96 h reduced the number of HEK 293 cells, non-transfected or transfected with α1β2γ2S $GABA_A$ receptors. The cytotoxic effect of ethanol was counteracted by co-administration of 1 μM gabapentin in transfected, but not in non-transfected cells. Exposure to 100 mM ethanol induced an increase in the number of binding sites for benzodiazepines and convulsants, while this effect was reversed by simultaneous exposure to 1 μM gabapentin, 100 μM bicuculline or their combination. Administration of ethanol, as well as bicuculline, induced functional allosteric uncoupling of the GABA and the benzodiazepine binding site, which was prevented by simultaneous gabapentin treatment. The mRNA expression levels of α1, β2 and γ2S $GABA_A$ receptor subunits were not influenced by the exposure to either ethanol or gabapentin, but only by their co-administration.
Discussion: Although our results support the hypothesis of an involvement of $GABA_A$ receptors in the actions of gabapentin, further research is needed to elucidate the mechanisms of gabapentin's protective effects against ethanol-induced cytotoxicity.

A2.10

Efficacy of the morphine–ketamine–magnesium sulphate combination in the tail-immersion test in rats

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.10
http://www.intrinsicactivity.org/2015/3/S2/A2.10

Background: *N*-methyl-D-aspartate (NMDA), ketamine and magnesium enhance the antinociceptive effects of opioid analgesics in different animal models of pain, as well as in humans. This study aimed at evaluating whether magnesium sulphate added to a morphine–ketamine combination produces a higher level of analgesia.
Methods: Analgesic activity was assessed by the tail-immersion test in male Wistar rats (200–250 g). The distal 5 cm of the tail was immersed in a warm water bath (55 ± 0.5 °C) and the time for tail withdrawal was measured as response latency.
Results: Magnesium sulphate (0.5–60 mg/kg, s.c.) and ketamine (5–30 mg/kg, i.p.) administered alone did not produce any effect. Magnesium sulphate (5 and 60 mg/kg) and ketamine (5 and 30 mg/kg) increased the antinociceptive effect of morphine (2.6 mg/kg, i.p.). Magnesium sulphate (5 mg/kg) increased the antinociceptive effect of the morphine (2.6 mg/kg)–ketamine (2.5 or 5 mg/kg) combination when magnesium sulphate was added to morphine after, but not before, ketamine. Magnesium sulphate also prolonged the duration of the antinociceptive effect of the morphine–ketamine combination. Low doses of morphine (2.6 mg/kg), ketamine (5 mg/kg) and magnesium sulfate (5 mg/kg) given together did not cause motor impairment, which was verified by the rotarod test. The antinociceptive effect of the triple combination was readily antagonized by naloxone (3 mg/kg, s.c.), a nonselective antagonist of opioid receptors, indicating that the effect is mediated via opioid receptors.
Discussion: These data suggest that the combined administration of low doses of ketamine and magnesium sulphate provides more profound effects without exceeding safe doses. This information may be useful for preventing or treating acute pain in several settings. However, interaction may also occur when magnesium sulphate is used as an electrolyte replenisher after morphine–ketamine analgesia. An additional bonus are the neuroprotective effects of ketamine and possibly magnesium. This study revealed that in the tail-immersion test in rats the efficacy of the morphine–ketamine–magnesium sulphate combination is influenced by the order of medication administration; a higher level of activity is demonstrated only when ketamine is added to morphine before magnesium sulphate.
Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant no. 175023).

A2.11

The Pro-Gly-containing dipeptidic cognitive enhancer noopept increases the DNA-binding activity of HIF-1

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.11
http://www.intrinsicactivity.org/2015/3/S2/A2.11

Background: Noopept (the ethyl ester of *N*-phenylacetyl-L-prolylglycine, GVS-111) was synthetized and studied pharmacologically at the Institute of Pharmacology. The experimental study of this orally active substituted dipeptide revealed a wide spectrum of cognitive improving and neuroprotective effects. Noopept was shown to increase the survival and to restore the memory damaged by hypobaric hypoxia, to diminish the volume of necrotic area on different models of stroke, to attenuate the degree of cognitive disturbances as well as NGF and BDNF deficits in a model of Alzheimer's disease (AD). *In vitro* experiments revealed noopept's ability to attenuate the manifestations of oxidative stress, to restore the calcium homeostasis, to stimulate the neurogenesis and to diminish tau-protein aggregation in the amyloid model of AD, to attenuate α-synuclein aggregation in a model of parkinsonism, to increase the survival of human cultivated cortical neurons from the fetus with prenatally diagnosed Down syndrome. Noopept increases the expression of NGF and BDNF in hippocampus and hypothalamus, inhibits the stress-induced kinases pSAPK/JNK and pERK. Meanwhile, looking for the interaction of noopept with more than 100 conventional receptors, we failed to reveal the primary target for this dipeptide. The aim of the present investigation was to evaluate the influence of noopept on DNA-binding activity of various transcriptional factors: CREB, NFAT, NF-κB, p53, STAT1, GAS, VDR, HSF1 and HIF-1.

Methods: Experiments were performed on HEK 293 cells, transiently transfected by luciferase reporter constructions containing sequences for CREB, NFAT, NF-κB, p53, STAT1, GAS, VDR, HSF1 and HIF-1.

Results: Noopept (10 μM) increased the DNA-binding activity of HIF-1 only, while lacking an ability to affect that of CREB, NFAT, NF-κB, p53, STAT1, GAS, VDR and HSF1. Being applicated in the condition of $CoCl_2$-induced HIF-1 stabilization, noopept provoked an additional increase of DNA binding of HIF-1. The degree of this HIF-positive effect was shown to be concentration-dependent. The common nootropic drug piracetam (1 mM) failed to significantly affect any of the transcriptional factors in this study. The results of molecular docking showed that the L-isomer of noopept, unlike its pharmacologically ineffective D-isomer, is able to bind with the active site of prolyl-hydroxylase 2, the enzyme responsible for HIF-1 degradation. The energy of enzyme–ligand binding for noopept and its metabolite phenylacetylproline was close to that for standard inhibitors of prolyl-hydroxylase.

Discussion: Taking into account the important role of genes activated by HIF-1 in the arrangement of adaptive reaction to hypoxia, data on noopept's ability to provoke a selective increase of DNA-binding activity of HIF-1 explain the wide spectrum of noopept's neurochemical and pharmacological effects revealed before. The results of this study suggest the HIF-positive effect as a primary mechanism of noopept's activity.

A2.12

Effects of hemantane on the activity of proline-specific endopeptidases in plasma of rats with experimental Parkinson's disease

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.12
http://www.intrinsicactivity.org/2015/3/S2/A2.12

Background: Proline-specific endopeptidases—DPP-4 (dipeptidyl peptidase 4; EC 3.4.14.5) and PEP (prolyl endopeptidase; EC 3.4.21.26)—and the peptides that they hydrolyse are involved in the pathogenesis of neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease and others. The aim of this study was to evaluate the levels of activity of DPP-4 and PEP in two models of experimental PD and assess the effects of hemantane (*N*-2-(adamantyl)-hexamethylenimine hydrochloride), a novel antiparkinsonian drug with potential neuroprotective activity, which was developed in the Zakusov Institute of Pharmacology.

Methods: PD was induced in rats by rotenone (2.75 mg/kg per day for 7 days, i.p.) and by injection of 6-hydroxydopamine (6-OHDA; 12 μg) into the left medial forebrain bundle (MFB). Blood plasma was taken on day 20 after the first rotenone injection and on day 35 after injection of 6-OHDA. Hemantane (10 mg/kg) was administered 10 min prior to rotenone during 7 days, or during 21 days daily starting from day 14 after injection of 6-OHDA. Detection of DPP-4 and PEP activity was carried out by fluorometric assay.

Results: In rats with rotenone-induced PD, a 44.3% increase of PEP activity was determined compared to intact animals ($p < 0.05$). Hemantane caused a 29.7% decrease of PEP activity compared to non-treated rats ($p < 0.05$). DPP-4 activity in this model of PD did not change; hemantane also had no effect on DPP-4 activity compared to non-treated animals. In rats with 6-OHDA-induced PD no changes in PEP activity were revealed as well as no effect of hemantane. In rats with 6-OHDA-induced PD a 17.2% increase of DPP-4 activity compared to sham-operated animals ($p < 0.05$) was determined. In rats which were treated with hemantane, a further increase of DPP-4 activity (by 18% compared to non-treated rats, $p < 0.05$) was found.

Discussion: PEP is known to promote α-synuclein aggregation. The rotenone model of PD is the only model where altered α-synuclein accumulation was reproduced. The ability of hemantane to reduce PEP levels suggests that the drug could possess PEP inhibitory properties. The PD model using 6-OHDA administration into the MFB is a model of more severe PD. In this model hemantane failed to decrease motor disturbances in previous studies as well as DPP-4 activity in the current assay.

A2.13

Oxidative phosphorylation in the healthy and in the epileptic mouse brain

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.13
http://www.intrinsicactivity.org/2015/3/S2/A2.13

Background: Mitochondrial dysfunction appears to be a common factor in neurodegenerative diseases and epilepsy. Strikingly,

neurodegenerative diseases show regional specificity in vulnerability and follow distinct patterns of neuronal loss. It is a challenge to understand how mitochondrial failure in particular brain regions contributes to specific pathological conditions.

Methods: High-resolution respirometry combined with specific pharmacological activation and inhibition protocols of elements of the respiratory system revealed significant differences of complex I- and II- (CI and CII)-linked oxidative phosphorylation (OXPHOS) capacity and coupling control between motor cortex, striatum, hippocampus and pons of naive mice.

Results: CI-linked respiration was highest in motor cortex. In contrast, CII-linked capacity was especially important in the striatum. Apparent excess capacities of the electron transfer system (ETS) over OXPHOS also differed between regions. In the kainic acid model of temporal lobe epilepsy in mice, we observed down-regulation of CI- and upregulation of CII-linked respiration in the injected dorsal hippocampus 3 weeks after treatment.

Discussion: In summary, respirometric OXPHOS analysis allows detailed analysis of mitochondrial function from small amounts of specific tissues (about 2 mg). It thus enables comparison of different brain tissues implicated in neurodegenerative diseases of the healthy mouse and disease models, while leaving enough material for further studies on the tissues. We propose that the presented differences may indicate risk factors for region-specific neuronal vulnerabilities. For example, a low apparent ETS excess capacity over OXPHOS capacity in the striatum together with the distinct pattern of respiratory control may contribute to the high vulnerability of striatal neurons in the presence of CII-inhibiting mutated huntingtin proteins.

Acknowledgements: Supported by the Austrian Science Fund (FWF), project W1206-B05.

A2.14

The G protein-biased kappa opioid receptor agonist 6′-GNTI blocks hippocampal paroxysmal discharges without inducing aversion

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.14
http://www.intrinsicactivity.org/2015/3/S2/A2.14

Background: Neuropsychiatric disorders are one of the main challenges of medicine with epilepsies representing some of the most frequent. Temporal lobe epilepsy (TLE) is the most common type of epilepsies and is often accompanied by marked neuronal degeneration. It was shown that the deletion of prodynorphin in mice and low expression in humans is associated with increased epilepsy vulnerability. Dynorphin targets opioid receptors and in particular the κ opioid receptor. Kappa receptors are located in strategically ideal places to control hippocampal excitability also in chronic TLE. The aim of this study was to investigate the potential of κ receptor agonists as antiepileptic drugs (AEDs) in a mouse model of drug-resistant TLE.

Methods: Fifteen C57BL/6N male mice were injected unilaterally with kainic acid (KA; 1 nmol in 50 nl saline; $n = 10$) into the dorsal hippocampus. Four-channel EEG traces were recorded from ipsi- and contralateral hippocampi and motorcortices applying depth- and surface electrodes from freely moving mice, respectively. The κ receptor-specific agonist U-50488H, saline or one the new AEDs oxcarbazepine, lamotrigine and levetiracetam were applied i.p., while the biased κ receptor partial agonist 6′-GNTI was delivered i.c.v. through a guide canula. Number and duration of EEG seizures were automatically evaluated for the 60 min preceding and following the injections. Another group of animals (20 male mice C57BL/6N) was tested in the conditioned place avoidance (CPA) paradigm for U-50488H and 6′-GNTI.

Results: Spike trains and hippocampal paroxysmal discharges (HPDs) in the ipsilateral hippocampus were observed starting from day 5 after KA injection. Application of either U-50488H or 6′-GNTI decreases both spike trains and HPDs caused by KA in a dose-dependent manner. The AEDs lamotrigine and oxcarbazepine only reduced spike trains. As expected, the CPA experiments revealed that the animals conditioned to U-50488H developed avoidance for the compartment paired with this drug. On the other hand the biased κ receptor agonist 6′-GNTI did not produce any avoidance.

Discussion: Our data demonstrate the anticonvulsant action of κ receptor agonists in the chronic phase of epilepsy, comparable to the effect of 2.5 mg/kg diazepam. Furthermore, we demonstrate that the biased κ receptor partial agonist 6′-GNTI does not induce place avoidance in the CPA paradigm. The absence of κ receptor-induced dysphoria is probably due to the fact that 6′-GNTI does not recruit the β-arrestin pathway.

Acknowledgements: This work was supported by the Austrian Science Fund FWF: W1206-BO5.

A2.15

Monitoring the movement of helix 1a of LeuT$_{Aa}$ in micelles versus liposome system by using luminescence resonance energy transfer (LRET)

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.15
http://www.intrinsicactivity.org/2015/3/S2/A2.15

Background: Solute carrier class 6 proteins (SLC6) have gained a great attention in terms of their pharmacological importance. Malfunctioning of SLC6 proteins results in numerous debilitating central and nervous system diseases. LeuT$_{Aa}$, a bacterial homologue of SLC6 protein, with various high-resolution crystal structures is serving to date as structural and functional paradigm to SLC6 proteins. Large-scale changes in helix 1a (TM1a) of LeuT$_{Aa}$ in solution have been investigated using single molecule FRET studies relating these movements to the substrate-releasing state of LeuT$_{Aa}$. We used LRET as a tool to study the movement of TM1a in micelles as well as in a more native lipid membrane environment.

Methods: Employing lanthanide-based resonance energy transfer (LRET) as a tool of trade, we measured the intramolecular distance changes in LeuT$_{Aa}$. Mutants were screened for their functional activity using scintillation proximity assay. These mutants were further characterized by accessing their uptake activity after successfully reconstituting them in POPC liposomes. LRET-based intramolecular distance measurements were done in DDM detergent micelles from purified pre-labeled proteins. In case of lipid membrane environment, pre-labeled protein was reconstituted into POPC liposomes. Ionic gradient was excluded during measurement in POPC proteoliposomes.

Results: The C-terminal LBT (R519-LBT-G520_LeuT) and its cysteine mutants (R519-LBT-G520_A9C_LeuT) showed substrate binding and transport activity comparable to the wild-type LeuT$_{Aa}$. Focusing TM1a movements in Na^+-bound (outward-open) and Na^+-free (inward-open) conformations of LeuT$_{Aa}$, LRET measurements were carried out. In case of DDM detergent micelles environment

TM1a was quite flexible in inward-open *vs.* outward-open conformations. In contrast to detergent micelle environment, lipid environment posed a great constrain over the flexibility of TM1a. These experimental results were also supported strongly with our *in silico* studies. In addition, $LeuT_{Aa}$ was reconstituted into giant unilamellar vesicles (GUVs) of defined composition to gain a gradient over the plasma membrane.

Discussion: Lipid membranes pose a constrained environment for TM1a movement in substrate-releasing conformation. While the constraint of TM1a movement in lipid environment is released for relatively flexible movements of TM1a in detergent micelles system. In-house $LeuT_{Aa}$ GUVs are quite stable and will provide a nice gradient over the plasma membrane.

A2.16

The impact of phosphatidylinositol-4,5-bisphosphate (PIP_2) on serotonin transport function

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Intrinsic Activity, 2015; **3**(Suppl.2):A2.16
http://www.intrinsicactivity.org/2015/3/S2/A2.16

Background: The serotonin transporter (SERT) plays a key function in the termination of serotonergic neurotransmission. SERT is the main pharmacological target in treating depressive disorders and also a target for various drugs of abuse. Drugs like amphetamine-type stimulants reverse the direction of transport which finally leads to an increased serotonin concentration in the synaptic cleft. Transmembrane proteins get in close contact with the lipid environment and are partitioned in specific lipid microdomains. In a previous study we already implicated the phosphatidylinositol PIP_2, a major signaling molecule, to influence amphetamine effects at SERT and elucidated a specific binding interface [1]. We explored a positively charged SERT area using a computational approach and identified a putative second binding site which is close to the inner leaflet of the plasma membrane.

Methods: ***Amino acid exchange*** was done by introduction of single point mutation into a YFP-tagged human SERT (wild-type) construct. ***Uptake and release assays:*** 0.2 µM [^{3}H]5-HT at increasing 5-HT concentrations (1–60 µM) was added for 1 min; 10 µM paroxetine was used to determine nonspecific uptake. 30 µM m-3M3FBS and 30 µM PAO or DMSO respectively 10 µM Pal-peptide were incubated for 20 min at room temperature. Substrate efflux was measured after cells were preloaded with 0.1 µM [^{3}H]MPP+ for 20 min at 37 °C. Cells were then transferred into chambers and a stable baseline was established by superfusion with Krebs-Ringer-Hepes buffer for 40 min. Efflux was induced using 3 µM *para*-chloramphetamine (*p*CA). Two-minute fractions were collected and samples were counted in a beta counter. ***Cell surface biotinylation:*** After 4 h starvation, cells were incubated with sulfo-NHS-SS-biotin (1 mg/ml). Excessive biotin was quenched (100 mM glycine). 100 µg protein was loaded on 30 µl streptavidin-agarose beads. Samples were analysed via western blot. ***Lipid overlay assay:*** PIP strips (Avanti®) were blocked with 3% BSA (fatty acid free) and incubated with protein in TBS o/n at 4 °C.

Results: Manipulating cellular PIP_2 levels had an effect on amphetamine-induced substrate efflux. Neutralizing this positively charged SERT area led to a loss of this effect.

Discussion: We could show that both binding sites are necessary for a stable PIP_2–SERT interaction. Neutralization of positive charges within the binding sites abolished PIP_2 modulation of amphetamine-induced efflux. By drastically reducing intracellular PIP_2 levels we could show a decreased amphetamine-induced efflux in SERT. This effect could not be observed in mutant SERT, indicating a loss of PIP_2-mediated effect on substrate efflux. Furthermore we could show that SERT not only interacts with PIP_2 but also with other phosphatidylinositol species. This interaction is almost lost upon neutralization of both binding sites.

Acknowledgements: The work of was financially supported by the Austrian Science Fund FWF (P23658-B11).

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A2.17

Functional and physical interactions between P2Y receptors and ion channels

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Intrinsic Activity, 2015; **3**(Suppl.2):A2.17
http://www.intrinsicactivity.org/2015/3/S2/A2.17

Background: Neuronal P2Y receptors, *i.e.* nucleotide-sensitive G protein-coupled receptors (GPCRs), are known to control various voltage-gated ion channels, in particular K_V7 potassium and $Ca_V2.2$ calcium channels. The differential modulation of these ion channels via GPCRs was shown to rely on the presence or absence of scaffolding proteins. Since scaffold proteins are believed to bring GPCRs and ion channels in close proximity to guarantee efficient G protein-mediated modulation, this project evaluated whether a tight contact between P2Y receptors and ion channels is a prerequisite for their functional interaction.

Methods: P2Y receptors and ion channels were labeled either with CFP or YFP. For all experiments, a CFP/YFP pair of receptor and channel was transfected transiently into tsA201 cells. Channel modulation by nucleotides was determined by patch-clamp recordings. The fluorescence microscopy techniques FRET (Förster resonance energy transfer) and DRAP (donor recovery after acceptor photobleaching) were used to determine the protein–protein interaction between receptors and channels. Furthermore, FRAP (fluorescence recovery after photobleaching) was performed to elucidate the mobility of the receptors and channels in the membrane.

Results: Activation of $P2Y_1$, but not of $P2Y_{12}$, receptors by ADP inhibited the K^+ currents in a concentration-dependent manner by up to 20.5 ± 1.9%. Conversely, activation of both, $P2Y_1$ and $P2Y_{12}$, receptors reduced the Ca^{2+} currents by up to 60.1 ± 7.4% and 76.3 ± 4.2%, respectively. FRET and DRAP experiments showed that $P2Y_1$ has a protein–protein interaction with both, $K_V7.2/7.3$ (NFRET 0.32 ± 0.02, DRAP recovery 10.3 ± 3.0%) and $Ca_V2.2$ (NFRET 0.37 ± 0.02, DRAP recovery 10.1 ± 1.8%). On the other hand, $P2Y_{12}$ has an interaction only with $Ca_V2.2$ (NFRET 0.39 ± 0.03, DRAP recovery 12.7 ± 1.0%) but not with the $K_V7.2/7.3$ channels. FRAP experiments revealed that the mobility of the ion channels alone is higher than that of the receptors. The coexpression of the P2Y receptors significantly reduced the mobility of the $Ca_V2.2$ channel by 50% (from 3.3 sec to 6.2 sec). In the case of $K_V7.2/7.3$ channels, the τ values were not significantly changed by the presence of P2Y.

Discussion: The functional control of K_V7 by $P2Y_1$ and $Ca_V2.2$ by $P2Y_1$ and $P2Y_{12}$ receptors relies on a close apposition of receptors and channels. In the case of $Ca_V2.2$ and P2Y12 this is even accompanied by a physical interaction.

Acknowledgements: This study is supported by the FWF-funded doctoral program CCHD (W1205).

A2.18

"Second generation" mephedrone analogs, 4-MEC and 4-MePPP, differentially affect monoamine transporter function

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.18
http://www.intrinsicactivity.org/2015/3/S2/A2.18

Background: The increase in the use of synthetic psychoactive "designer drugs" followed by the ban of 4-methyl-*N*-methylcathinone (mephedrone) is a cause for grave concern. This newly emerging threat of "second generation" mephedrone analogues including 4-methyl-*N*-ethylcathinone (4-MEC) and 4-methyl-α-pyrrolidino-propiophenone (4-MePPP) are skillfully designed to evade law and require thorough investigation to understand their physiological effects and pharmacological action on their targets, the monoamine transporters.

Methods: An array of techniques was used to analyse the effects of 4-MEC and 4-MePPP including molecular, cellular and whole animal methods. *In vitro* transporter assays served the purpose to elucidate the inhibitory and release properties of the drugs at the serotonin transporter (SERT) and dopamine transporter (DAT). Microdialysis was used to assess the *in vivo* neurochemistry. Transporter-mediated currents were detected in oocytes expressing SERT. Computational docking was used as a tool to shed light to understanding the differences in their pharmacological profile.

Results: 4-MEC displayed a "hybrid" profile acting as a SERT substrate and DAT blocker. It also produced a large increase in extracellular 5-HT, a small increase in dopamine and very minimal motor stimulation. It also evoked inward current in SERT-expressing oocytes. 4-MePPP is a blocker for both SERT and DAT, produced selective increase in dopamine levels and robust motor stimulation. The inability of 4-MePPP to influence the SERT was supported by computational docking of the two drugs at the binding pocket of SERT and DAT revealing subtle differences in their binding mode at the SERT binding pocket.

Discussion: The above findings reflect the importance of understanding the pharmacology of newly emerging drugs and highlight the central role of structure–activity relationship of the drugs and its profound influence on the pharmacology. For the full publication of the data see [1].

Acknowledgements: This research was supported by the Intramural Research Program of the NIDA, NIH, grant DA000523-07 to M.H.B. and the Austrian Research Fund FWF, grants F3506 and W1232 to H.H.S.

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A2.19

The effect of magnesium sulfate in carrageenan-induced inflammatory pain in rats

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.19
http://www.intrinsicactivity.org/2015/3/S2/A2.19

Background: Magnesium is the fourth most abundant essential ion in the human body and plays a fundamental role in many cellular functions, such as storage, metabolism and energy utilization. Additionally, magnesium acts as a blocker of voltage-dependent *N*-methyl-D-aspartate (NMDA) receptor ion channels. It has been demonstrated to enhance the effects of opioids and general and local anaesthetics. Magnesium has analgesic efficacy against neuropathic pain, but reports on its effects on inflammatory pain are controversial. This study aimed at evaluating the systemic and local effects of magnesium sulfate on the inflammatory somatic pain after systemic and local administration in rats.

Methods: Hyperalgesia was induced in male Wistar rats by injection of 0.5% carrageenan (0.1 ml) into the paw. $MgSO_4$ was given s.c. either 5 min before the injection of carrageenan or co-injected with carrageenan. Hind paw withdrawal threshold to mechanical stimuli was measured six hours after intraplantar injection of carrageenan using the von Frey anesthesiometer test.

Results: Pretreatment with systemic $MgSO_4$ resulted in a dose-independent increase in the mechanical paw withdrawal threshold after carrageenan injection. Subcutaneous $MgSO_4$ at doses of 0.5, 5, 15 and 30 mg/kg, reduced the hyperalgesia by 44.4 ± 8.8%, 68 ± 8.4%, 24.6 ± 6.9% and 45.3 ± 6.7%, respectively. The effect lasted up to 3 h. $MgSO_4$ at doses of 0.05, 0.1 and 0.5 mg/paw, co-injected with carrageenan had no influence on hyperalgesia. A dose of 0.1 mg/paw injected into the contralateral (non-inflamed) paw also had no effect on carrageenan induced hyperalgesia.

Discussion: The present study shows that magnesium sulfate is effective against pain associated with inflammation after systemic, but not after local peripheral administration. The absence of any effect of $MgSO_4$ following local, peripheral administration, and the presence of an effect after systemic administration, might suggest that this effect is mediated by a central mechanisms. Low doses of systemic $MgSO_4$ may thus be useful in the treatment of somatic inflammatory pain.

Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant no. 175023).

A2.20

Kinetic interrogation of substrate binding and transport in the serotonin transporter

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Intrinsic Activity, 2015; **3**(Suppl. 2):A2.20
http://www.intrinsicactivity.org/2015/3/S2/A2.20

Background: The serotonin transporter (SERT) controls serotonin signaling by reuptake of serotonin from the extracellular space. Moreover, SERT (together with the other monoamine transporters) is a prominent target of a variety of psychoactive drugs, ranging from

illicit to therapeutic substances. Some of these drugs are inhibitors, whereas others are substrates. These substances, and their action on SERT, have been the subject of intense study [1]. However, kinetic knowledge on the mechanism by which SERT orchestrates substrate binding and translocation has been lacking because of technical limitations.

Methods: We thus utilized the high temporal resolution of the whole-cell patch-clamp technique [2, 3] to unravel the kinetic determinants of serotonin transporter substrate selectivity and substrate transport. Moreover, we developed a refined kinetic model of SERT function that accounts for the experimental data.

Results: We show that our approach is suitable to measure substrate-binding kinetics without the need of any radioligands as surrogate, and with a temporal resolution that is not achievable by conventional biochemical methods.

Discussion: Our findings may foster attempts of rational drug design by adding kinetic knowledge to available structural data.

Acknowledgements: This work was supported by the Austrian Science Fund / Fonds zur Förderung der Wissenschaftlichen Forschung (grant F3506 to H. H. S. and grant F3510 to M. F.) and the MD / PhD program of the Medical University of Vienna (to P. S. H.).

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A2.21

Phosphorylation of $K_V7.2$ regulates its PIP_2 sensitivity

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.21
http://www.intrinsicactivity.org/2015/3/S2/A2.21

Background: K_V7 channels are a subfamily of voltage-gated K^+ channels that play a major role in the regulation of neuronal excitability. G_q-coupled receptors like the M_1 acetylcholine receptor can regulate K_V7 channel function by affecting the levels of PIP_2, which is required for channel opening. On the other hand, phosphorylation is also involved but an interaction of these pathways has not been well explored.

Methods: We used liquid chromatography–mass spectrometry to identify phosphorylation sites from rat brain and transfected heterologous cells. To evaluate the effect of phosphorylation on PIP_2-mediated $K_V7.2$ regulation, we generated the dephosphomimetic A^5 mutant (S427/436/438/446/455A^5) and reduced the PIP_2 levels by activating the voltage-sensitive phosphatase Dr-VSP. *In vitro* phosphorylation assays were performed to determine the responsible kinases phosphorylating the sites in the PIP_2-binding domain. Cells were treated with a mixture of the respective kinase inhibitors (roscovitine, SB 203580, KN-62, H-7). Rat primary neurons of the superior cervical ganglia (SCG) were cultured and treated with the inhibitor mix before evaluating the effect of PIP_2 depletion by activating the M_1 receptor with increasing concentrations of oxotremorine methiodide (Oxo-M).

Results: We identified 13 phosphorylation sites in immunopurified $K_V7.2$. Among them, five phosphorylation sites were clustered in one of the putative PIP_2-binding domains. Dr-VSP needed longer to inhibit the current in the dephosphomimetic mutant compared to wild-type $K_V7.2$. CDK5, p38 MAPK, CaMKII and PKA were found *in vitro* to phosphorylate the identified sites in the PIP_2-binding domain. Thus, we inhibited the kinases with the respective inhibitors and observed a similar Dr-VSP response as with the dephosphomimicking mutant. Furthermore, pretreating SCG neurons with the inhibitor mix and depleting PIP_2 by using increasing concentrations of Oxo-M resulted in a similar decrease of M current inhibition.

Discussion: Our results suggest that phosphorylation of $K_V7.2$ in the putative PIP_2-binding domain determines its PIP_2 sensitivity.

Acknowledgements: This project was funded by the Austrian Science Fund FWF (grant P23670).

A2.22

Analgesic action of *Androctonus crassicauda* venom: evidence for new analgesic peptides

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.22
http://www.intrinsicactivity.org/2015/3/S2/A2.22

Background: Scorpions are living more than 400 million years by the help of their powerful venom against almost all animals and mammals including humans. *Androctonus crassicauda* (Olivier 1807), known as black scorpion in Turkey, is one of the venomous scorpions and most toxic for mammals and humans. *Mesobuthus gibbous* (Brulle 1832), known as yellow scorpion is known as less toxic than *A. crassicauda* and reported to have ethnomedical use for limited inflammatory diseases. The aim of this study was to investigate analgesic actions of the whole venoms of *A. crassicauda* and *M. gibbous* in mice.

Methods: The venoms of *A. crassicauda* gathered from Southeast Turkey and *M. gibbous* gathered from Southwest Turkey were obtained by mild electrical stimulation of telsons and were lyophilized. Samples of dried whole venom were diluted by 0.9 % NaCl and used for analgesic tests (0.001 mg/kg, i. p.). Application of a bulldog clamp on the tail of Balb/c albino mice of either sex was used as mechanical algesic stimulus, and 52 °C water for thermal algesia. Cut-off time ($t_{\text{cut-off}}$) was 15 sec; pre-drug and post-drug withdrawal latencies ($L_{\text{pre-drug}}$, $L_{\text{post-drug}}$) were used to calculate percent analgesia as follows:

$$\%\ analgesia = \left(\frac{L_{post\text{-}drug} - L_{pre\text{-}drug}}{t_{cut\text{-}off} - L_{pre\text{-}drug}}\right) \times 100$$

Venom peptide sequences were downloaded from the Uniprot protein databank. R/Bioconductor packages were used for alignment of proteins, statistical evaluation and plotting. Differences between values were tested using Student's *t*-test; the null hypothesis was rejected when p was < 0.05.

Results: Analgesic activity on mechanical algesia was observed for *A. crassicauda* venom but not for the venom of *M. gibbous*. Both

venoms were inactive on thermal stimulation. Alignment of the proteins of *A. crassicauda* and *M. gibbous* showed considerable differences, especially for the tyrosine amino acid residues.
Discussion: To the best of our knowledge, only *M. gibbous* has ethnomedical use in Turkey and the eastern Mediterranean regions. Alignment of toxins of the whole venom of *A. crassicauda* showed the 5th and 42th amino acids were tyrosine in toxins named SCX8 and the 41th was tyrosine in SCX5. Because of the importance of these located tyrosine amino acids on analgesic actions of toxins, SCX8 and SCX5 are new candidates for analgesic peptides.

A2.23

Gender-specific analgesic action of thymol

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.23
http://www.intrinsicactivity.org/2015/3/S2/A2.23

Background: Thymol is a volatile monoterpene being one of the major compounds of volatile oils of several plants like the Labiatae family which have ethnomedical use since antiquity for various diseases including pain and inflammatory diseases. Thymol is reported as antioxidant, antimicrobial, hepatoprotective, positive allosteric modulator of $GABA_A$ receptors and to activate transient receptor potential (TRP) ion channels, TRPA1 and TRPV3. The aim of this study was to investigate the gender-specific action of thymol in mice.
Methods: Thymol was commercially obtained and diluted in DMSO (100 mg/kg). Mechanical and thermal algesic stimuli were used in the experiments. Application of a bulldog clamp on the tail of Balb/c albino mice of either sex was used as mechanical algesic stimulus, and 52 °C water for thermal algesia. Cut-off time ($t_{cut\text{-}off}$) was 15 sec; pre-drug and post-drug withdrawal latencies ($L_{pre\text{-}drug}$, $L_{post\text{-}drug}$) were used to calculate percent analgesia as follows:

$$\%\ analgesia = \left(\frac{L_{post\text{-}drug} - L_{pre\text{-}drug}}{t_{cut\text{-}off} - L_{pre\text{-}drug}}\right) \times 100$$

The R statistical package was used for the statistical evaluation and plotting. Differences between values were tested using Student's *t*-test; the null hypothesis was rejected when p was < 0.05.
Results: Analgesic action of thymol was observed in male mice but there was no effect in female mice. Differences on mechanical and thermal algesic stimuli were observed (p values were 0.005 and 0.040, respectively).
Discussion: Thymol is a small compound composed of a benzene ring substituted with methyl, isopropyl and hydroxyl groups. Although being a simple, hydrophobic volatile compound, and having similarity to propofol it was not surprising to see GABA-mimetic activity of thymol. TRPA1- and TRPV1-binding properties of thymol make it a compound having multiple sites of actions. To the best of our knowledge, the present results are the first to report a gender-specific action of thymol in analgesic tests. Male but not female animals will be appropriate in order to evaluate the actions of thymol and similar monoterpenes.

A2.24

Quercetin uptake into neonatal rat astrocytes

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.24
http://www.intrinsicactivity.org/2015/3/S2/A2.24

Background: Quercetin is a flavonoid widely distributed in fruits and vegetables, and is a potent antioxidant with neuroprotective activity. In animal transgenic models of Alzheimer's disease, quercetin decreased astrogliosis and microgliosis in the hippocampus and the amygdala. The open question remains if the quercetin activity on astrocytes is extracellular or intracellular. Thus, the aim of this study was to investigate the uptake of quercetin into astrocytes.
Methods: We isolated astrocytes form the cerebral cortex of neonatal rats, and grown them into monolayer cultures. We determined the time dependence and concentration dependence of [^{3}H]quercetin uptake into the cultured astrocytes at 37 °C (total uptake) and at 4 °C (non-specific uptake). To study the role of membrane proteins, we pre-incubated the cells with PMSF and DTNB, which form an irreversible link in the active site of membrane proteins with serine and cysteine, respectively. To study the energetic role of uptake, we (i) inhibited the respiratory chain by pre-incubation with KCN, NaN_3 and $NaVO_3$; and (ii) inhibited glycolysis by pre-incubation with NaF. We also studied the involvement of OATPs (organic anion-transporting polypeptides) and SGLT1 (sodium-dependent glucose co-transporter 1) transporters in the uptake of [^{3}H]quercetin by co-incubation with their substrates or inhibitors.
Results: We found that the uptake of quercetin is mediated by facilitated diffusion by comparing the uptake at 37 °C and at 4 °C, where we have obtained no kinetic differences (K_m = 4,5 μM; V_{max} = 94 pmol/mg protein/min).The inhibition of the cell energy production in astrocytes did not affect the uptake of quercetin, thus confirming that there is no active transport. The transport was inhibited by PMSF and DTNB pre-incubation, showing the importance of membrane proteins. Moreover, we showed that both OATPs and SGLT1 are involved in the uptake of quercetin.
Discussion: Uptake of quercetin is mediated by facilitated diffusion involving several membrane transporter systems. Our study opens the perspective of studying flavonoid-mediated neuroprotective activity by focusing on astrocytes and other glial cells.

A2.25

Role of the central kappa opioid system in modulation of salt appetite through central and basolateral amygdala

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.25
http://www.intrinsicactivity.org/2015/3/S2/A2.25

Background: It has been documented that endogenous opioid peptides, dynorphins, are important for maintaining hydro-mineral balances, by modulating thirst and salt appetite through central kappa opioid receptors. We have previously shown that an osmotic stimulus, such as dehydration, up-regulates the expression of the transcriptional factor Giot1, which in turn increases the expression of mRNA encoding the dynorphin precursor preprodynorphin in the hypothalamic paraventricular and supraoptic nucleus. These hypothalamic nuclei are directly projecting to the amygdala. Therefore, the aim of the present study was to investigate the role of kappa opioid receptors, located in the central amygdala and the basolateral amygdala, in the regulation of thirst and salt appetite.
Methods: Experiments were performed in 12-weeks-old male Wistar rats. Rats were bilaterally cannulated in the central amygdala or the

basolateral amygdala for infusion of the selective kappa opioid receptor antagonist norbinaltorphimine (nor-BNI, 20 nmol) or saline (2 µl). Rats were randomized into two experimental groups and subjected to a salt loading protocol and a water deprivation–partial repletion protocol (WD-PR). In the salt loading protocol rats pre-treated with nor-BNI were offered hypertonic saline solution for 7 days. In the water deprivation–partial repletion protocol rats were dehydrated for 36 hours and then partially rehydrated for 2 hours. This was followed by 2-hours-long salt appetite test.

Results: In salt-loaded rats, nor-BNI infused into the basolateral amygdala significantly decreased consumption of hypertonic saline solution, while infusion of nor-BNI into the central amygdala decreased the consumption of hypertonic saline in the WD-PR protocol.

Discussion: Under physiological conditions basolateral and central amygdala increase salt intake. After bilateral lesions of the basolateral amygdala sodium intake is inhibited. However, bilateral lesions of the central amygdala reduce spontaneous sodium intake, while water intake is unchanged. Our results show that central kappa opioid receptors in the amygdala nuclei, basolateral and central, modulate salt appetite and water intake in different manners in response to different osmotic stimuli, dehydration and salt loading. It follows that the central kappa opioid system triggers different mechanisms in different parts of the amygdala tuning salt and water intake, behavior associated with maintenance of hydro-mineral balance.

Acknowledgements: This work was supported by the Biotechnology and Biological Sciences Research Council BBSRC, UK (grant no. BB/J015415/1), and the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant no. III 41013).

A2.26

Ethyl-acetate extract of *Artemisia herba-alba* decreases locomotor activity and exhibits muscle-relaxant properties in rats

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.26
http://www.intrinsicactivity.org/2015/3/S2/A2.26

Background: *Artemisia herba-alba* Asso (AHA) is distributed throughout the Mediterranean region and is traditionally used for its antispasmodic, antiseptic, antiparasitic and blood glucose-lowering properties. It is also reported that AHA can be used in the treatment of some neurological disorders. *In vitro* studies have shown that the ethyl-acetate extract of AHA contains flavonoids which have affinity for $GABA_A$ receptor. The purpose of our study was to investigate the effects of AHA ethyl-acetate extract on motor behaviour in rats.

Methods: Experiments were performed in adult male Wistar rats weighing 250–280 g. Increasing doses (10, 30, 100 mg/kg) of an ethyl-acetate extract of AHA were applied intraperitoneally to animals before submitting them to motor behaviour testing. An open-field arena was used to assess ambulatory behaviour, while muscle strength and coordination were estimated using the grip-strength test and rotarod test, respectively. Control groups were treated with saline containing 5% Tween 80 or diazepam.

Results: During a five-minute exposure to an open-field arena, rats treated with all doses of AHA showed a decline in both vertical and horizontal activity, reflected as a decrease in the number of supported and non-supported rears and a reduced number of total squares crossed compared to the control group treated with saline. The strength-grip test showed decreased muscle strength in forelimbs of rats treated with 30 mg/kg and 100 mg/kg AHA ethyl-acetate extract compared to saline-treated rats and this decrease was comparable to one induced by diazepam. Only diazepam-treated rats spent less time on the rotarod when compared to the saline-treated control group.

Discussion: This is the first *in vivo* study that examined effects of *Artemisia herba-alba* on rodent motor behaviour. Our results show that the ethyl-acetate extract of *A. herba-alba* reduces locomotor activity and induces muscle relaxation without affecting coordination. These results may be useful for the development of new drugs for the treatment of neurological disorders characterized by increased muscle tone.

A2.27

Effects of testosterone treatment on hypothalamic microstructure in female-to-male transsexuals

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Intrinsic Activity, 2015; **3**(Suppl. 2): A2.27
http://www.intrinsicactivity.org/2015/3/S2/A2.27

Background: An increasing number of neuroimaging studies indicates that sex hormones modulate human brain structure and function [1, 2]. Recently, we showed that testosterone treatment in female-to-male transsexuals (FtM) elevated the binding of cerebral serotonin transporters, an important protein regulating serotonergic neurotransmission [1]. Furthermore, structural modifications after testosterone treatment were substantiated in gray and white matter using voxel-based morphometry and tractography, respectively (data submitted). As head ganglion of the endocrine system, the hypothalamus plays a key role in hormone function, with its structure and cell assembly being shaped by steroid hormones [3, 4]. Here, our aim was to closer examine microstructural neuroplastic changes in the hypothalamus by investigating the effect of hormone treatment on gray matter microstructure in FtM transsexuals using diffusion tensor imaging (DTI).

Methods: Twenty-three FtM transsexuals were included in this longitudinal study (age: 27.3 ± 6.3; mean ± SD). Transsexuals were measured before start of treatment, after 4 weeks, and after about 4 months of treatment start. Treatment consisted of 1000 mg testosterone undecanoate every 12 weeks and in two cases additionally 10 mg lynestrenol daily. Transsexuals were scanned on a 3 T Tim Trio Scanner (Siemens Medical, Germany). DTI acquisition was performed with an isotropic resolution of 1.6 mm^3 acquiring diffusion-weighted images in 30 directions with a *b* value of 800 s/mm^2. Calculation of mean diffusivity (MD) maps was done in FSL [5] after eddy current correction. Spatial normalization of MD maps was carried out with deformation fields obtained from segmentation of baseline T1-weighted images with the VBM8 toolbox. Repeated-

measures ANOVA and post-hoc pairwise comparisons were done using SPM. Correlations between changes in MD and changes in bioavailable testosterone plasma levels were calculated. The statistical threshold was set at $p < 0.05$ FDR cluster-corrected.

Results: Results: DTI analysis of whole brain gray matter revealed significant differences in MD maps between the three time points in bilateral posterior hypothalamus (x = 6, y = −7, z = −15, F = 10.3; and x = −4, y = −7, z = −15, F = 10.2), as well as in left fusiform and middle temporal gyrus (k ≥ 47 cluster size, corresponding to expected voxels per cluster of k = 47, ANOVA, $p < 0.001$ uncorrected). Post-hoc pairwise comparisons revealed significant MD reductions in bilateral posterior hypothalamus (x = 6, y = −7, z = −17, T = 4.0; and x = −4, y = −7, z = −15, T = 4.3) after 4 weeks of treatment ($p = 0.046$, corrected), and a more pronounced reduction after four months of treatment (x = 9, y = −6, z = −8, T = 4.78; and x = −7, y = −7, z = −13; T = 3.63; $p < 0.001$, corrected). After four months of treatment, correlation analysis revealed a significant negative association between MD changes in the right hypothalamus (x = 9, y = −6, z = −8; *i.e.* peak voxel of the post-hoc *t*-test) and increases in bioavailable testosterone ($r = −0.64$; $p = 0.017$).

Discussion: Our results indicate that testosterone treatment leads to microstructural changes in hypothalamic tissue of FtM transsexuals. Several post-mortem studies have indicated that pre- and perinatal testosterone surges in the womb shape hypothalamic nuclei. These processes were proposed to underlie a person's gender identity [3,4]. Here, we propose that also changes in adult testosterone levels affect hypothalamic microstructure. Microstructural changes in hypothalamic nuclei, as seen in our study, may reflect adaptive changes in endocrine function after prolonged exogenous administration of testosterone in FtM transsexuals.

Acknowledgements: This research was funded by the Austrian Science Fund FWF (grant P23021 to R.L.).

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A2.28

Changes in progesterone levels correlate with changes in subcortical brain structures in male-to-female transgender subjects after acute high-dose cross-sex hormone administration

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Intrinsic Activity, 2015; **3**(Suppl.2):A2.28
http://www.intrinsicactivity.org/2015/3/S2/A2.28

Background: Sex steroid hormones exert widespread effects on the brain and the body. They are involved in sexual differentiation, development and behaviour and play a pivotal role in the development and function of the central nervous system. Transgender subjects, undergoing hormone therapy, deliver a unique model to study these effects in the living human brain. Male-to-female subjects (MtF) regularly receive high-dose estradiol and anti-androgen treatment to achieve feminization of the body. Studies are scarce, but results already point towards decreases in brain volume in MtFs after acute cross-sex hormonal treatment, which was mainly observed due to increases in the ventricular system. The aim of the investigation was to corroborate these prior findings and to test whether changes in hormonal levels are correlated with changes in brain volume in subcortical brain structures.

Methods: Fourteen MtF subjects (mean age ± SD = 26.9 ± 6.1) were measured at baseline and after a period of 4 months of high-dose estradiol and anti-androgen treatment. Blood hormonal levels were assessed at each time point. Structural MRI was carried out at 3 T (Siemens Tim Trio) using a 32-channel head coil (MPRAGE, T1; 256×240 matrix, 160 slices, voxel size 1×1×1.1 mm, TE = 4.21 ms, TR = 2,300 ms; TI = 900 ms; α = 9°). Subcortical assessment of brain structures was done with FreeSurfer [1] (version 5.1.0) using the longitudinal processing stream. Subsequently, correlations were calculated for changes in hormonal levels and significant volumetric changes in subcortical structures between pre and post treatment (TP1 *vs.* TP2). Due to missing hormonal assessment, one subject had to be excluded from the correlation analysis.

Results: Blood hormonal levels of testosterone, estradiol and progesterone changed significantly after the 4-months period of estradiol and anti-androgen treatment ($p < 0.01$). While an increase in estradiol (TP1: 29.77 ± 14.42 pg/ml; TP2: 133.54 ± 121.33 pg/ml) was observed, testosterone (TP1: 5.48 ± 2.05 ng/ml; TP2: 0.97 ± 1.84 ng/ml) and progesterone (TP1: 0.76 ± 0.28 ng/ml; TP2: 0.53 ± 0.19 ng/ml) levels decreased as expected. The structural assessment of subcortical brain regions showed significant ($p < 0.05$, uncorrected) volumetric increases in the entire ventricular system and bilateral decreases in the hippocampus, amygdala and in the right caudate and putamen after the 4-months treatment period. Furthermore, changes in hormonal blood levels and changes in subcortical regions revealed that decreasing progesterone levels were associated with increases in the left ($r = 0.65$; $p = 0.02$) and right lateral ventricle ($r = 0.55$; $p = 0.05$) and the third ventricle ($r = 0.57$; $p = 0.04$) and with decreases in the right caudate ($r = 0.65$; $p = 0.02$) and hippocampus ($r = 0.63$; $p = 0.02$).

Discussion: Acute high-dose estradiol and anti-androgen treatment in MtF subjects seems to be related to volumetric gray matter decreases in the brain. We observed increases in the ventricles and decreases in several subcortical brain regions. These results are in line with prior studies, indicating decreases in gray matter volume in MtF subjects after cross-sex hormonal treatment. Furthermore, our analysis indicate that progesterone is strongly involved in this process, as changes in progesterone levels correlated with changes in subcortical brain areas.

Acknowledgements: This research was supported by a grant of the Austrian Science Fund (FWF; P23021) to R.L.

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Immunopharmacology and Infection

A3.1

Neutrophil effector responses are fully suppressed by secretory phospholipase A_2-modified HDL

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Intrinsic Activity, 2015; **3**(Suppl. 2):A3.1
http://www.intrinsicactivity.org/2015/3/S2/A3.1

Background: Secretory phospholipase A_2 ($sPLA_2$) generates bioactive lyso-phospholipid products implicated in atherosclerosis. In patients with acute coronary syndrome, the $sPLA_2$ inhibitor varespladib surprisingly increased the risk of myocardial infarction. High-density lipoprotein (HDL) is the main source of phospholipids and the major substrate for $sPLA_2$ in plasma. Therefore, we investigated the effects of $sPLA_2$-mediated modification of HDL on neutrophil function, a critical player in atherosclerosis and inflammation.
Methods: Human neutrophils were isolated from peripheral blood of healthy human volunteers. The neutrophil shape change, CD11b activation and Ca^{2+} flux were measured by flow cytometry. Neutrophil adhesion was measured under flow conditions using the flow-chamber assay. Lipid rafts were stained with FITC–cholera toxin B and its abundance was assessed by flow cytometry and fluorescent microscopy. Cholesterol efflux was measured from neutrophils pre-loaded with [^{3}H]cholesterol.
Results: Treatment of HDL with $sPLA_2$ ($sPLA_2$-HDL) resulted in the formation of palmitoyl-lysophosphatidylcholine (LPC 16:0) as the most prominent LPC species. $sPLA_2$-HDL rapidly prevented neutrophil shape change, Ca^{2+} flux, CD11b activation, adhesion, migration and formation of neutrophil extracellular traps (NETs). Moreover, $sPLA_2$ treatment of HDL markedly increased cholesterol efflux capability of HDL associated with a rapid disruption in cellular cholesterol-rich microdomains (lipid rafts). Native HDL showed no significant effects and removing LPC products from $sPLA_2$-HDL abolished all anti-inflammatory activities towards neutrophils, whereas enrichment of native HDL with LPC 16:0 mimicked $sPLA_2$-HDL effects.
Discussion: Overall, our studies suggest that the increased cholesterol-mobilizing activity of sPLA-HDL and suppression of rise in intracellular Ca^{2+} levels are the likely mechanism that counteracts agonist-induced activation of neutrophils. Our results raise the possibility that $sPLA_2$-induced modification of HDL composition and function modulates neutrophil trafficking and effector responses during inflammation.
Acknowledgements: The work was supported by the Austrian Science Fund FWF (P22976 and P22521) and the Austrian National Bank (no. 14853)

A3.2

Safety, pharmacokinetics, pharmacodynamics and immunogenicity of a new anti-TNFα monoclonal antibody (GSK2800528)

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Intrinsic Activity, 2015; **3**(Suppl. 2):A3.2
http://www.intrinsicactivity.org/2015/3/S2/A3.2

Background: GSK2800528 is an anti-TNFα monoclonal antibody. It has an identical amino acid sequence to adalimumab, the market leading anti-TNFα, except for three amino acid substitutions in the Fc portion of the molecule (YTE = M252Y/S254T/T256E) designed to increase antibody recycling, to reduce clearance and increase half-life. This is the first study of GSK2800528 in humans.
Methods: Fourty-five healthy volunteers, male or female between 18 and 65 years old, took part in a single-centre study (NCT01899755) conducted at Hammersmith Medicines Research. Subjects were split in to 4 cohorts: cohorts 1–3 received either a single dose of GSK2800528 (10, 40 or 160 mg) or placebo; cohort 4 received a single dose of adalimumab (40 mg). Safety and tolerability was closely monitored throughout, and blood samples were taken for assessment of drug concentration, anti-drug antibodies and pharmacodynamic markers (including an *ex vivo* whole-blood assay measuring IL-8 release in response to exogenous TNFα). A population PK analysis was performed on GSK2800528 and adalimumab PK data using NONMEM 7.1.2.
Results: There were no serious adverse events (SAEs), significant AEs, or AEs of special interest. There were no clinically significant changes in biochemical parameters, urinalysis parameters, vital signs, or ECG parameters in any treatment group. The PK of GSK2800528 was linear over the 10 to 160 mg range. A two-compartment model with first-order absorption and elimination was identified to describe both GSK2800528 and adalimumab data. The population-predicted apparent systemic clearance (CL/F) of GSK2800528 and adalimumab was 7.07 ml/h (10.3% RSE) and 18.4 ml/h (12.3% RSE) respectively, resulting in a mean fold reduction in CL/F of 2.6 (1.84–3.5). All subjects in the 40 mg GSK2800528 and 40 mg adalimumab cohorts showed inhibition of IL-8 release at day 7 post-dose. By day 56, IL-8 levels in the adalimumab cohort had returned to approximately baseline whereas IL-8 levels in the GSK2800528 cohort remained inhibited. All subjects dosed with GSK2800528 ($n = 27$) had detectable anti-drug antibodies by day 84. 23 of 27 (85%) subjects dosed with GSK2800528 had neutralizing anti-GSK2800528 antibodies. All subjects dosed with adalimumab ($n = 9$) had detectable anti-drug antibodies by day 140. Neutralizing antibodies were detected in 8 of the 9 (89%) subjects dosed with adalimumab.
Discussion: GSK2800528 was well tolerated and the PK profile showed the expected increase in half-life. Modelling suggested that GSK2800528 40 mg dosed every 4 weeks would provide similar exposure to adalimumab 40 mg dosed every 2 weeks. The *ex vivo* IL-8 assay demonstrated sustained levels of pharmacologically active drug on day 56 in the GSK2800528 cohort in contrast to the adalimumab cohort. The incidence and titer of anti-drug antibodies following a single dose of GSK2800528 or adalimumab were comparable.
Acknowledgements: This study was funded by GlaxoSmithKline.

A3.3

Transmembrane proteins of *Fasciola hepatica:* identification and characterization of new putative drug targets

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Intrinsic Activity, 2015; **3**(Suppl. 2):A3.3
http://www.intrinsicactivity.org/2015/3/S2/A3.3

Background: *Fasciola hepatica*, a parasitic flatworm (phylum Platyhelminthes, class trematode, subclass Digenea, family Fasciolidae), is the cause of one of the most important diseases affecting animal health all over the world: liver fluke disease (fascioliasis). Triclabendazole (TCBZ) is the drug of choice for more than 25 years because of its high activity against both adult and juvenile flukes. However, there are an increasing number of reports on drug resistance against TCBZ in *F. hepatica*.
Methods: We performed next-generation sequencing (NGS) to identify new ABC transporters of *F. hepatica* and mutations in these transporters that could confer resistance to TCBZ. For this approach, TCBZ-resistant and susceptible adult flukes from Northern Ireland and Lower Austria were used. In parallel, we also generated antibodies against putative ABC transporters of *F. hepatica*. Additionally, cells were transfected with ABC transporters to perform cell viability assays (CVA).
Results: Next generation sequencing data provided us about 60 ABC transporters in *F. hepatica*. We found *F. hepatica* multidrug resistance transporter (MDR) involved in TCBZ efflux by CVA. As seen by CVA, *F. hepatica* MDR might be a candidate to elicit drug resistance.
Discussion: The results from both the bioinformatics part and the functional analysis will probably shed light on how flukes became resistant.
Acknowledgements: Supported by AIT (1.G8.00016.0.0).

A3.4

Human bile reduces antimicrobial activity of selected antibiotics against *Escherichia coli* and *Enterococcus faecalis* *in vitro*

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Intrinsic Activity, 2015; **3**(Suppl. 2):A3.4
http://www.intrinsicactivity.org/2015/3/S2/A3.4

Background: In antimicrobial drug development and clinical routine antibiotics are tested in standardised culture media. The impact of biological fluids like urine, cerebrospinal fluid or plasma, *i.e.* the site of bacterial infection, on antimicrobial activity was previously demonstrated. The present *in vitro* experiments investigated the effect of bile on bacterial killing of ciprofloxacin (CIP), meropenem (MEM), tigecycline (TGC) and linezolid (LZD) against *Escherichia coli* and *Enterococcus faecalis*.
Methods: Human bile was obtained from 11 patients who underwent cholecystectomy because of cholecystitis or cholecystolithiasis and sterilisation was achieved by gamma radiation. Time–kill curves of CIP, MEM and TGC against *E. coli* ATCC 25922, as well as LZD and TGC against *E. faecalis* ATCC 29212 were performed in pooled human bile and in Mueller-Hinton broth (MHB). For each compound and strain at least 4 concentrations were tested. Minimal Inhibitory concentrations (MICs) determined by broth microdilution method were conducted in MHB only.
Results: Human bile did not negatively affect bacterial growth over 24 hours. Bacterial counts (in CFU/ml after 24 hours) of bile growth controls were approximately equal to MHB growth controls for *E. coli* and 2.5-fold greater for *E. faecalis* indicating a promotion of bacterial growth for the latter strain. Bile reduced killing of CIP, MEM and TGC against *E. coli* and killing of LZD against *E. faecalis* considerably. This effect was strongest for TGC against *E. coli*.
Discussion: The present data indicate that bile inhibits antimicrobial activity of CIP, MEM, TGC and LZD against *E. coli* and *E. faecalis*, respectively. These findings may have important implications for the treatment of bacterial infections of the gallbladder and biliary tract, and should be explored in more detail.

Gastrointestinal and Reproductive Pharmacology

A4.1

Investigation of European medicinal plants to influence small intestinal motility *in vitro*

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Intrinsic Activity, 2015; **3**(Suppl. 2):A4.1
http://www.intrinsicactivity.org/2015/3/S2/A4.1

Background: For millennia plants have been used for medicinal purposes. We investigated extracts of 10 plants traditionally used in Austria against gastrointestinal complaints, whether alterations of small intestinal motility might contribute to their apparent beneficial effects. The choice was based on the list of plants published by Vogl *et al.* [1] as well as Wichtl *et al.* [2] and Bradley [3].
Methods: The following herbs were investigated: Melissae folium, Origani herba, Betonicae herba, Angelicae radix, Levistici radix, Imperatoriae radix, Petroselini radix, Ribis nigri folium, Euphrasiae herba, and Chelidonii herba. Additionally, Belladonnae folium was used as a positive control. Extracts were prepared with 60 % ethanol (v/v) using accelerated solvent extraction or the Soxhlet method, and dried under nitrogen. For the motility experiments, segments of guinea-pig ileum were mounted longitudinally in organ baths containing Tyrode solution gassed with 95 % O_2/5 % CO_2. Motor responses of full-thickness strips were recorded under isotonic conditions, whereas longitudinal muscle/myenteric plexus (LMMP) preparations were mounted under isometric conditions and stimulated electrically at 0.05 Hz. Such a stimulation has been shown to yield regular contractions that are mostly cholinergic in nature. The dried extracts were reconstituted in 50 % DMSO (v/v) in distilled water at 20 mg/ml and further diluted with distilled water as needed. All preparations were first stimulated with a maximally effective concentration of bethanechol (100 µM) followed by increasing concentrations of extracts. Changes in tension were evaluated as % of the response to bethanechol for unstimulated ileal preparations and as % change of the response to electrical stimulation before drug addition in the case of electrically stimulated LMMP strips. Finally, all extracts were analysed by thin-layer chromatography using the methods published in the European Pharmacopoeia (8th edition 2014).
Results: The various extracts at final concentrations of 12.5–200 µg/ml did not evoke any significant dose-dependent ileal contractions nor did they inhibit the electrically evoked contractions to a meaningful extent. Only the Belladonna extract inhibited electrically evoked contractions by approximately 90 % already at a concentration of 6.25 µg/ml. The chromatographic analysis showed that the extracts contained characteristic ingredients as described in the European Pharmacopoeia.
Discussion: The results show that none of the tested extracts directly influences small intestinal motility except for Belladonna, which inhibited the electrically evoked contractions as expected. This, however, does not imply that these herbs are useless against gastrointestinal complaints because *in vivo* several additional modes of actions can come into effect. Alterations of bile secretion, intestinal water and electrolyte transport or carminative actions have been shown to underlie the beneficial effects of a number of medicinal herbs used against gastrointestinal problems. Furthermore, in many instances fresh herbs or tea preparations are administered, which

may contain compounds that are not recovered in an ethanolic extract.

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A4.2

Effects of Ca^{2+}-activated K^+ channel modulators on the contractility of the rat gastric fundus

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Intrinsic Activity, 2015; **3**(Suppl. 2):A4.2
http://www.intrinsicactivity.org/2015/3/S2/A4.2

Background: Ca^{2+}-activated K^+ (K_{Ca}) channels are important regulators of excitability of the gastrointestinal smooth muscle cells. $K_{Ca}1.1$ and 2 channels (also called large- and small-conductance Ca^{2+}-activated K^+ [BK and SK] channels, respectively) regulate Ca^{2+} entry from the extracellular fluid. In addition, the opening of $K_{Ca}2$ channels mediates the effects of some inhibitory neurotransmitters. The aim of our study was to investigate the effects of K_{Ca} channel modulators on the contractility of the proximal stomach.

Methods: Longitudinal muscle strips were prepared from the rat gastric fundus and were suspended under isotonic conditions (9.8 mN load) in Krebs solution maintained at 37°C, bubbled with carbogen and containing atropine (1 μM) and guanethidine (5 μM) (to obtain nonadrenergic, noncholinergic [NANC] conditions) inside 5-ml organ baths. The relaxations were studied in strips precontracted by the TP receptor agonist U46619 (0.1 μM). The effects of iberiotoxin (IBTX), UCL1684 and TRAM-34 (selective blockers of $K_{Ca}1.1$, 2 and 3.1 channels, respectively) and NS1619 and NS309 (activators of $K_{Ca}1.1$, 4 and 5.1 channels, and 2 and 3.1 channels, respectively) were investigated. Responses are expressed as percentages of maximal strip relaxations induced by papaverine (300 μM).

Results: IBTX (50 nM), UCL1684 (0.3 μM) and TRAM-34 (1 μM) did not affect basal muscle contractility ($n = 4$ each). When their effects were evaluated on U46619 (0.1 μM)-precontracted strips, UCL1684 (0.3 μM) and IBTX (50 nM) further contracted the strips by 3.8 ± 2.2% ($n = 6$) and 16.1 ± 3.0% ($n = 9$), respectively. NS1619 (30 and 60 μM) relaxed the strips by 9.1 ± 1.0% ($n = 11$) and 25.3 ± 3.6% ($n = 5$), respectively. NS309 (0.3–30 μM) induced concentration-dependent relaxations with pD_2 and E_{max} of 5.32 ± 0.53 and 90.5 ± 2.6% ($n = 8$), respectively. TRAM-34 (1 μM) did not significantly affect NS309 (0.3–30 μM)-induced relaxations ($n = 4$). On the contrary, the relaxations induced by NS309 (10 μM) were greatly reduced by UCL1684 (1 μM) (71.4 ± 2.1%, $n = 12$, and 17.6 ± 2.5%, $n = 4$, $p < 0.001$, without and with UCL1684, respectively).

Discussion: The results of our study suggest that K_{Ca} channels have no role in controlling resting membrane potential of smooth muscle cells of rat gastric fundus. A small number of $K_{Ca}1.1$ and 2 seems to open in response to U46619-induced effects. The activation of $K_{Ca}2$ channels produces significant relaxations of the proximal stomach, suggesting that $K_{Ca}2$ channel openers could be considered as useful relaxant agents of the gastric smooth muscle.

Acknowledgements: Supported by Fondi di Ateneo of the Catholic University of the Sacred Heart, Rome.

A4.3

The relaxation of myometrium by the natural polyphenols resveratrol and naringenin

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Intrinsic Activity, 2015; **3**(Suppl. 2):A4.3
http://www.intrinsicactivity.org/2015/3/S2/A4.3

Background: Natural polyphenols are present in a large number of plant species. Special sources of resveratrol are grapes and wine, as well as its products, but also for naringenin grapefruits, its juice, hop and beer. During the last decade, resveratrol was in the focus of the scientific and wider public as a substance that slows aging or has anti-cancer, anti-inflammatory and cardioprotective properties. A large number of cellular structures have been shown as possible sites of action, thus resveratrol is labeled as "one molecule – many targets". Unlike resveratrol, naringenin belongs to a group being studied less, flavonoids. Its mechanism of inhibition of the contraction of uterine smooth muscle has not been studied. The aims of this study were to investigate the possible inhibitory effect of polyphenols in several experimental models of pregnant and non-pregnant uterus.

Methods: The animals used in the experiments were virgin female Wistar rats. Myometrial samples were obtained from non-laboring women (37–39 weeks of gestation) undergoing elective cesarean sections. Samples were mounted into organ baths for recording isometric tension. Resveratrol (1 μM – 100 μM) and naringenin (1 μM – 1 mM) were added cumulatively to the bath for isolated organs. The effects of polyphenols were investigated on the spontaneous rhythmic contractions, oxytocin-induced phasic (0.2 nM) and tonic (20 nM) contractions of rat uterus and oxytocin-induced (2 nM) contractions of human uterus. The effects of synthetic openers of K^+ channels, pinacidil and NS1619, were tested and compared to the effects of polyphenols.

Results: The results show that resveratrol exerts potent inhibitory effect on spontaneous and induced contractions of non-pregnant rat uterus and human pregnant myometrium. Naringenin inhibited contractions of animal and human myometrium in a concentration-dependent manner. Resveratrol showed a statistically significantly higher potency than naringenin in all contraction models. Mean effective concentrations of naringenin were similar for all models, which was not the case for resveratrol.

Discussion: Based on the results presented in this work, it is acceptable to conclude that resveratrol and naringenin have great potential to be used in the prevention and treatment of abnormal and undesirable uterine contractility, as in the case of dysmenorrhea and premature births.

Acknowledgements: Our work has been supported by scientific research grant no. 31020 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

Oncology

A5.1

The influence of detoxification agents on the intensity of side effects caused by medium-high doses of methotrexate in children with acute lymphoblastic leukemia: case series

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Intrinsic Activity, 2015; **3**(Suppl. 2):A5.1
http://www.intrinsicactivity.org/2015/3/S2/A5.1

Background: The treatment of childhood acute lymphoblastic leukemia (ALL) in Serbia is conducted according to protocol ALL IC BMF-2009. The therapy includes the application of cytostatic drugs methotrexate and 6-mercaptopurine, and drug detoxifying calcium folinate. At the moment, 80% of affected children could be cured with the current treatment, but resistance to the therapy and its toxic effects remain serious clinical problems. The aim of the study was to investigate the influence of detoxification agents (calcium folinate, silymarin and ursodeoxycholic acid) on the side effects of methotrexate, applied in this protocol.

Methods: A modified acute toxicity form (GPOH) was used for the monitoring of side effects. The research included children with either standard or intermediate risk ALL in the consolidation therapy phase, who were hospitalised at the Institute for Child and Youth Health Care of Vojvodina in Novi Sad during the period from July 2013 to February 2014.

Results: The most frequent side effect after 40 applications of methotrexate in ten children was bone marrow depression. Methotrexate caused: leukopenia in 10 patients, thrombocytopenia in 5 patients; after the use of folic acid, platelet count increased in 8 patients, leukocyte count in 2 patients. Less frequent side effects: increased serum transaminase activity, fever, bronchopneumonia, diarrhoea with mild cramps, and hypercalcaemia.

Discussion: The application of calcium folinate, silymarin and ursodeoxycholic acid prevented the occurrence of severe adverse effects caused by medium-high doses of methotrexate. Observed adverse effects were of mild to moderate intensity, reversible, and did not significantly disturb the quality of life in treated patients.

Acknowledgements: This study was funded by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina (grant no. 114-451-1105/2014-03).

A5.2

The role of EGFR mutations in lung cancer: molecular basis of targeted therapy

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Intrinsic Activity, 2015; **3**(Suppl. 2):A5.2
http://www.intrinsicactivity.org/2015/3/S2/A5.2

Background: The epidermal growth factor receptor (EGFR), a tyrosine kinase (TK) receptor, represents the crucial component of cell signalling pathways. In numerous malignancies, including non-small-cell lung cancer (NSCLC), the intracellular TK activity of EGFR may be deregulated due to somatic mutations of the EGFR gene, increased gene copy number and/or EGFR protein overexpression, thus representing a therapeutical target. EGFR mutation testing in advanced NSCLC nowadays is an essential step in decision on treatment with tyrosine kinase inhibitors (TKI).

Methods: During seven months (from June to December 2014), 175 patients were included in testing. Histological and cytological specimens were processed and genomic DNA was isolated using the Cobas® DNA Sample Preparation Kit. Measurement of DNA concentration was performed using the Qubit® dsDNA BR Assay Kit and the Qubit® Fluorometer. The target DNA was amplified and detected on the Cobas® z 480 analyzer using a real-time PCR test provided in the Cobas® EGFR Mutation Test Kit.

Results: All tested patients were of Caucasian descent and had the adenocarcinoma subtype of NSCLC, stage IIIb (27.4%) or IV (72.6%). Among 175 patients, 68% were males and 32% females, the median age was 61.5 (range 29–87 years). EGFR mutations were detected in 16 patients (9.1%), the wild-type gene was detected in 158 patients (90.3%) while in 1 patient (0.6%) the amplification was not achieved due to an inadequate sample. The types of detected mutations were as follows: deletions in exon 19 were detected in 9 patients (56.3%), exon 21 L858R point mutation was detected in 4 patients (25%), whereas exon 18 and exon 20 point mutations were found in 3 samples (18.7%).

Discussion: The prevalence of EGFR mutations in our patients with advanced lung adenocarcinoma is 9.1%. Regarding the type of mutations, deletions in exon 19 are the most frequent EGFR mutations, which is in concordance with previously published data. According to literature data, the EGFR mutation rate in the Caucasian population is approximately 10% for NSCLC, which corresponds to our results. We suggest that one of the strategies to improve the detection of mutations could be a better patient selection and stratification. The investigations of the molecular basis of cancer may bring better understanding of cancerogenesis and a further development of targeted therapies, thus providing a higher efficiency and lower toxicity compared to conventional treatment options.

A5.3

The influence of pretreatment with antioxidants on cytotoxicity of the epigenetic agent vorinostat towards HT-29 colon cancer cells

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Intrinsic Activity, 2015; **3**(Suppl. 2):A5.3
http://www.intrinsicactivity.org/2015/3/S2/A5.3

Background: Vorinostat is a histone deacetylases (HDAC) inhibitor that promotes apoptosis of malignant cells by several mechanisms. Multiple studies have failed to show an efficacy of vorinostat as a monotherapy against solid tumors, but it has shown a great potential to act synergistically with various chemotherapeutics. Conflicting results were obtained regarding the role of oxidative stress in antitumor effects of HDAC inhibitors, and therefore the aim of our

study was to analyze the influence of antioxidants on the cytotoxic activity of vorinostat towards colon cancer cells.
Methods: Human colon adenocarcinoma HT-29 cells were used to assess the cytotoxicity of vorinostat, alone or in combination with the antioxidant agents *N*-acetyl-cysteine (NAC) and α-tocopherol (TOC), using the colorimetric MTT assay. Multiple drug effects were examined by calculating the combination index (CI) using the CompuSyn software: CI < 1 is evidence for synergy, whereas CI > 1 is evidence of antagonism.
Results: Vorinostat exhibited a modest cytotoxic activity against HT-29 cells, in a concentration-dependent manner. The IC_{50} value of vorinostat was 5.1 μM, while the clinically relevant concentrations are between 1 and 2 μM. In combination studies, HT-29 cells were treated with 1 μM and 2 μM vorinostat, 30 minutes after being treated with 10 mM NAC and 3 μM TOC that displayed negligible antiproliferative effects. Both NAC and TOC managed to sensitize cells towards the activity of vorinostat, especially in a concentration of 2 μM. Calculated CIs of 0.1981 and 0.0803 for NAC, and CIs of 0.3566 and 0.0774 for TOC, in combination with 1 μM and 2 μM vorinostat respectively, suggest their synergistic effects in concentrations that can be achieved *in vivo*. The effect of NAC pretreatment was more pronounced than that of TOC for a lower concentration of vorinostat, while it was similar for a higher concentration of vorinostat.
Discussion: The response to vorinostat may be improved by combining it with antioxidants. The mechanisms responsible for this synergistic effect should be investigated more in-depth.
Acknowledgements: This work is supported by the Ministry of Education, Science and Technological Development of Serbia, grant no. III 41012.

A5.4

Effects of titanium dioxide nanoparticles on growth of malignant melanoma cells

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Intrinsic Activity, 2015; **3**(Suppl.2):A5.4
http://www.intrinsicactivity.org/2015/3/S2/A5.4

Background: Malignant melanoma (MM) is one of the most common cancers worldwide. At present, the (pharmaco)therapy is far from being optimal. Titanium dioxide (TiO_2) is one of the most widely used nanomaterials in everyday life and has emerged as a potential killer of malignant cells. Extensive studies have shown that it can cause cell toxicity under *in vitro* and *in vivo* conditions. Accordingly, the aim of our study was to investigate the influence of nano-TiO_2 on the growth of MM cells.
Methods: The human metastatic MM cell line WM 266-4 (ATCC) was used to obtain dose- and time-depended responses. The MTT assay was carried out to measure the cells' metabolic activity and viability. In addition, an LDH cytotoxicity assay was performed. The cells (3x 103) in the 7th passage were seeded into 24-well culture plates in duplicates, incubated overnight in ATCC-formulated EMEM medium and then treated with various concentrations of nano-TiO_2 (250, 100, 20, 10, 1 μg/mL) for 24, 48 and 120 hours without changing the media.
Results: The MTT test showed a significant increase in the MM cells' metabolic activity and viability after 48 hours of exposure regardless of the nano-TiO_2 concentration. After 120 hours of exposure, only in case of 250 and 100 μg/mL nano-TiO_2 concentrations, a marked decrease in the cells' metabolic activity and viability was observed. The LDH test confirmed findings from the MTT test; cytotoxic effects of nano-TiO_2 on MM cells were higher at higher nano-TiO_2 concentrations and longer times of exposure.
Discussion: In conclusion, our results suggest that nano-TiO_2 may markedly impair the growth of WM 266-4 cells and thus might open a new window in treatment modalities of MM. However, a significantly increased MM metabolic activity and viability after 48 hours of exposure was observed. This discrepancy raises questions which have to be answered before a potential clinical use of nano-TiO_2.

A5.5

The influence of *N*-acetylcysteine on the cytotoxicity of single-walled carbon nanotubes in human lung carcinoma cells

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Intrinsic Activity, 2015; **3**(Suppl.2):A5.5
http://www.intrinsicactivity.org/2015/3/S2/A5.5

Background: Single-walled carbon nanotubes (SWCNTs) have been reported to induce cytotoxicity in different cell lines. Although the mechanisms underlying cytotoxicity are not fully understood, accumulation of reactive oxygen species (ROS) and oxidative damage is considered to be a likely contributing factor.
Methods: Human lung carcinoma cells, A549, and human fetal lung fibroblasts, MRC-5, were used to assess the cytotoxicity of SWCNT in the presence and absence of a redox status regulator, *N*-acetyl-cysteine (NAC), via the MTT assay.
Results: At ≤ 250 μg/ml, SWCNT induced a nearly three-fold greater loss of viability in A594 *vs.* MRC-5 cells. SWCNT cytotoxicity at higher concentrations was similar for both cell lines, while NAC alone was non-toxic. The cytotoxicity to A549 cells of SWCNT (250 μg/ml) in combination with NAC was significantly decreased at the lowest NAC concentration (1.5 g/ml), and was similar to NAC treatment alone at that concentration. Higher concentrations of NAC in combination with SWCNT (250 μg/ml) resulted in increased cytotoxicity in both A549 and MRC-5 cells.
Discussion: A549 malignant lung cells are more susceptible to low concentrations of SWCNT *vs.* normal lung cells, and low concentrations of *N*-acetylcysteine appear to be cytoprotective, possibly due to its antioxidant properties.
Acknowledgements: Ministry of Science and Technological Development of the Republic of Serbia, grant no. 173014 "Molecular mechanisms of redox signaling in homeostasis: adaptation and pathology".

A5.6

Stage-dependent interleukin-6 component in statin-induced apoptosis of metastatic melanoma cells unmasked by tocilizumab

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Intrinsic Activity, 2015; **3**(Suppl. 2): A5.6
http://www.intrinsicactivity.org/2015/3/S2/A5.6

Background: Context-dependent, interleukin 6 (IL-6) has pro- and anti-inflammatory effects. The IL-6 neutralizing antibody tocilizumab is used to attenuate the pro-inflammatory action in arthritis patients and has been associated with rapid progression of melanoma in two case reports. The molecular mechanisms behind these observations are not clear at the moment.
Methods: ELISA was used to detect secreted IL-6 in human melanoma cells. IL-6 signalling was investigated by Western blots, while apoptosis, proliferation and migration were measured by caspase 3 activity, annexin V staining, cell-cycle analyses and cell gap-closure assay.
Results: Human metastatic melanoma cells A375 and 518A2 secrete high amounts of IL-6, in contrast to early stage WM35 cells. Canonical IL-6 signalling is intact in these cells, documented by transient phosphorylation of STAT-3. Although WM35 cells are highly resistant to simvastatin-induced apoptosis, co-administration with IL-6 enhanced the susceptibility to undergo apoptosis. This pro-apoptotic effect of IL-6 might be explained by a down regulation of Bcl-XL, and cell-cycle arrest observed only in WM35 cells. Metastatic A375 and 518A2 melanoma cells are highly susceptible to simvastatin-induced apoptosis, but coadministration of IL-6 had no additive effect. Interestingly, simvastatin enhanced IL-6 secretion in these cells. The IL-6 receptor-blocking antibody tocilizumab did not trigger apoptosis or migration in a transwell assay *per se*. However, co-administration with simvastatin unmasked an IL-6-sensitive proportion in the simvastatin-induced caspase 3 activation and in gap-closure assays with metastatic melanoma cells, but not in WM35 cells from the radial growth stage.
Discussion: High plasma levels of IL-6 correlate with poor outcome in late-stage melanoma patients. This observation correlates with high secretion of IL-6 from A375 and 518A2 cells and the induction of proliferation. However, in the presence of simvastatin these metastatic melanoma cells undergo severe apoptosis. The coadministration of simvastatin and tocilizumab unmasks now an IL-6 component behind the simvastatin-induced effects. It is therefore conceivable that tocilizumab contributes to accelerated gap-closure and reduced levels of apoptosis which may explain the rapid onset of melanoma in some individuals receiving this medication. Tocilizumab-related safety concerns might be considered and further investigated by *in vivo* melanoma models. Such an approach might also shed new light on the molecular switch, which regulates IL-6 signalling in metastatic melanoma cells with possible implications on the tumour microenvironment.
Acknowledgements: This work was supported by a grant from the Herzfelder'sche Familienstiftung and the Austrian Science Funds FWF (P-22385) to M. H.

A5.7

Characterization of STAT5 serine phosphorylation as a drug target in BCR-ABL1$^+$ leukemia

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Intrinsic Activity, 2015; **3**(Suppl. 2): A5.7
http://www.intrinsicactivity.org/2015/3/S2/A5.7

Background: In Philadelphia chromosome-positive leukemia, STAT5 is essential for signaling down-stream of the BCR-ABL1 oncogene and mediates resistance towards current tyrosine kinase inhibitor (TKI) therapy. STAT5A has two serine sites at position 725 and 779 in the transactivation domain. Serine phosphorylation is required for leukemic transformation with a constitutive active STAT5A mutant but dispensable for normal hematopoietic development. We investigated the role of serine phosphorylation in BCR-ABL1-induced leukemia and screened for the putative upstream kinase(s).
Methods: We generated STAT5A with single serine site mutations (STAT5A^{S725A} or STAT5A^{S779A}) or double mutants (STAT5A^{SASA}) and transfected stable BCR-ABL1$^+$ cells. We conducted leukemic mouse model experiments and analyzed survival and disease onset of mice. To identify the upstream kinase(s) we conducted a chemical compounds screen with kinase inhibitors using BCR-ABL1$^+$ cells that overexpress wild-type or phospho-mimetic STAT5A mutants (STAT5A^{SDSD}). Positive hit compounds were validated via immunoblotting with antisera specific for pSTAT5. Lentiviral shRNA-mediated knock-down was performed in BCR-ABL1$^+$ cells.
Results: Expression of the double mutant STAT5A^{SASA} hampered cell proliferation and significantly delayed disease onset in mice. When investigating the single point mutations STAT5A^{S725A} or STAT5A^{S779A} we observed enhanced survival for the STAT5A^{S725A}-expressing group and an even further prolonged leukemic onset for the STAT5A^{S779A} cohort when compared to the wild-type STAT5A. Determination of the subcellular location of YFP-tagged STAT5A^{S779A} in BCR-ABL1$^+$ cells showed that mutated STAT5 failed to enter the nucleus. We identified group I PAK kinases regulating the phosphorylation of STAT5^{S779}. Similarly, blocking of PAK kinase activity with kinase inhibitors significantly reduced the levels of STAT5 in the nucleus of mouse and human BCR-ABL1$^+$ cells. The single knock-down of PAK1 or PAK2 alone did not affect the proliferation of human BCR-ABL1$^+$ cells, whereas the successive knock-knock of both PAK kinases killed leukemic cells.
Discussion: We demonstrated the importance of STAT5 serine phosphorylation in BCR-ABL1-induced leukemia and showed that PAK-dependent phosphorylation of STAT5A^{S779} is required for nuclear translocation. Recent work in FLT3- and KIT-driven leukemias confirmed the regulation of STAT5 by PAK1 and upstream factor FAK. It is conceivable that our findings are relevant for other cancers than hematopoietic malignancies—wherever STAT5 is the master mediator of aberrant signaling.
Acknowledgements: This work was supported by the Austrian Science Fund FWF (SFB 28 to V. S. and R. M., and P24295-B23 to A. H. K.) and GEN-AU (PLACEBO; BMWF-70.081/0018-II/1a/2008 to V. S.).

A5.8

CDK4 and CDK6 cooperate in counteracting the INK4 family of inhibitors during murine leukemogenesis

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Intrinsic Activity, 2015; **3**(Suppl. 2): A5.8
http://www.intrinsicactivity.org/2015/3/S2/A5.8

Background: Cyclin-dependent kinase (CDK) 4 and 6 are key players mediating G_1 progression of the cell cycle. When bound to D-type cyclins they become active and phosphorylate their substrates,

most importantly the retinoblastoma protein (Rb). High CDK6 levels are frequently observed in human malignancies and CDK4/6 inhibitors show promising efficacy against different types of tumors in clinical trials. INK4 proteins negatively regulate CDK4/6 activity and are frequently inactivated upon transformation.

Methods: We used knock-in mice that express a CDK6 (CDK6-R31C) or CDK4 (CDK4-R24C) mutant insensitive to INK4-mediated inhibition. Spontaneous tumor formation was analyzed over a period of two years and leukemic cell lines were generated by transducing freshly isolated bone marrow cells with BCR-ABLp185. These leukemic cell lines harboring different genotypes were compared in transplantation experiments using NSG mice, investigated biochemically and studied in microarray analysis.

Results: Mice harboring both mutant alleles (CDK6-R31C and CDK4-R24C) developed predominantly spontaneous hematopoietic and endocrine tumors and showed a drastic reduction in life span compared to the individual single mutants. Using BCR-ABL-transformed cells as model system we found that CDK6-R31C causes increased binding of p16INK4a to the remaining wild-type CDK4. In the presence of both INK4-insensitive kinases we observed accelerated disease onset that can be explained by hyper-phosphorylated Rb and significant alterations in the transcriptional profile.

Discussion: The importance of CDK4/6 for tumor formation is reflected by the emerging success of CDK inhibitors, such as palbociclib, which has been shown to significantly prolong progression-free survival of breast-cancer patients and hence has been designated as a breakthrough therapy of the year 2013 by the FDA. Our observations reveal that CDK4 and CDK6 cooperate in hematopoietic tumor development. In the presence of at least one functional INK4 protein, the concomitant overexpression of both CDK4 and CDK6 may be required to overcome the limited phosphorylation of Rb that is inflicted by increased binding of the inhibitor to the remaining wild-type CDK. Our study underlines the importance of simultaneous targeting of CDK4 and CDK6 in hematopoietic tumors in which INK4 proteins are inactivated.

Acknowledgements: This work was supported by the Austrian Science Fund FWF (grant P24297 and SFB F47).

Toxicology

A6.1

Non-linear dose-dependent distribution of tariquidar to the human liver measured with PET

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Intrinsic Activity, 2015; **3**(Suppl. 2):A6.1
http://www.intrinsicactivity.org/2015/3/S2/A6.1

Background: The investigational third-generation P-glycoprotein (ABCB1) inhibitor tariquidar (XR9576) has been used in clinical trials in tumour patients in combination with anticancer drugs, such as vinorelbine, paclitaxel, docetaxel and doxorubicin, in order to overcome multidrug resistance of tumours [1]. In these clinical trials increased systemic exposure to anticancer drugs was observed in patients receiving tariquidar leading to dose-limiting toxicities, which has been attributed to inhibition of ABCB1 in tissues other than the tumour tissue. In the present study we used positron emission tomography (PET) imaging to study the *in vivo* distribution of [^{11}C]tariquidar to the liver of healthy volunteers.

Methods: Four healthy male volunteers underwent two consecutive 60-min dynamic abdominal PET scans with [^{11}C]tariquidar, a first scan after administration of only a microdose of [^{11}C]tariquidar (< 20 µg) and a second scan during continuous i. v. infusion of unlabelled tariquidar (3.75 mg/min). In parallel to PET imaging arterial blood sampling was performed and radioactivity in plasma was measured in a gamma counter. The liver was delineated as a region of interest on MR-co-registered PET images and distribution of [^{11}C]tariquidar to the liver was expressed as the liver-to-plasma area under the time-activity curve ratio (AUC_{liver}/AUC_{plasma}) and as uptake clearance from blood into liver ($CL_{uptake,liver}$), which was estimated by a previously described graphical analysis approach (integration plot) [2].

Results: Following i. v. injection of [^{11}C]tariquidar, high radioactivity uptake was observed in the liver. In PET scan 2, which was performed during infusion of unlabelled tariquidar, AUC_{plasma} was 44.7 ± 21.1 % higher than in PET scan 1, in which only a microdose of [^{11}C]tariquidar was administered (scan 1: 13.3 ± 3.1, scan 2: 18.9 ± 4.0, $p = 0.012$, paired *t*-test). AUC_{liver}/AUC_{plasma} was reduced by 32.0 ± 8.7 % (scan 1: 25.7 ± 5.1, scan 2: 18.3 ± 4.0, $p = 0.01$) and $CL_{uptake,liver}$ was reduced by 27.1 ± 9.0 % in scan 2 as compared to scan 1 (scan 1: 0.49 ± 0.08 ml/min/g, scan 2: 0.36 ± 0.09 ml/min/g, $p = 0.002$).

Discussion: We observed non-linearity in [^{11}C]tariquidar distribution to the human liver. Liver distribution was lower and plasma exposure was higher for a pharmacological dose as compared with a microdose of [^{11}C]tariquidar pointing to dose-dependent inhibition by tariquidar of basolateral uptake transporters in hepatocytes, *i. e.* organic anion transporting polypeptides (OATPs). This suggests that tariquidar is substrate and inhibitor of human OATPs. Inhibition of OATPs in the liver may have also contributed to increased plasma concentrations of anticancer drugs observed in previous clinical trials with tariquidar.

Acknowledgements: Funded by the Austrian Science Fund FWF, grant F3513-B20.

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A6.2

LC-MS analysis of phenolic compounds and antioxidant activity of dietary supplement formulations based on edible mushrooms

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Intrinsic Activity, 2015; **3**(Suppl. 2):A6.2
http://www.intrinsicactivity.org/2015/3/S2/A6.2

Background: There is an increasing number of food supplements with perceived and real health benefits on the market. As food supplements are one of the most easily accessible complementary and integrative therapies, they are widely used in modern diets. Thus, the aim of the present work was to determine the antioxidant potential of methanol extracts made from commercial preparations of

Cordyceps sinensis (Berk.) Sacc., *Ganoderma lucidum* (Curtis) P. Karst. and *Coprinus comatus* (O. F. Müll.) Pers. mushrooms.

Methods: Antioxidant properties were determined using four different test systems, namely 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (HO) and nitric oxide (NO) radical scavenging assays and ferric-reducing antioxidant power assay (FRAP), in addition to the determination of their total phenolic contents and LC-MS analysis of the concentration of the main phenolic compounds found in mushroom species.

Results: All the assessed extracts were able to reduce DPPH in a dose-dependent manner with IC_{50} values ranging from 172 to 483 μg/ml. In two other tests for measuring the antioxidant activity, the methanolic extract of *C. sinensis* showed the best properties. The assay of reducing power showed that the most active mushroom is *C. sinensis* again with an absorbance value of 1.392 ± 0.009. The same was seen for the analysis of selected phenolic compounds; *C. sinensis* was found to have the highest content.

Discussion: The commercial preparations of *C. sinensis* and *C. comatus* can be considered to be suitable food supplements included in well-balanced diets as a rich source of antioxidants. On the other hand, the commercial preparation of *G. lucidum* needs to be studied further. It can be concluded that other dietary supplements, not only plants, can reduce the amount of free radicals and be potent and safe antioxidants.

Acknowledgements: The results presented here were obtained in project no. 41012 supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, and project no. 114-451-3551/2013 supported by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina.

A6.3

Transcriptomic effects of ursodeoxycholic acid treatment on adriamycin-induced oxidative liver injury

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Intrinsic Activity, 2015; **3**(Suppl. 2): A6.3
http://www.intrinsicactivity.org/2015/3/S2/A6.3

Background: Adriamycin (ADR) is a potent anticancer drug; however, hepatotoxicity commonly overshadows its anti-neoplastic effectiveness. The overproduction of free radicals is considered as a significant cause of this side effect. The aim of our study was to evaluate the influence of ursodeoxycholic acid (UDCA), a bile acid with antioxidative properties, on the expression of genes involved in antioxidative response to ADR treatment.

Methods: Eighteen male Wistar rats were divided in three groups. Animals were treated with vehicle (saline i. p.), ADR (3 mg/kg i. p. every other day for 3 doses in total) or both with ADR and UDCA (25 mg/kg p. o. every other day for 3 doses in total, starting one day before administering ADR). On day 28, animals were euthanized and total RNA was isolated from liver tissue and reversely transcribed into complementary DNA. Gene expression was determined by qRT-PCR and results were analyzed using the $2^{-\Delta\Delta C_T}$ method.

Results: The relative expression of genes encoding Sod, Cat, Gpx and Gr in the liver of animals treated with ADR was 4.3-, 5.5-, 12.4- and 6.2-fold decreased, respectively. Co-treatment with UDCA resulted in 1.7-, 2.8-, and 12.0-fold increased expression of Sod, Cat and Gpx and 1.8-fold decreased expression of Gr, compared to the group treated with ADR only. In addition, the expression of pro-apoptotic Bax was 1.7-fold increased in the livers of animals treated with ADR, whereas co-administration of UDCA reduced Bax expression to control values.

Discussion: According to its ability to enhance the antioxidative defence system at the transcriptomic level as well as to decrease expression of pro-apoptotic Bax mRNA in the liver, UDCA may be considered as an agent with potentially hepatoprotective properties against oxidative liver injury induced by high doses of ADR.

Acknowledgements: Supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant no. III 41012.

A6.4

Hyperbaric oxygen protects neurotrophic activity of carbon monoxide-exposed astrocytes

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Intrinsic Activity, 2015; **3**(Suppl. 2): A6.4
http://www.intrinsicactivity.org/2015/3/S2/A6.4

Background: Carbon monoxide (CO) poisoning causes neuronal and glial apoptosis that can result in delayed neurological symptoms. The damage of brain cells can be prevented by oxygen therapy. Recently we reported that CO/normoxia caused a progressive decline of viability and mitochondrial function accompanied by caspase and calpain activation. Impairment in astrocyte function was time-dependently reduced by hyperbaric, but not normobaric, oxygen. Due to the central role of astrocytes in maintaining neuronal function by offering neurotrophic support we investigated toxic effects of CO/normoxia on intrinsic neurotrophic activity in these cells and evaluated possible protective influence of oxygen treatment against CO poisoning.

Methods: Cultured rat astrocytes were exposed to 3.000 ppm CO in air for different time periods (0.5–24 h) followed by 24 h of normoxia. Following an 8-hour exposure to CO that significantly affected astrocytic cellular function the cultured cells were exposed during 24 h of normoxia for 1 h in different time periods (0–7 h) after CO to 100% normobaric oxygen (NBO) or 100% oxygen at a pressure of 3 bar (HBO). Real-time PCR was performed to examine the expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). Specific two-site enzyme immunoassays were utilized to determine protein synthesis and secretion of the examined neurotrophins.

Results: CO/normoxia caused a progressive decline of gene expression, synthesis and secretion of NGF, BDNF and NT-3 with different intensity. A maximal response was seen after 8 h in CO. Subsequent 1-hour treatment with oxygen disclosed pressure- and time-dependent efficacy in restoring astrocytic neurotrophic activity. The protective effect was evident when the cells were exposed to HBO 1–5 h after CO but not if they were exposed to HBO immediately after incubation in CO. A diminished efficiency of HBO in enhancement of neurotrophin synthesis was observed 7 h after CO exposure. In contrast, NBO showed no protective influence on CO-poisoned cells.

Discussion: The neuroprotective role of oxygen therapy in CO-exposed astrocytes is pressure- and time-dependent. In addition to

preventing mitochondrial dysfunction and apoptotic processes our present results indicate that HBO, but not NBO, restores astrocytic neurotrophic support that may possibly dictate the short- and long-term neuronal survival as well as the maintenance and retraction of synaptic connections. In order to prevent the occurrence of late neuropsychological sequelae our study opens the way to consider time and pressure regimens of oxygen therapy in the clinical management of CO poisoning.

A6.5

Influence of apigenin on the biochemical serum parameters and histological changes of liver tissue in rats exposed to oxidative stress

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Intrinsic Activity, 2015; **3**(Suppl. 2): A6.5
http://www.intrinsicactivity.org/2015/3/S2/A6.5

Background: With the high beneficial potential of flavonoids, today's modern medicine focuses on the effects of apigenin, which is present in everyday food ingredients. In several researches conducted on laboratory animals, apigenin was found to have certain antioxidative effects by reducing liver damage in laboratory animals exposed to oxidative stress. The research confirms the influence of apigenin on biochemical parameters, on indicators of hepatic and renal function, as well as on alterations in liver structure in white mice exposed to oxidative stress induced by toxic doses of paracetamol.
Methods: The research was conducted on sexually mature white male Wistar laboratory rats, divided into four groups of 6 animals each. During 6 days, the animals were orally pretreated with apigenin and physiological saline (10 mg/kg). The rats were decapitated, completely autopsied and the collected blood was further used in the assessment of biochemical parameters.
Results: The application of toxic doses of paracetamol increased the activity of hepatic transaminases in serum compared to controls ($p < 0.05$). In animals pretreated with apigenin, the serum activity of aspartate transaminase was a quarter lower as compared to controls, while the activity of alanine transaminase was 5 times lower compared to controls. The direct bilirubin concentration was significantly lower in rats pretreated with apigenin compared to controls ($p < 0.05$). The serum urea level in rats treated with paracetamol was significantly lower when compared to other groups ($p < 0.05$).
Discussion: The application of toxic doses of paracetamol leads to a significant disorder of biochemical parameters, indicators of hepatic and renal function, in the serum of laboratory rats. Pathohistological liver tissue changes induced by toxic paracetamol doses were less present in those animals pretreated with apigenin. Observed hepatoprotective and nephroprotective properties of apigenin provide insight into potential beneficial effects of apigenin.
Acknowledgements: This work is supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project no. 41012) and by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina (project no. 114-451-3551/2015-03).

A6.6

Forensic significance of determination of the alcohol elimination rate through analysis of blood samples at two times

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Intrinsic Activity, 2015; **3**(Suppl. 2): A6.6
http://www.intrinsicactivity.org/2015/3/S2/A6.6

Background: Determination of the relevant blood alcohol concentration (BAC) at the time of incriminating events is a constant challenge in the daily work of forensic experts. It is indirectly related to the alcohol elimination rate, which is used to calculate the relevant BAC at the time of critical events.
Methods: In this regard, taking blood samples at two times to determine the elimination rate of alcohol is a medico-legal doctrine. The present study was conducted with eighty-eight subjects whose blood samples were taken at two times. Then, the alcohol concentration was determined and also the value of the elimination rate and its correlation with the following variables: gender, age, body weight, height, body mass index, level of the BAC, disease, injury, bleeding, infusions.
Results: The correlation of beta elimination rate and these variables was determined in order to facilitate understanding of the given values of elimination rate and also greater objectivity. In the phase of alcohol elimination there were eighty subjects (10 women and 70 men), in which the alcohol elimination rate and its correlation with the observed parameters was observed. The minimum rate of elimination of the subjects was 0.09 g/kg/h, while the maximum elimination rate was 0.55 g/kg/h; the average elimination rate was 0.18 g/kg/h. The results showed a strong positive correlation between anthropometric parameters (weight, height, BMI) of patients and the elimination rate of alcohol from the blood. The body weight had the largest impact on the rate of alcohol elimination with a value of variance of 61%. Also, there was a statistically significant effect BAC on the alcohol elimination rate, as well as a strong correlation between eliminatuion rate and whether the subjects had received infusions or had injuries and bleeding. There was no statistically significant relationship between beta elimination rate and other variables (gender, age and disease).
Discussion: Taking blood sample twice to determine the individual alcohol elimination rate can significantly increase the precision of retrograde calculation of alcohol levels at the time of an event for forensic purposes.

A6.7

Evaluation of fluoride concentration in tapped, bottled and filtered water available in Croatia

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Intrinsic Activity, 2015; **3**(Suppl. 2): A6.7
http://www.intrinsicactivity.org/2015/3/S2/A6.7

Background: Fluoride is a chemical element that has been shown to cause significant effects on human health through drinking water. International standards for drinking water have been established by organizations such as the World Health Organization (WHO). However, local conditions as well as diet and exercise play a large role in body fluoride intake during the day. Fluoride administered in optimal concentration is caries-protecting, while excessive amounts

of fluoride can cause dental fluorosis, skeletal fluorosis and osteoporosis. To adjust the right amount of fluoride to patient, one needs to know the daily intake of fluoride through drinking water since it is the main source of fluoride for the human body. The aim of this study was to determine fluoride concentrations in waters most frequently used domestically, which are tapped, bottled and filtered water.

Methods: Samples of tapped waters were obtained from different homes that were supplied from all five main water wells of Zagreb, Croatia. Samples of filtered water were taken after running through two main types of water filtration systems: silver-impregnated activated carbon and ion-exchange filters and filters based on reverse osmosis and ultrafiltration. Samples of bottled water were acquired from three supermarkets, all of eight commercially available brands in Zagreb. All samples were tested with a combination fluoride-ion-selective electrode (Orion, 96-09-00, MA, USA) and the average read-out of two tests was recorded.

Results: The mean fluoride content of the tapped water samples was 0.032 mg F/l with a range from 0.027 to 0.037 mg F/l. The mean fluoride content of filtered water samples was 0.022 mg F/l with a range from 0.003 to 0.037 mg F/l. The mean fluoride content of the bottled water samples was 0.083 mg F/l with a range from 0.015 to 0.301 mg F/l.

Discussion: Caries prevention is done on an individual basis. In the context of a prevention program it is important to investigate the source of drinking water. Individuals using water filtered with filters based on reverse osmosis and ultrafiltration as main source of drinking water should receive a more intense caries prevention program.

A6.8

Fluoride content of bottled waters commercially available in Zagreb, Croatia

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Intrinsic Activity, 2015; **3**(Suppl. 2):A6.8
http://www.intrinsicactivity.org/2015/3/S2/A6.8

Background: The role of fluoride in the prevention of dental caries is well known. Optimal daily intake of fluoride should be 0.05–0.07 mg F/kg body weight. On the other hand, excessive fluoride intake can cause dental fluorosis and hypomineralisation of enamel characterized by greater surface porosity. To avoid the risk of dental fluorosis daily intake should not exceed a daily level of 0.10 mg F/kg body weight. Current studies showed that fluoride content in water may be highly variable, and water is considered as main source of fluoride. The aim of this study was to determine the fluoride content in bottled waters commercially available in Zagreb, Croatia.

Methods: Thirty brands of bottled water were obtained from three supermarkets in Zagreb, Croatia. Bottled waters were divided in three groups: carbonated water, non-carbonated water and flavoured water. Following calibration, two tests were conducted on each bottle using a combination fluoride ion-selective electrode (Orion, 96-09-00, MA, USA). The average reading for each brand was calculated and also compared with the fluoride content printed on the label, if available.

Results: The mean (± SD) fluoride content of the carbonated bottled water samples were 0.338 ± 0.328 mg F/l with a range from 0.014 to 1.150 mg F/l. The fluoride content of the non-carbonated bottled water samples were 0.083 ± 0.097 mg F/l with a range from 0.015 to 0.301 mg F/l. The fluoride content of the flavoured bottled water samples were 0.225 ± 0.348 mg F/l with a range from 0.023 to 0.927 mg F/l. Out of the brands tested, 43 % (n = 13) mention the fluoride content on the label.

Discussion: Even though the fluoride concentrations in the tested samples were in the safe range it is recommended to list fluoride content on labels of all bottled waters. The decision about fluoridation treatment should be designed having in mind the amount of fluoride intake from beverages and their possible cumulative influence, so the optimal caries-preventive effect can be obtained and the risk of dental fluorosis reduced.

Drug Research

A7.1

Development of flow methods for the determination of *N*-acetyl-L-cysteine in pharmaceutical formulations

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Intrinsic Activity, 2015; **3**(Suppl. 2):A7.1
http://www.intrinsicactivity.org/2015/3/S2/A7.1

Background: *N*-acetyl-L-cysteine (NAC) is a synthetic aminothiol antioxidant that has been in clinical use for more than 40 years, primarily as a mucolytic agent and in the management of paracetamol (acetaminophen) poisoning. There is a need for a new, robust, inexpensive, and rapid method of determination of NAC in pharmaceutical formulations, in order to assure proper quality control (QC). Patient safety during therapy, on the other hand, is indirectly linked to proper QC of the medication. Novel flow methods for the determination of NAC, such as the flow-injection method (FIA) or the sequential-injection method (SIA), have been developed and validated. Both methods are kinetic methods, which means that the measurement of the analytical signal is made under dynamic conditions in which the concentrations of reactants and products are changing as a function of time. The proposed flow methods of analysis (FIA and SIA) are interesting alternatives in NAC determinations instead of conventional batch methods and chromatography with different detectors. The advantages afforded by the flow methods of analysis are high sample frequency, low consumption of sample and reagents, low contamination risks, and significant reproducibility that provides high precision and enhanced selectivity as a result of the kinetic nature of the recorded analytical signal. Furthermore, the proposed flow methods of analysis require very limited laboratory bench space and necessary instrumentation.

Methods: The proposed methods are based on the reduction of Cu(II)-neocuproine reagent to Cu(I)-neocuproine with the analyte, in a Britton-Robinson buffer solution (pH 3.0). The non-steady-state absorbance of the formed yellow Cu(I)-neocuproine complex is measured at 458 nm. For the flow-injection method the three-line manifold with one reaction coil was used. Optimization of manifold parameters and experimental conditions were carried out by means of univariate method. The sequential-injection manifold consisted of a Cheminert® M50 pump (VICI Valco), a syringe-free stepper motor-driven pump, a 10-port selection valve model (C25-3180D) with a multiposition actuator control module (EMHCA-CE; VICI Valco). Both flow systems use a spectrophotometric detector.

Results: Using a flow-injection method of analysis, a linear calibration curve is established in a concentration range of 6×10^{-7} to 4×10^{-5} mol/l NAC with a detection limit of 9.4×10^{-8}. On the other hand, using the sequential-injection method of analysis, linearity was obtained in the concentration range of 4×10^{-6} to 3×10^{-4} mol/l. The detection limit

was found to be 1.2×10^{-6} mol/l. The proposed methods are simple, rapid, sensitive and reproducible (FIA: RSD 0.9%, $n = 100$; SIA: RSD 1.9%, $n = 100$). In addition, the proposed methods are sensitive enough to enable determination of near-nanomole amounts of NAC without expensive instruments with an analytical frequency of 120/h (FIA) and 60/h (SIA).

Discussion: The proposed methods can be applied for the determination of NAC in pharmaceutical preparations. Therefore, they could be useful for QC of NAC-containing medications, indirectly improving patient safety during therapy (*e.g.* by reducing the risk of under- or over-dosage).

A7.2

A pharmacological and computational study on sewarine, a naturally derived alkaloid, as a new ligand interacting with the κ opioid receptor

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Intrinsic Activity, 2015; **3**(Suppl. 2): A7.2
http://www.intrinsicactivity.org/2015/3/S2/A7.2

Background: The kappa opioid receptor (KOR), a member of the opioid receptors family, has received extensive attention in recent years, and nowadays it emerges as a potential target for the treatment of a variety of human disorders, including pain, affective disorders, drug addiction, and psychotic disorders. The KOR is distributed throughout the brain, the spinal cord and various peripheral tissues. The structure of the KOR was elucidated by X-ray crystallography, giving insights into the binding pocket of the KOR. Applying a pharmacophore-based virtual screening strategy, we have recently reported on a novel KOR ligand, sewarine. It is a naturally derived alkaloid from the plant *Rhazya stricta*, used in traditional medicine against human diseases such as cancer, rheumatism, skin diseases, or pain. Herein we present a comparative pharmacological study on the interaction and signaling of sewarine at the KOR from guinea-pig and human origin. In addition, the binding mode of sewarine to the crystal structure of the human KOR is described.

Methods: Binding and activity at the KOR were determined using radioligand binding, [^{35}S]GTPγS functional and forskolin-induced cAMP accumulation assays. Molecular docking in the human KOR crystal structure was performed using GOLD 5.1 software.

Results: In *in vitro* binding studies, sewarine showed high KOR selectivity with similar binding affinities to the KOR in the guinea-pig brain and CHO cells expressing the human KOR. While in guinea-pig brain, sewarine displayed KOR antagonism, in CHO-hKOR cells it acted as a KOR partial agonist. The relatively low stimulatory effect of sewarine at the human KOR was fully reversed by the selective KOR antagonist nor-BNI. The apoptotic effect of sewarine in human leukemia CEM-C7H2 cells was also demonstrated to involve the KOR, based on the significant antagonism of nor-BNI. The structural features that promote binding of sewarine to the human KOR were investigated by molecular docking studies. Similar to well-known KOR ligands, the salt bridge between the protonable nitrogen in sewarine and Asp138 was maintained, and hydrophobic contacts were established with Val108, a residue responsible for KOR selectivity.

Discussion: Through combination of biochemical, pharmacological and computational approaches, we highlight the outcomes on the selective interaction and signaling of sewarine via the KOR in neuronal and cellular systems expressing KOR. The present findings provide insights into the binding mode and signaling at the KOR of sewarine as a novel KOR ligand of plant origin, which may represent a promising lead molecule for optimization towards superior probes targeting the KOR, and ultimately for the development of new therapeutics for human neurological and other disorders.

A7.3

Deuteration changes the binding of some histaminergic agonists to the histamine H_2 receptor in astrocytes

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Intrinsic Activity, 2015; **3**(Suppl. 2): A7.3
http://www.intrinsicactivity.org/2015/3/S2/A7.3

Background: A crucial step in the binding of histaminergic ligands, *e.g.* histamine to the H_2 receptor, is the formation of three hydrogen bonds between amino acid residues (Asp^{98}, Asp^{186} and Thr^{190}) present in the third and the fifth transmembrane α-helices and three nitrogen atoms of the histamine molecule. In order to estimate the relevance of hydrogen bonds in the process of binding of ligands to the H_2 receptor we compared the binding properties of [^{3}H]tiotidine to histamine H_2 receptor binding sites in cultured neonatal rat astrocytes in control and deuterated medium.

Methods: To test this hypothesis we performed saturation and inhibition binding studies using [^{3}H]tiotidine as a biomarker in cultured glial cells. We modeled changed binding affinity upon deuteration of histamine in conjunction with quantum chemical calculations and quantization of nuclear motion of the protons involved in hydrogen bonding.

Results: [^{3}H]tiotidine binds in a reversible and saturable manner to a single population of binding sites with maximal binding-site density (B_{max}) of 22.0 ± 3.2 fmol/mg protein and equilibrium dissociation constant (K_d) of 6.3 ± 1.9 nM. Histamine, 2-methylhistamine and 4-methylhistamine displaced the radioligand with pIC_{50} values of 7.6 ± 0.14, 8.5 ± 0.16, and 7.4 ± 0.25, respectively. Binding characteristics changed upon deuteration: the B_{max} dropped nonsignificantly to 17.4 ± 5.2 fmol/mg protein; the K_d of [^{3}H]tiotidine changed to 8.6 ± 5.0 nM ($p > 0.05$; determined by Student's *t*-test; $n = 6$); the pIC_{50} values for histamine, 2-methylhistamine and 4-methylhistamine in deuterated conditions were 8.0 ± 0.15, 6.8 ± 0.16 ($p < 0.05$; Student's *t*-test; $n = 6$) and 7.7 ± 0.13, respectively ($n = 6$). The experimental data show that deuteration significantly attenuated binding free energy of 2-methylhistamine (2.15 kcal/mol), but decreased binding free energy for 4-methylhistamine (−0.51 kcal/mol) and histamine (−0.78 kcal/mol). *Ab initio* calculations of the isotope effect were performed for the endogenous ligand histamine for transfer of monoprotonated histamine ion from the aqueous environment to the receptor binding site. Implicitly quantized NH and OH motion revealed that the changes can be rationalized by attenuated strength of hydrogen bonding upon deuteration which is known as Ubbelohde effect.

Discussion: Replacing hydrogen atoms involved in binding of histamine ligands to H_2 receptor binding sites with deuterium atoms results in different length of intermolecular and intramolecular distances. This leads to a structural change of ligand and receptor binding sites which significantly affects the binding affinities of methylhistamines The effects of deuteration on the affinity is the difference between the interaction free energy receptor–ligand and water–ligand giving rise to increased or decreased values. Our study offers a simple and practical approach how to treat nuclear quantum effects in drug–receptor binding and will hopefully help reaching a distant goal that is *in silico* discrimination between agonists and antagonists.

Acknowledgements: The work was supported grants no. J1-2014, P3-067 and P1-012 of the Slovenian Research Agency.

A7.4

Oxyresveratrol as a promising drug candidate for metabolic diseases: a pharmacoinformatics study

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Intrinsic Activity, 2015; **3**(Suppl. 2): A7.4
http://www.intrinsicactivity.org/2015/3/S2/A7.4

Background: Resveratrol is a polyphenol with demonstrated cardioprotective, chemopreventive, anti-inflammatory and antioxidant effects. Recently it was shown that resveratrol binds to the PPAR-γ receptor and that it can reduce insulin resistance associated with obesity. Low solubility in water is the major limiting factor for widespread pharmaceutical use of resveratrol. Therefore, the aim of our study was to identify analogues of resveratrol with improved pharmacokinetic properties and higher binding affinities towards the PPAR-γ receptor.
Methods: 3D structures of resveratrol and its analogues were retrieved from the ZINC database, while the PPAR-γ structure was obtained from the Protein Data Bank. Docking studies were performed using the Molegro Virtual Docker software. Molecular descriptors relevant to solubility and pharmacokinetics were calculated from ligand structures using the VolSurf+ software.
Results: Using a structural similarity search method in the ZINC database, 57 compounds were identified and subjected to docking analyses. Binding energies (MolDock scores) ranged from −136.69 to −90.89 kcal/mol. The MolDock score for resveratrol was −118.04 kcal/mol. Sixteen compounds exerted lower binding energies, *i.e.* higher affinities towards PPAR-γ. Calculated values of the SOLY descriptor, as logarithm of intrinsic solubility, ranged from −5.05 to −3.24, and 23 studied compounds were found to be more soluble in water than resveratrol. By combining these results it was revealed that only two tetrahydroxy stilbene derivatives, piceatannol and oxyresveratrol, had both better solubility and affinity towards PPAR-γ. Calculated pharmacokinetic parameters showed that both these compounds were more stable metabolically and more widely distributed in the body than resveratrol, but only oxyresveratrol had a higher value of the amphiphilic moment, which determines the ability to permeate membranes and absorption.
Discussion: The results of our study demonstrate that oxyresveratrol is a promising drug candidate that should be investigated more in-depth for a potential use in metabolic diseases.
Acknowledgements: This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, project no. III 41012.

A7.5

The role of bile acids in drug penetration through biological membranes

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Intrinsic Activity, 2015; **3**(Suppl. 2): A7.5
http://www.intrinsicactivity.org/2015/3/S2/A7.5

Background: One of the greatest challenges in the pharmaceutical industry is the development of new technologies that enable poorly membrane-permeable drugs to effectively penetrate biological membranes. Currently, bile acids as compounds that may facilitate transport of drugs across various membranes are the topic of extensive research [1]. Therefore, the aim of this work is to emphasize the role of bile acids in this field.
Methods: The relevant original and review articles published from 2000–2015 in various databases were analysed.
Results: There has been a growing interest in using bile acids for modification of drug absorption and drug delivery due to their ability to act as a drug carrier system in the form of mixed micelles, bilosomes and chemical conjugates with drug molecules. The role of bile acids in promoting drug permeation has been experimentally illustrated in various pharmaceutical formulations for oral, nasal, ocular, buccal, pulmonary and rectal administration route. Due to amphiphilic properties, bile acids can interact with biological membranes, thus disturbing their functioning. The final outcome of bile acids on the cell membrane depends on many factors including type and structure of bile acids and membrane characteristics. Bile acids have an ability to enhance the epithelial transport of hydrophilic drugs through the paracellular route and that of hydrophobic compounds through both paracellular and transcellular routes.
Discussion: The unique and distinguishable structure and specific physicochemical properties of bile acids have enabled them to be used in the development of drugs, as pharmaceutical tools and potential drug carrier systems that could improve, control and localise drug delivery. The available information will probably yield, in the near future, new drug formulations with improved pharmaceutical properties.
Acknowledgements: This work is supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant no. III 41012.

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A7.6

The role of bile acid derivatives in transport of drugs through biological membranes

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Intrinsic Activity, 2015; **3**(Suppl. 2): A7.6
http://www.intrinsicactivity.org/2015/3/S2/A7.6

Background: In recent years oxo derivatives of bile acids have been intensively investigated as compounds with amphiphilic properties which contribute to the transport of drugs trough biological membranes. As a system of drug carrier, bile acid derivatives express potential regarding the transport of large molecules like macrolide antibiotics. Macrolide antibiotics are in widespread use for the treatment of bacterial infections caused by Gram-positive, and to a limited extent by Gram-negative, bacteria. Because of the voluminosity of their molecules, macrolide antibiotics exhibit limited penetration into brain tissue which is often the target of bacterial infections.

Methods: The aim of this study is to determine which bile acid derivatives better provide transport of erythromycin into brain tissue. In view of this, the present work is concerned with the application of the chromatographic parameter R_M^0 obtained by normal-phase thin-layer chromatography in the solvent system toluene/butanol and silica gel as stationary phase to describe the hydrophobicity of bile acids.

Results: Table 1: Parameters of hydrophobicity of bile acids

Bile acids	Log *P*	cLog *P*	R_M^0 (T/E)[1]	R_M^0 (T/B)[2]
Deoxycholic acid	4.20	4.51	1.23 ± 0.08	1.46 ± 0.07
Chenodeoxycholic acid	4.13	4.51	1.20 ± 0.06	1.44 ± 0.08
Cholic acid	3.04	2.43	0.85 ± 0.03	1.03 ± 0.05
12-oxo-lithocholic acid	4.69	4.11	1.04 ± 0.03	1.25 ± 0.05
3,7,12-trioxo-cholanoic acid	4.01	2.33	0.48 ± 0.01	0.65 ± 0.02

R_M^0 determined in [1] toluene/ethanol and [2] toluene/butanol

Discussion: The increase in the number of oxo groups in the molecule is accompanied with a decrease in the hydrophobicity of the convex side of the steroid skeleton of the bile acid derivatives. Increasing hydrophobicity of both the macrolide and the bile acid strengthen this interaction.

Acknowledgements: The authors acknowledge the financial support of the Ministry of Science and Technological Development of the Republic of Serbia (project no. 41012).

A7.7

A method for quantification of echinocandins in body fluids

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Intrinsic Activity, 2015; **3**(Suppl.2):A7.7
http://www.intrinsicactivity.org/2015/3/S2/A7.7

Background: Invasive fungal infections (IFIs) pose a major threat to immuno-compromised and critically ill patients. Anidulafungin and micafungin belong to the echinocandins, which are active against *Candida* and *Aspergillus*. Target-site concentrations of antimicrobials are crucial for eradication of pathogens and for the clinical outcome. Therefore, we established and validated a method for quantification of anidulafungin and micafungin in various body fluids (plasma, ascites, pleural effusion, cerebrospinal fluid and bile).

Methods: Anidulafungin and micafungin were measured by high-performance liquid chromatography (HPLC) and UV detection. Sample preparation was performed by protein precipitation with acetonitrile. Gradient elution was done with ammonium acetate and acetonitrile at a flowrate of 1.0 ml/min. Anidulafungin was detected at 306 nm, micafungin at 273 nm. Quantification was validated according to the European Medicine Agency (EMA) and the International Conference on Harmonisation (ICH) guidelines, including intra- and interday variability and repeatability.

Results: The lower limit of quantification was 0.1 µg/ml for anidulafungin and micafungin in human plasma, ascites, pleural effusion, artificial cerebrospinal fluid and porcine bile. Intra- and interday variability in these body fluids was within the required range (< 15%). The recovery of the echinocandins showed differences between the body fluids (*e.g.* for a concentration of 10 µg/ml about 50% in bile, about 90% in pleural effusion). Repeatability was within the required range for all body fluids for anidulafungin and micafungin. Coefficient of determination (R^2) of the calibration curves was > 0.99 and fits therefore European guidelines of values higher than 0.99.

Discussion: Measurement of anidulafungin and micafungin by HPLC and UV detection was reproducible and sufficiently sensitive. Therefore, this method appears to be suitable for quantification of anidulafungin and micafungin in pharmacokinetic studies on echinocandin penetration into human body fluids.

Acknowledgements: We thank Pfizer for financial support.

Pharmacoepidemiology and Pharmacovigilance

A8.1

Treatment of respiratory tract infections in primary practice: common mistakes in Health Centre Novi Sad

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Intrinsic Activity, 2015; **3**(Suppl.2):A8.1
http://www.intrinsicactivity.org/2015/3/S2/A8.1

Background: Controlled, rational use of antimicrobial drugs is, for now, the most effective way to maintain the level of antibiotic resistance at an acceptable level while providing optimal patient care. Inappropriate prescribing of antibacterial drugs in primary practice is commonly associated with treatment of respiratory tract infections. The aim of research was to analyse treatment of respiratory tract infections in primary practice in Health Centre Novi Sad.

Methods: The research was designed as a cross sectional study. Data were collected from medical records for a period of 12 months (01.07.2013 – 30.06.2014) in Health Center Novi Sad. Data on medical diagnosis, chosen treatment and sensitivity of isolated bacteria (if applicable) were collected.

Results: During the observed period approximately 190,000 prescriptions were issued. The most common diseases treated were sore throat, acute bronchitis, common cold, sinusitis, otitis etc. Except for common cold (35%) and acute bronchitis (65%), approximately 75% of patients with respiratory tract infections were prescribed antibacterial drugs. In most of the cases prescribing was empirical, without isolation of bacteria.

Discussion: The most common mistakes in treatment of respiratory tract infections in Health Centre Novi Sad were: (i) antibacterial treatment of infections with predominantly viral aetiology (common cold, acute bronchitis), (ii) empirical antibacterial treatment in conditions with possible viral aetiology (sore throat), (iii) empirical treatment in conditions with possible bacterial aetiology without isolation of bacteria and determination of sensitivity to antibacterial drugs, (iv) low prescribing of narrow-spectrum antibacterial drugs such as natural penicillins.

Acknowledgements: This work was supported by the Ministry of Education, Science and Technology Development of the Republic of Serbia (grant no. III 41012).

A8.2

An analysis of expired medications in Serbian households

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Intrinsic Activity, 2015; **3**(Suppl. 2): A8.2
http://www.intrinsicactivity.org/2015/3/S2/A8.2

Background: Expired medicines accumulating in households is a universal problem worldwide. The potential presence of expired medications in households has recently been receiving attention due to its implications regarding health outcomes, heath care cost, and patient and environmental safety. The aim of this study was to determine the amount and structure of expired medications in Serbian households and to determine the therapeutic groups and clinical areas which generate most waste.
Methods: This was an observational, cross-sectional study conducted in households in the city of Novi Sad, Serbia. The study was performed over an 8-month period (December 2011 – July 2012) and consisted of personal insight into the inventory drugs in households. In order to obtain a calculated sample size, systematic random sampling was performed.
Results: Of 1,008 families, 383 agreed to participate and complete the questionnaire (38.3% response rate). In almost half of households (44.4%), expired medications were maintained. The amount of expired medications was 402 items, corresponding to 9.2% of total medications present in surveyed households. Of all expired medications, 70.4% were prescription drugs. The majority of expired medications (64.7%) were in solid dosage (tablets, capsules, granules, lozenges), following semisolid (ointments, creams, gel, suppositories) and liquid dosage forms (drops, syrups). Expired drugs in the households belonged mostly to 3 categories: antimicrobials for systemic use (16.7%), dermatological preparations (15.9%) and drugs for alimentary tract and metabolism (14.2%).
Discussion: Our findings were mostly consistent with other studies in terms of percentage of expired medications, but varied in the therapeutic groups of expired drugs. The differences are potentially attributable to the difference in the demographic characteristics of the investigated households, different health-seeking habits or different supply routes of medications. Serbia must consider the issue of medication wastage seriously. Part of this wastage can be prevented, and considering the limited resources of the country, it is prudent to start taking action. Finally, public services should promote awareness raising and educational campaigns targeting different age groups and using various communication routes.
Acknowledgements: This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia, project no. 41012.

A8.3

Guidelines adherence for prescription of oral antidiabetics in Serbia

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Background: Prescription of an appropriate antihyperglycemic agent depending on the standard guidelines has an important role in controlling diabetes and improving patient health. The aim of the study is to follow-up the adherence to the standard guidelines for the prescription of oral antidiabetics (OADs) in Serbia.
Methods: The study examined consumption of OADs in 2013. The data were retrieved from the annual reports of the Agency for Drugs and Medical Devices of the Republic of Serbia. Consumption was calculated using the ATC/DDD methodology and results were expressed in DDD/1000 inhabitants/day (DDDs/TID).
Results: The total consumption of OADs was 79.97 DDDs/TID. Sulphonylureas were the most frequently used class of OADs during the examined year (35.32 DDDs/TID) and among them glimepiride was the most frequently used drug with 20.26 DDDs/TID. Biguanides were the next frequently used class, represented only by metformin (30.85 DDDs/TID). The use of thiazolidinediones, DPP-4 inhibitors, meglitinides as well as acarbose remained marginal.
Discussion: Diabetologists and clinical pharmacologists should explain causes leading to the higher consumption of sulphonylureas than metformin, which is a preferred OAD according to the standard guidelines, in order to enable the optimal utilization of OADs in Serbia.
Acknowledgements: This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia, project no. 41012.

A8.4

Use of drugs for the treatment of diabetes mellitus in the Republic of Serbia

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Background: Diabetes mellitus is one of the leading chronic non-communicable diseases and in Serbia is the fifth leading cause of death. According to the latest classification of WHO, modified for the needs of the national guide for diabetes Republic of Serbia, there are four main groups of diabetes mellitus. The aim of this study was to analyze the consumption of serum antidiabetic drugs used in diabetes mellitus therapy in Serbia from 2007 to 2012, and to compare these data with Norway and Finland, countries with developed pharmacotherapeutic practice.
Methods: The data about the use of antidiabetic drugs in Serbia from 2007 to 2012 were taken from the Agency for Drugs and Medical Devices of the Republic of Serbia, for Norway they were taken from the official website of the Norwegian Institute for Public Health and for the use of antidiabetic drugs in Finland they were taken from the official webisite of the Agency for Drugs of Finland.
Results: In Serbia the use of antidiabetics is continuously increasing. The most commonly prescribed drugs are oral antidiabetic drugs and sulfonylurea derivatives, while Norway and Finland record the highest consumption of a biguanide and the next on the list are sulfonylurea derivatives. Sulfonylurea derivatives are the most frequently used drugs for the treatment of diabetes mellitus in Serbia (2007: 20.5 DDD; 2008: 38.1 DDD; 2009: 25.9 DDD; 2010: 15.7 DDD; 2011: 10.1 DDD; 2012: 34.1 DDD) as compared to Norway and Finland. The use of these drugs is increasing in Serbia. On the other hand, the use of biguanides in Serbia (2007: 12.5 DDD; 2008: 12.5 DDD; 2009: 15.0 DDD; 2010: 19.1 DDD; 2011: 22.2 DDD; 2012: 26.6 DDD) is significantly lower as compared to Norway and Finland.
Discussion: Analyzing the consumption of antidiabetic drugs in Serbia, Norway and Finland in the period from 2007 to 2012, Serbia is a country between Norway and Finland. Norway shows a uniform consumption while Finland shows a progressive increase in the consumption of antidiabetic drugs.
Acknowledgements: This research was supported by the Provincial Secretariat for Science and Technological Development of the Autonomous Province of Vojvodina (project no. 114-451-2458/2011)

and by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project no. 41012).

A8.5

Prescription of flunitrazepam in Austria, 2006–2014

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Background: Flunitrazepam is an intermediate acting benzodiazepine that causes strong anterograde amnesia. It has seen a long history of abuse, as a date-raping drug in the 1980s and as a combination to illicit opioids and amphetamines to ensure a "soft landing" after the "high". Authorities in many countries have tried to overcome the illicit use of flunitrazepam. Since December 2012, the prescription of flunitrazepam requires schedule II standards (*Suchtgiftrezept*) in Austria.
Methods: Data from all prescriptions filled in Austrian pharmacies on public expense by outpatients (2006–2014) were obtained from the Federation of Austrian Social Insurance Institutions (*Hauptverband der österreichischen Sozialversicherungsträger*) and were analyzed for precriptions of flunitrazepam using the WHO drug statistic methologies.
Results: After a steady rise to 5.32 million daily doses in 2011, the number of daily doses filled after the introductions of restricions on prescribing has significantly decreased to 2.06 million. These figures demonstrate the efficacy of the measures by the authorities.
Discussion: The dramatic fall in flunitrazepam prescription does not only demonstrate the illicit use of flunitrazepam but also the willingness of physicians to prescribe a potential drug of abuse without due diligence. To further decrease flunitrazepam abuse, further measures by the authorities (such as closing "back doors") are required—many countries have banned flunitrazepam completely.

A8.6

Prescription and savings potential of RAAS inhibitors in Austria, 2012

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Background: Drugs acting on the renin–angiotensin–aldosterone system (RAAS) are the most frequently prescribed drugs in pharmacotherapy of the cardiovascular system: in 2012, 800 million dose equivalents worth € 190 million were prescribed on public expense in Austria. The variety of therapeutic principles and the variety of substances calls for in-depth analysis and evaluation of prescription data for compliance of the prescription practice with medical guidelines and pharmacoeconomic recommendations.
Methods: Data from all prescriptions filled in Austrian pharmacies on public expense by outpatients (2006–2012) were obtained from the Federation of Austrian Social Insurance Institutions (*Hauptverband der österreichischen Sozialversicherungsträger*) and analyzed for prescriptions of drugs acting on the RAAS using modified WHO drug statistic methologies. Savings potential calculations are based on recommendations and regulations of Austrian and German authorities.
Results: 490 million dose equivalents of ACE-Inhibitors (ACEIs) worth € 79.2 millions and 310 million dose equivalents of angiotensin receptor blockers (ARBs) worth € 111 million were prescribed on public expense in 2012. In contrast to guideline recommendations, the majority of ACEIs and ARBs were prescribed as combinations (mainly with thiazide diuretics). Prescription rate for generics was 54.9% for ACEIs and 15.5% for ARBs. The calculated saving potential was € 110 million (57%).
Discussion: Prescriptions of ACEIs and ARBs only partially reflect the recommendations of guidelines and authorities; regulatory efforts to lower medication prices have shown a limited effect. Prescription rates of generics are relatively low, prescription rates of "me-too" substances and expensive combinations are high. The enormous savings potential calls for optimization of medical prescription practice and prescription regulations by the authorities.

A8.7

Nalmefene and concomitant opioid therapy: a systematic analysis of the global WHO pharmacovigilance database

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Intrinsic Activity, 2015; **3**(Suppl. 2): A8.7
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Background: Nalmefene (Selincro®) is a selective opioid receptor antagonist, which was licensed in February 2013 in Europe and in April 2014 in Switzerland for the reduction of alcohol consumption in adults with a high drinking risk level.
Methods: 200 reports of adverse drug reactions of nalmefene have been documented worldwide in the global WHO pharmacovigilance database. Reports were analysed regarding concomitant opioids therapy.
Results: The majority of patients (105; 53%) was aged between 45 and 64 years, 135 patients (68%) were male. In 21 cases (10.5%) nalmefene and an opioid were administered concomitantly. In 13 patients (69%) nalmefene was combined with methadone, in 1 with morphine, in 1 with fentanyl, in 2 with buprenorphine, in 2 with codeine and in 2 with oxycodone. Only 3 patients, who had any of these combinations were female (14%), the median age was 44 years (min. 28, max. 66). In 15 cases the terms "opiate withdrawal symptoms", "withdrawal syndrome" or "drug withdrawal syndrome" were coded. Symptoms included tachycardia, agitation, diarrhoea, abdominal pain. Until now, the regional pharmacovigilance center in Zurich received 4 cases of nalmefene combined with opioids.
Discussion: The combination of nalmefene with opioids should be avoided as this interaction may cause withdrawal symptoms by competitive binding at the opioid receptors. A detailed medical history or a toxicological screening is recommended.

Education and Training

A9.1

Interprofessional pharmacology workshop: intervention to close the gap between physicians' and pharmacists' attitudes toward their mutual collaboration

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Background: The aging societies, the increasing number of new drugs on the market and widespread consumption of OTC drugs are leading to a higher incidence of drug interactions and reduced compliance of patients. Better collaboration between health-care professionals has been recognized as both a reasonable and effective strategy in reducing this unwanted process. Previous studies have shown that physicians have a significantly less positive attitude toward interdisciplinary collaboration than pharmacists. Therefore, the aim of the present study was to close the gap between physicians' and pharmacists' attitudes toward their mutual collaboration by organizing an interprofessional pharmacology workshop.
Methods: The three-hour workshop was organized at the University of Dubrovnik as a form of a continuous, lifelong learning workshop. Participants were physicians (n = 18) and pharmacists (n = 23). Three complex clinical cases were presented to health-care professionals during the workshop: hypertension, asthma and metabolic syndrome. Each participant had to identify drug-related problems (DRP) and suggest the changes of pharmacotherapy and lifestyle in order to achieve the desired therapeutic goal for patients described in the clinical cases. There were three groups of information about each clinical case: (i) general information that was available to all participants, (ii) specific information available only to physicians (clinical guidelines, physiological measurements, laboratory values, etc.), and (iii) specific information available only to pharmacists (OTC and phytomedicine intake, drug compliance, lifestyle, etc.). Participants were not allowed to exchange their specific information in the first case. After they solved the first case independently, they realized that limitations of available information, due to lack of interprofessional collaboration resulted in limited identification of DRPs and misjudged actions for achieving the therapeutic goal. Therefore, participants spontaneously engaged to collaborate in order to detect all DRPs and to achieve the therapeutic goal for the other two patients. To determine attitudes toward collaboration, participants had to complete a validated questionnaire ("Scale of Attitudes Toward Collaboration Between Pharmacists and Physicians", SATCP^2) at the beginning and at the end of the workshop. The total SATCP^2 score (TS) was calculated and data are expressed as mean ± SD. The data were analyzed by non-parametric statistical tests and the results were considered statistically significant at $p < 0.05$.
Results: Pharmacists showed a more positive attitude toward collaboration than physicians before the workshop (52.1 ± 4.1 *vs.* 48.3 ± 3.9). However, the attitude of physicians increased significantly after the workshop (52.1 ± 6.1 *vs.* 48.3 ± 3.9) and reached the values of pharmacists' attitude after the workshop (52.4 ± 5.2).
Discussion: The interprofessional pharmacology workshop successfully closed the gap between physicians' and pharmacists' attitudes toward their mutual collaboration. It seems that interprofessional workshops represent an efficient approach in promoting collaboration between health care professionals.

A9.2

Interprofessional students' pharmacology workshop: intervention to improve health profession students' attitudes toward physician–pharmacist collaboration

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Background: The rapid advancement in pharmacotherapy concomitantly increases the possibility of medical errors and leads to the increase of total costs of health care. Collaboration between physicians and pharmacists is recognized as an important factor for reducing medical errors and improving patient outcomes. Previous studies have shown that medical students have significantly less positive attitude toward interdisciplinary collaboration than pharmacy students. Therefore, the aim of the study was to increase the attitude in medical and pharmacy students toward collaboration between pharmacists and physicians by organizing an interprofessional students' pharmacology workshop.
Methods: The three-hour workshop was organized at the University of Split School of Medicine. Participants were medical (n = 42; 4th–6th year) and pharmacy students (n = 38; 4th–5th year). Inclusion criteria for participation in the workshop were completed courses of internal medicine and pharmacology for medical students, and general and specialized pharmacology for pharmacy students. Three complex clinical cases were presented to students during the workshop: hypertension, asthma and metabolic syndrome. Each student had to identify drug related problems (DRP) and suggest the changes of pharmacotherapy and lifestyle in order to achieve the desired therapeutic goal for patients described in the clinical cases. There were three groups of information about each clinical case: (i) general information that was available to all students, (ii) specific information available only to medical students (clinical guidelines, physiological measurements, laboratory values, etc.), and (iii) specific information available only to pharmacy students (OTC and phytomedicine intake, drug compliance, lifestyle, etc.). Medical and pharmacy students were not allowed to exchange their specific information in the first case. After they solved the first case independently, they realized that limitations of available information, due to lack of interprofessional collaboration resulted in limited identification of DRPs and misjudged actions for achieving the therapeutic goal. Therefore, participants spontaneously engaged to collaborate in order to detect all DRPs and to achieve the therapeutic goal for the other two patients. To determine attitudes toward collaboration, students had to complete a validated questionnaire ("Scale of Attitudes Toward Collaboration Between Pharmacists and Physicians", SATCP^2; [1]) at the beginning and at the end of the workshop. The total SATCP^2 score (TS) was calculated and data are expressed as mean ± SD. The data were analyzed by non-parametric statistical tests and the results were considered statistically significant at $p < 0.05$.
Results: Pharmacy students showed a more positive attitude toward collaboration than medical students, both before (58.8 ± 3.7 *vs.* 48.1 ± 7.3) and after (60.1 ± 4.0 *vs.* 52.9 ± 8.4) the workshop. However, there was a statistically significant increase of TS in both groups after the workshop (+1.3% *vs.* +2.2% for pharmacy students and +4.8% *vs.* +10% for medical students, as relative change from baseline value). Gender did not influence the results in any group.
Discussion: The interprofessional students' pharmacology workshop significantly improved attitudes toward collaboration between physicians and pharmacists in both students' groups, with more marked changes observed in medical students.

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A9.3

Innovative education and training: a major step forward for Europe through the imi-train initiative

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Intrinsic Activity, 2015; **3**(Suppl. 2): A9.3
http://www.intrinsicactivity.org/2015/3/S2/A9.3

Innovative education and training: a major step forward for Europe through the imi-train initiative

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Background: Well-trained scientists and professionals can only stay at the leading edge of developments in their fields through continuing professional development (CPD). To safeguard Europe's global competitiveness in medicines research a strong environment for innovative education and training is an imperative which must aim at developing and maintaining competence and competencies. imi-train [1], through its education and training sub-projects, has taken up this challenge and developed solutions related to these goals.

Methods: imi-train is a European partnership between EMTRAIN [2], Eu2P [3], PharmaTrain [4], SafeSciMET [5] and the associated EUPATI [6] education and training programmes funded by the Innovative Medicines Initiative (IMI) [7]. Together they have tackled a series of gaps and issues in the European postgraduate education and training arena: on-course® [8], a searchable online course catalogue, was developed to display Europe's Master, PhD and CPD programmes in the biomedical sector [9]. LifeTrain [10] was initiated as an open community with a unifying goal: driving lifelong learning for biomedical professionals [11]. As a service to course providers, quality standards have been established [12] and a repository of teaching methodologies was compiled which describes their pros and cons (to be made available shortly). In the area of PhD training, a framework for public–private partnership PhD programmes was conceived and a four-day PhD workshop was designed, aiming at creating industry awareness among PhDs. New course programmes have been developed in the areas of pharmacovigilance and pharmacoepidemiology (Eu2P) [13], medicines development sciences (PharmaTrain), safety sciences (SafeSciMET) and training for patients (EUPATI): all are run in public–private partnership, focus on personalised, innovative teaching approaches, apply modular structures combined with e-learning components and comply with the defined IMI quality standards. They support defined competency profiles thus focussing on competence rather than on pure knowledge acquisition; including implementation of the Specialist in Medicines Development.

Results: on-course® currently contains around 7000 programmes and is steadily growing. LifeTrain, through four ground-breaking workshops, have brought together the major stakeholder groups: course providers, professional/scientific bodies, employers, and individuals, for an intensive dialogue. More and more competency profiles are being developed and implemented, including most recently the core competencies for pharmaceutical physicians and drug development scientists of the PharmaTrain Federation [14] or the European Certified Pharmacologist (EuCP) programme of EPHAR and EACPT [15]. These and more than 15 other best practice examples have been presented at the 4th LifeTrain Workshop [16]. Around 80 organisations have formally signed up as signatories of LifeTrain, the majority being professional bodies.

Discussion: Despite the numerous ground-breaking achievements, there is still a long way to go. Europe requires a radical change of the post-graduate/professional educational system to foster lifelong learning. There is a need for a researcher training infrastructure similar to the ESFRI Research Infrastructures. imi-train has taken the first major step in that direction.

Acknowledgements: The authors acknowledge the support by the Innovative Medicines Initiative and the imi-train Project Consortia.

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A9.4

on-course®: a major upgrade of this course catalogue enhances its service to the biomedical community

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Intrinsic Activity, 2015; **3**(Suppl. 2): A9.4
http://www.intrinsicactivity.org/2015/3/S2/A9.4

Background: on-course® is the most comprehensive postgraduate biomedical course database in Europe [1]. Launched in February 2012, it has rapidly grown in size and functionalities since then. Based on feedback from the user base and in order to make use of newest technologies, on-course® was re-launched in October 2014 and presents a number of new and/or improved functionalities.

Methods: Since 2012, feedback from users has continuously been collected and analysed. In parallel, the on-course® curators team has developed many new ideas for additional functionalities and services, but has also faced limitations in handling the fast growing amount of data most effectively. Thirdly, IT experts provided advice regarding new technologies and solutions relevant to on-course®. These sources of information have been used to define the enhancements of the newly launched on-course® resource.

Results: on-course® was re-developed using the Django open-source content management system. This allows the on-course® team more independence from IT programmers and higher flexibility to react more quickly and effectively to customers' demands and habits. It also creates more visibility on internet search engines. The look-and-feel of on-course® has been improved based on user feedback and observations of user behaviours. Course seekers are now offered a 'google-like' free-text search functionality which, combined with the advanced search filters, increases the relevance of search results. The new bookmarking function allows users to add courses into comparison lists. Registered users can define their search preferences in their user profiles for repeated use. The amount and types of data fields have been adapted to a structure [2] which in future will allow automated data feeds from course providers' data bases more easily; pilots will be launched shortly to build reference cases. Course providers benefit from a simpler and better guided data entry and editing system. Course providers will soon be offered guidance for their choice of the right teaching methodology. This resource includes a learning-style quiz as well as a repository of existing teaching methodologies which aims to optimise selection. The on-course® platform now also provides more background information to users including statistics, relevant publications, graphs, information about gaps and trends and other facts and figures relevant to biomedical education and training [3]. In the back-end, the 'course management system' has been improved. The on-course® curators can run effective queries to monitor the status of course information. This will further foster the quality of the data. New functionalities have also been implemented in support of the research on on-course® data. This will allow more effective screening, analyses and interpretation of data with regard to trends, gaps, and other relevant findings.

Discussion: The IT world is developing rapidly. Thus on-course® will also continue to develop to make use of newest technologies which appear on the market daily. The next steps include real-time visualisations of statistics and trends as well as the linkage between courses and competency profiles. The popularity of on-course® is growing rapidly with Google analytics showing the number of on-course® users and visits doubling since November 2014.

Acknowledgements: The authors acknowledge the support by the Innovative Medicines Initiative and the EMTRAIN Consortium.

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A9.5

The EPHAR European Certified Pharmacologists (EuCP) programme: an update

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Intrinsic Activity, 2015; **3**(Suppl. 2): A9.5
http://www.intrinsicactivity.org/2015/3/S2/A9.5

Background: EPHAR has initiated the European Certified Pharmacologists (EuCP) Programme to identify experts in the field of pharmacology whose competency profile, in addition to their personal specialised scientific expertise, covers expert knowledge in all major fields and who have experiences and practical awareness in a wide spectrum of pharmacological techniques. Since the formal launch of the programme (July 2014), seventeen EPHAR member societies have declared their active participation in the EuCP programme.

Methods: The member societies were called to set up transparent rules for their national EuCP schemes which must meet all requirements of the EuCP Guidelines; these rules must consist of a clear catalogue of criteria with respect to knowledge, practical awareness and skills, as well as general rules including rules for final assessment of candidates. Before a national pharmacological society can begin to certify individuals as qualified for EuCP certification, the national EuCP scheme must be accredited by the EuCP Committee.

Results: As of the time of this meeting, three member societies (France, Austria, the Netherlands) have submitted national EuCP schemes. The programmes differ in structure and reflect the flexibility of the EuCP Programme with respect to the respective national conditions. The Italian programme is based on a catalogue of criteria, which have to be certified by individual applicants; the Austrian programme is based on a legally regulated medical specialisation in pharmacology and toxicology (guidelines for non-medical pharmacologists will follow); the Dutch programme describes rules for training of pharmacologists who shall be certified as EuCPs.

Discussion: Thus, these first submitted national programmes can also serve as 'case studies' for other participating member societies to develop their national EuCP rules. All three programmes are currently under evaluation by the EuCP Committee and, once approved, will be published on the EuCP website [1].

Reference

1. http://www.eucp-certification.org

Author Index (Numbers refer to abstract no.)

Intrinsic Activity

www.intrinsicactivity.org

An Online Open-Access Publication of the Austrian Pharmacological Society (APHAR)