Background: The G protein-coupled receptor 55 (GPR55) has recently been suggested to target smoking and has been marketed to induce weight loss and reduce smoking. However, due to severe side effects after prolonged use, rimonabant (SR141716A), which until that date was known to be an inverse agonist/antagonist on the CB1 receptor-associated sorting protein 1 (GASP-1) levels were induced by high K+ and G1 (0.01 – 100 µM) produced a concentration-dependent activation of adenylate cyclase and downstream signaling. This work provides tangible evidence that GPR55 is degraded after prolonged agonist stimulation and this mechanism is regulated by the G protein-coupled receptor-associated sorting protein 1.

Methods: All experiments were performed using Human Embryonic Kidney (HEK293) cells and HEK293 cells stably expressing FLAG-tagged GPR55. Knock-down of endogenous GASP-1 levels were induced by infection with Lenti-shGASP-1 (shGASP-1) or Lenti-shScrambled (shScr) virus. The post-endocytic trafficking of GPR55 and its regulation by GASP-1 was elucidated by means of immunocytochemistry and biotinylation degradation experiments.

Results: By a variety of approaches, we demonstrated that GPR55 directly interacts with GASP-1 and is targeted to the degradative pathway via GASP-1 in a recombinant HEK293 cell model. For instance, knockdown of endogenous GASP-1 in HEK293 cells using siRNA silencing changes the trafficking properties of GPR55.

Conclusions: This work provides tangible evidence that GPR55 is degraded after prolonged agonist stimulation and this mechanism is regulated by the G protein-coupled receptor-associated sorting protein 1.
Conclusions: Adenosine induced endothelium-independent relaxation of carotid artery irrelevant of artery occlusion. Conversely, it appears that in a pathological setting with ischemia the signal mechanism of potassium channel activation is significantly reduced.

Acknowledgements: The Ministry of Science, Serbia, supported this research with grant 1450158.

A3
Resveratrol reduces myofibroblast arrhythmogenicity
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BMC Pharmacology 2010, 10(Suppl 1):A3

Background: Among grape skin polyphenols, trans-resveratrol (RES) has been reported to slow the development of cardiac fibrosis and to affect myofibroblast (MFB) differentiation. Because MFBs induce slow conduction and ectopic activity following heterocellular gap junctional coupling to cardiomyocytes, we investigated whether RES and its main metabolites affect arrhythmogenic cardiomyocyte-MFB interactions.

Methods: Experiments were performed with patterned growth strands of neonatal rat ventricular cardiomyocytes coated with cardiac MFBs. Impulse propagation characteristics were measured optically using voltage-sensitive dyes. Long-term video recordings served to characterize drug-related effects on ectopic activity. Data are given as means ± S.D. (n = 4–20).

Results: Exposure of pure cardiomyocyte strands to RES at concentrations up to 10 µmol/L had no significant effects on impulse conduction velocity (V) and maximal action potential upstroke velocities (dV/dtmax). By contrast, in MFB-coated strands exhibiting slow conduction, RES enhanced V with an EC50 of ~10 nmol/L from 226 ± 38 to 344 ± 24 mm/s and dV/dtmax from 48 ± 7 to 69 ± 2 APA/ms, i.e., to values of pure cardiomyocyte strands (347 ± 33 mm/s; 75 ± 4 APA/ms). Moreover, RES led to a reduction of ectopic activity over the course of several hours in heterocellular preparations. RES is metabolized quickly in the body; therefore, we tested the main known metabolites for functional effects and found them similarly effective in normalizing conduction with EC50s of ~10 nmol/L (3-Ο-RES), ~20 mmol/L (RES-3-O-β-glucuronide) and ~10 mmol/L (RES-sulfate), respectively. At these concentrations, neither RES nor its metabolites had any effects on MFB morphology and α-smooth muscle actin expression. This suggests that the antiarrhythmic effects observed were based on mechanisms different from a change in MFB phenotype.

Conclusions: The results demonstrate that RES counteracts MFB-dependent arrhythmogenic slow conduction and ectopic activity at physiologically relevant concentrations. Because RES is rapidly metabolized following intestinal absorption, the finding of equal antiarrhythmic effectiveness of the main RES metabolites warrants their inclusion in future studies of potentially beneficial effects of these substances on the heart.

A4
A ligand-based 3D pharmacophore model for the µ opioid receptor: application to the morphinan class of opioids
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BMC Pharmacology 2010, 10(Suppl 1):A4

Background: Opioid receptors belong to the rhodopsin subclass within the superfamily of G protein-coupled receptors (GPCR), which are characterized by the presence of seven transmembrane (7TM) helices. They interact with morphine and related opioid alkaloids as well as with endogenous opioid peptides. There are three main types of opioid receptors (µ, δ, κ), which are differently implicated in opioid function. Ligands specifically targeting each opioid receptor type are of high interest both as research tools and potential therapeutic agents. Activation of the µ opioid receptor produces many other effects, besides its main involvement in pain control, including immunomodulation, respiratory depression, constipation, tolerance and physical dependence. With the lack of an experimental 3D structure of the µ opioid receptor, discovery of 3D pharmacophores for the receptor that can explain the activity of a series of ligands represents an important approach in drug discovery.

Materials and methods: A new ligand-based pharmacophore model for the µ opioid receptor was generated using the LigandScout program. A database consisting of morphinan derivatives was generated to provide the basis for virtual screening and validation studies. Opioid receptor binding activity data earlier published by our group were included in this in-house opioid library and a general structure-activity relationship for morphinan compounds was established.

Results: A merged feature ligand-based pharmacophore model for the µ opioid receptor was generated using a highly-active training set of morphinans (in-house opioid library). The model was optimized, validated and was shown to be able to identify highly active µ opioid ligands within a certain range, and excellent enrichments were achieved. The pharmacophore model indicated that the important features for the binding activity with the µ receptor are the presence of at least three hydrogen bond acceptors, one aromatic ring and one positive ionisable feature.

Conclusions: The availability of the present pharmacophore model is expected to provide a more rational hypothetical picture of the primary chemical features responsible for activity, to be a valuable tool for 3D virtual screening and thus to facilitate the design of novel active candidates targeting the µ opioid receptor.

Acknowledgements: Supported by the Austrian Research Fund FWF: P21350 and TRP 16-818.

A5
Enhanced fear expression in a psychopathological mouse model of trait anxiety: pharmacological interventions
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BMC Pharmacology 2010, 10(Suppl 1):A5

Background: The propensity to develop an anxiety disorder is thought to be determined by genetic and environmental factors. Here we investigated the relationship between an extreme genetic predisposition to trait anxiety and anxiety-related behaviour on the elevated plus maze.

Methods: Male CD-1 mice selectively bred for either high (HAB) or normal (NAB) anxiety-related behaviour on the elevated plus maze were subjected to classical fear conditioning.

Results: Both mouse lines learned to fear an initially neutral stimulus (CS) being indicated by increasing freezing levels. 24 h later, HAB mice displayed more pronounced freezing responses to both the context and cue CS compared with NAB mice, suggesting that trait anxiety determines stronger fear memory and/or a weaker ability to inhibit fear responses in the HAB line. Interestingly, already 1 h and 6 h after fear conditioning, freezing levels were high in HAB mice but not in NAB mice. The enhanced fear response of HAB mice was attenuated by treatment with either the a2,3,5-subunit-selective benzodiazepine partial agonist L-836,417, corticosterone or the selective neurokinin-1 receptor antagonist L-822,429.

Conclusions: Overall, the HAB mouse line may represent an interesting model (i) for identifying biological factors underlying misguided conditioned fear responses and (ii) for studying novel pharmacotherapies for patients with anxiety disorders, including post-traumatic stress disorder and phobias.

Acknowledgements: Supported by the Austrian Science Fund FWF NFN-S-102 (NS).
A6
The interplay of excitatory and inhibitory coupling modes is crucial for the regulation of neuronal electrical activities by L-type calcium channels
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BMC Pharmacology 2010, 10(Suppl 1):A6

Background: Neuronal L-type voltage-gated calcium channels (LTCCs) have long been implicated in the regulation of excitability. This function appears to be related to the coupling of LTCC-mediated Ca2+ influx to Ca2+-dependent conductances, such as Kca channels, e.g. Kv2.x (SK), and nonspecific cation (CAN) channels. However, despite numerous data related to the molecular functioning of LTCCs, little is known about the actual role of these channels in cellular electrical excitation. In this study, we examined how activation of LTCCs affects neuronal depolarizations and analyzed the contribution of Ca2+-dependent potassium and cation conductances.

Methods: Using hippocampal neurons in primary culture, pulse current injections were applied in the presence of TTX for stepwise depolarization, and the availability of LTCCs was modulated by Bay K8644 and isradipine.

Results: Varying pulse length and current strength, we found that weak depolarizing stimuli tend to be enhanced by LTCC activation, whereas in the course of stronger depolarizations LTCCs counteract excitation. Both effect modes appear to involve the same channels that mediate afterdepolarizations (ADPs) and afterhyperpolarizations (AHPs), respectively. Indeed, ADPs were activated at lower stimulation levels than AHPs. In the absence of TTX, activation of LTCCs prolonged or shortened burst firing, depending on the initial burst duration, and invariably augmented brief unprovoked (such as excitatory postsynaptic potentials) and provoked electrical events.

Conclusions: Hence, instantaneous regulation of membrane excitability by LTCCs involves activity of both excitatory and inhibitory Ca2+-activated ion channels. The overall enhancing or dampening effect of LTCC stimulation on excitability does not only depend on the presence of the respective coupling partner, but also on the stimulus intensity. These findings might have important implications for the usability of LTCC inhibitors in the treatment of various forms of abnormal neuronal electrical activities.

Acknowledgements: Supported by the FWF Austrian Science Fund (P19710).

A7
Cross-talk of PGD2 receptors: the DP receptor modulates signaling and trafficking of CRTH2
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BMC Pharmacology 2010, 10(Suppl 1):A7

Background: Prostaglandin (PG) D2 is substantially involved in allergic inflammation and signals via the seven transmembrane (7TM) spanning/G protein-coupled receptors (GPCRs) chemoattractant receptor homologous molecule expressed on TH2 cells (CRTH2) and D-type prostaglandin (DP) receptor. Both receptors are co-expressed in eosinophils among other immune cells and have emerged as therapeutic targets in allergic diseases. While a proinflammatory function of the CRTH2 receptor is well recognized, the role of the DP receptor in allergic inflammation, however, remains unclear. As it has been shown for many other 7TM/GPCRs, we recognized, the role of the DP receptor in allergic inflammation, however, which seems to be unlikely from its structure. Because the lipophilic thymoquinone exhibits a structural similarity with the natural mitochondrial electron carrier, ubiquinone, it was of interest whether the suggested antioxidant effect of thymoquinone in cells can be explained by its interaction with the mitochondrial respiratory chain.

Materials and methods: Intracellular Ca2+ release in HEK293 cells stably expressing CRTH2 (HEK-CRTH2), DP (HEK-DP) or both receptors (HEK-CRTH2 +DP) was examined by Flow Excitation (FLEX). Ca2+ flux in human eosinophils was measured by FLEX and flow cytometry. Agonist-induced receptor internalization was determined by confocal imaging and flow cytometry. Receptor interaction studies were performed by co-immunoprecipitation.

Results: The DP receptor is dominantly involved in mediating Ca2+ mobilization following CRTH2 activation. Agonist-induced DP receptor desensitization, which is a rapid mechanism to shut down cellular responses, also blocked CRTH2 signaling. Binding of the DP antagonist to its receptor abolished the cross-activation of the DP receptor by CRTH2. Co-immunoprecipitation studies revealed the formation of CRTH2/DP heteromers which might explain the pharmacological interaction between CRTH2 and DP receptors.

Conclusions: For the first time we show that the DP receptor seems to function as an interface molecule which translates the activation of the CRTH2 receptor to intracellular signal transduction pathways.

A8
Signalling and function of the human G protein-coupled receptor 55
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BMC Pharmacology 2010, 10(Suppl 1):A8

Background: We have recently shown that the G protein-coupled receptor 55 (GPR55) responds to some of the cannabinoid and non-cannabinoid ligands in addition to the classical cannabinoid 1 (CB1) and 2 (CB2) receptors. Here we show multiple signaling pathways triggered by GPR55 in response to its agonists. In addition the cytoskeleton rearrangement mediated by GPR55 is investigated.

Materials and methods: HEK-293 cells stably expressing the human GPR55 receptor were characterized in terms of signaling properties. To this end, reporter gene, dynamic mass redistribution (DMR), mitogen-activated protein kinases (MAPK) activation and phallolidin actin staining assays have been performed.

Results: Here we show that GPR55 is activated by lysophosphatidylcholine (LPC), AM251, SR141716A (rimonabant) and AM281. GPR55 activation induces NF-κB, NFAF and CREB activation. Stimulation of GPR55 induces F-actin formation under the control of Gx13, RhoA and ROCK. We also show the suitability of Corning® Epic® DMR assay for GPR55 ligand screening. Furthermore, GPR55 activation leads to phosphorylation of extracellular signal-regulated kinase 1/2 (ERK 1/2).

Conclusions: GPR55 as the novel cannabinoid receptor triggers distinct signaling pathways in response to LPC and some classical CB1 receptor inverse agonists/antagonists. Stress fiber formation mediated by GPR55 might indicate the probable function of this receptor in vivo.

A9
Is thymoquinone an antioxidant?
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BMC Pharmacology 2010, 10(Suppl 1):A9

Background: Thymoquinone is one of the active ingredients of black cumin (Nigella sativa L.) essential oil possessing anti-inflammatory, antineoplastic, neuro- and hepatoprotective properties. Some of these properties were attributed to an antioxidant effect of thymoquinone, which seems to be unlikely from its structure. Because the lipophilic thymoquinone exhibits a structural similarity with the natural mitochondrial electron carrier, ubiquinone, it was of interest whether the suggested antioxidant effect of thymoquinone in cells can be explained by its interaction with the mitochondrial respiratory chain.

Materials and methods: Antioxidant activities were determined spectrophotometrically by means of the 2,2-diphenyl-1-picrylhydrazyl-radical (DPPH) assay (516 nm) as well as by the O2−-dependent xanthine / xanthine oxidase / cytochrome c assay (550 nm). NADH and
succinate/thymoquinone oxidoreductase activities of KCN-blocked (1 mM) submitochondrial particles (0.01 mg/ml) from bovine heart were determined at 340 and 257.5 nm, respectively.

Results: With the DPPH assay thymoquinone was shown to be hardly antioxidant. In contrast, thymohydroquinone was even more active than the vitamin E analog pentamethylichromanol (rate constants for the reaction with DPPH: 283.7 ± 1.9 M⁻¹ s⁻¹ (3) vs. 236.2 ± 4.7 M⁻¹ s⁻¹ (6); data are means ± SEM (n). The cytochrome c system turned out to be unsuitable for the evaluation of the antioxidant activity of thymoquinone. Thymoquinone concentration-dependently (20–1000 µM) stimulated the NADH (150 µM) oxidation of submitochondrial particles to V₅₅₅ values of around 400 nmol per min per mg protein, K₅₀ values were 77.4 µM for thymoquinone and 6.4 µM for NADH. The thymoquinone-stimulated NADH oxidation was sensitive to inhibitors of the mitochondrial electron transfer (90% inhibition with 0.2 µM rotenone, 49% inhibition with 1 µM antimycin A, 82% inhibition with 1 µM antimycin A + 1 µM myxothiazol; 100 µM thymoquinone). In addition to its stimulatory effect on NADH: thymoquinone oxidoreductase, thymoquinone (50 µM) was reduced to its hydroquinone by rotenone- (0.2 µM) and KCN-inhibited submitochondrial particles when succinate (10 mM) was used as substrate. This reduction was sensitive to antimycin A and myxothiazol, inhibitors of mitochondrial complex III.

Conclusions: Thymoquinone (oxidized form) possesses a very low antioxidant activity while its reduced form (thymohydroquinone) exerts a high radical-scavenging capacity, comparable to that of pentamethylichromanol, a short-chain tocopherol analog. We assume that the mitochondrial respiratory chain is significant for the antioxidant properties of thymoquinone in the cell by converting the administered thymoquinone into its hydroquinone.

A10
Toxicity of ascaridole from Chenopodium ambrosioides in mammalian mitochondria
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BMC Pharmacology 2010, 10(Suppl 1):A10

Background: Chenopodium ambrosioides has been used in traditional American medicine against parasitic diseases. Its essential oil (EO) is still used to treat leishmaniasis although it exhibits toxic effects in mammalian cells. Therefore, we studied the toxic mechanism of EO and its major pure ingredients (carvacrol, carvophyllene oxide and ascaridole) in mammalian cells and mitochondria.

Methods: Ascaridole was synthesized from alpha-terpinene and characterized by NMR and IR spectroscopy. The toxic effects of these compounds on macrophages from BALB/c mice and on the bioenergetics of submitochondrial particles from bovine heart (SMP) and rat liver mitochondria (RLM) were studied. Toxic radical intermediates arising from the endoperoxide ascaridole were characterized by ESR spectroscopy.

Results: The MTT assay, which relies on mitochondrial function, revealed that carvacrol (IC₅₀ = 9.7 ± 4 µM) and ascaridole (IC₅₀ = 32 ± 8 µM) inhibited the survival of peritoneal macrophages from BALB/c mice in vitro more than the EO. In SMP we observed that all products inhibited mitochondrial respiration stronger for complex I than for complex II substrates. Most active in this resp was carvophyllene oxide, which preferably inhibited the complex I activity (IC₅₀ = 92 ± 6 µM). The pure compounds were more inhibitory for oxidative phosphorylation in RLM than in EO. In the absence of Fe²⁺, ascaridole (IC₅₀ > 612 µM) was less toxic to RLM than other major ingredients. However, it was shown that Fe²⁺ potentiated the toxicity of EO and ascaridole on oxidative phosphorylation of RLM. Evidence for the formation of carbon-centered radicals in the presence of Fe²⁺ has been obtained by ESR/spin trapping. To explore the route of ascaridole activation different iron-containing proteins were tested by ESR/spin trapping. Neither reduced nor oxidized mitochondrial cytochrome c as well as oxidized hemin were able to cleave ascaridole significantly. However, reduced hemin efficiently produced carbon-centered radicals from ascaridole. Since detoxification of ascaridole by mammalian antioxidative enzymes is rather slow, hemin-mediated ascaridole cleavage contributes to its toxicity.

Conclusions: These data suggest that the toxicity of the essential oil from Chenopodium ambrosioides is partially related to the inhibition of the respiratory chain preferably by carvophyllene oxide while the toxicity of the antiparasitic agent ascaridole is dependent on the availability of reox-active iron.

A11
Concentrative ER export of the serotonin transporter relies exclusively on an interaction with Sec24C
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BMC Pharmacology 2010, 10(Suppl 1):A11

Background: The transporters for serotonin (SERT), dopamine (DAT) and noradrenaline (NET) form the monoamine neurotransmitter transporter family. They are primarily responsible for the termination of neurotransmission via rapid reuptake of neurotransmitters from the synaptic cleft. We have previously shown that the C-terminus of SERT plays a key role in trafficking and folding of the transporter. Mutations in this region of the protein (specifically at sites P261S and R270L) cause intracellular retention of SERT, hence abolishing substrate uptake and reducing inhibitor binding. In the current study, we explored isoform-specific interaction of COPII component Sec24 proteins with monoamine transporters, to study the mechanistic nature of their ER export.

Methods: Our initial studies involving mass spectrometry revealed that SERT directly interacts with Sec24C. To confirm these data, we subsequently used the siRNA approach to individually knock down the four mammalian Sec24 isoforms A, B, C or D in immortalised cervical cancer cells (HeLa). Forty-eight hours subsequent to siRNA transfections, the cells were transfected with YFP-tagged transporter plasms and substrate uptake assays were performed after an additional 24h.

Results: While gene silencing of Sec24A, B or D led to no changes in uptake, reduction of Sec24C significantly. However, reduced hemin efficiently produced carbon-centered radicals from ascaridole. Since detoxification of ascaridole by mammalian antioxidative enzymes is rather slow, hemin-mediated ascaridole cleavage contributes to its toxicity.

Conclusions: These data suggest that the toxicity of the essential oil from Chenopodium ambrosioides is partially related to the inhibition of the respiratory chain preferably by carvophyllene oxide while the toxicity of the antiparasitic agent ascaridole is dependent on the availability of reox-active iron.

A12
Long-term regulation of ATP-binding cassette transporters on mRNA and protein level by simvastatin in human rhabdomyosarcoma and neuroblastoma cells
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BMC Pharmacology 2010, 10(Suppl 1):A12

Background: Resistance against chemotherapeutics is still a major problem in cancer therapy. One of the mechanisms of multidrug resistance is up-regulation of ATP-binding cassette (ABC) transporters.
HMG-CoA reductase inhibitors have been shown to directly inhibit the main representative, ABCB1 (P-glycoprotein). We have previously shown in rhabdomyosarcoma (RD) and neuroblastoma (SH-SYSY) cells that simvastatin enhanced the intrinsic apoptotic potential of the anthracycline doxorubicin [1,2]. The aim of the study presented here is to investigate the long-term effect of simvastatin on the expression of ABC-transporters in order to evaluate an adjuvant therapeutic potential of statins in chemotheraphy.

Methods: FACS analysis was performed to quantify the accumulation of doxorubicin in RD cells in combinations with simvastatin or verapamil. Several ABC transporters were analyzed on mRNA and protein level using real-time PCR and Western blotting in human RD and SH-SYSY cells. The endogenous level of ABC transporters was also investigated from the livers of simvastatin treated mice.

Results: Here we show that simvastatin led to enhanced accumulation of doxorubicin in RD cells. This was comparable to the effect which was observed for the coadministration of verapamil, a first generation ABCB1 inhibitor, with doxorubicin. ABCB1 was monitored by Western blot analysis of RD and SH-SYSY cells upon continuous application of simvastatin. In a time and concentration-dependent manner the ABCB1 transporter was less expressed in both cell lines. Interestingly, real-time PCRs revealed a compensatory elevation of mRNAs for various ABC transporters (ABCB1, ABCG1, ABCB6 and ABCG2). Moreover, the augmented mRNA levels of ABCB1 do not result in more transporters on protein level. In vivo, the down-regulation of ABCB1 was confirmed in the liver of simvastatin-treated mice.

Conclusions: Based on our findings, we conclude that simvastatin is able to directly inhibit ABC transporters immediately, but also leads to a long-term down-regulation of various transporters. This feature makes simvastatin a promising candidate for adjuvant chemotherapy to impair transporter-mediated multidrug resistance.

Acknowledgements: This work was supported by the Herzfeldersche Familienstiftung and FWF (project P-22385).

References

A13 Interactions of peripheral and central µ opioid systems during emotional stress
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BMC Pharmacology 2010, 10(Suppl 1):A13

Background: Numerous studies support an important contribution of endogenous opioid peptide systems in the mechanisms of emotional behavior. It is well known that the structure of opioid receptors (OR) and endogenous opioid peptides in the CNS and in the periphery is identical, but the central and peripheral functions of endogenous opioid systems are considered different, because the blood-brain barrier (BBB) generally prevents the entry of peptides into the brain. We hypothesize that the central and peripheral components of the endogenous opioid system function in close relationship, interacting with each other. The aim of this work was to study an influence of a peripheral administration of the µ opioid receptor ligands, which do not penetrate the BBB, on behavioral parameters as well as on extracellular levels of l-endorphin (BE) in the cingulate cortex (CC) of rats during acute emotional stress.

Materials and methods: The behavioral parameters of male Wistar rats were estimated in the elevated plus maze (EPM) test and in the Porsolt forced swim test. Determination of BE in the CC of the midbrain of rats was performed using microdialysis technique with following immunohistochemical analysis. The agonist of µ opioid receptor agonist loperamide and the opioid receptor antagonist methylnaloxone were administered intragastrically using a special catheter 30 min before the experiments.

Results: Peripheral administration of the µ opioid receptor agonist loperamide produced mostly an anxiolytic effect, while a peripheral treatment with the antagonist methylnaloxone evoked a more depressive effect. The administration of loperamide and methylnaloxone produced opposite effects on the extracellular level of BE in the CC of rat brain. Thus, loperamide decreases, whereas methylnaloxone significantly increased output of BE from CC neurons of rats. Immobilization stress produced only slight elevation of BE release in CC. Peripheral administration of loperamide, but not methylnaloxone, significantly increased extracellular levels of the studied neuropeptide in the CC of rats subjected to immobilization stress.

Conclusions: These data support our hypothesis on reciprocal interactions between the central and peripheral components of the endogenous opioid system. The results may also explain the mechanism of anxiolytic effects of loperamide.

Acknowledgements: The work was supported by the RFBR grant no. 08-04-00780-a.

A14 Effects on cell proliferation and EPR spin trapping studies of Abnormal Savda Munziq and its chromatographic subfractions
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BMC Pharmacology 2010, 10(Suppl 1):A14

Background: Abnormal Savda Munziq (ASMq) is a herbal preparation of traditional Uighur medicine for the prevention or treatment of a variety of diseases (e.g. bowel cancer, diabetes, hypertension and others).

Methods: In addition to the beneficial therapeutic effects of ASMq we also expected possible toxic effects and therefore focussed our in vitro investigations on inhibition of cell proliferation and also investigated its free radical scavenging as well as generating properties using electron spin resonance spectroscopy (ESR). ASMq was tested as aqueous solution as well as different subfractions obtained by sequential extraction with different solvents, i.e. hexane, ethyl acetate, ethanol, methanol, methanol/water and finally water. Furthermore, the ethanolic fraction (containing the highest mass) was subsequently divided into chromatographic subfractions using semi-preparative HPLC. The whole aqueous and organic extracts as well as the ethanolic subfractions were subjected to determination of cell proliferative activity of murine hybridoma cells (YAC-1), which were treated with increasing concentrations of the respective extracts.

Results: We were able to demonstrate substantial effects of a set of subfractions of ASMq on cell proliferation with a significant correlation between decreasing proliferative activity and increasing lipophilic properties of the ASMq-subfractions, also correlating to an increased retention time on semi-preparative C-18 columns in our HPLC experiments. In our ESR experiments we observed significant antioxidant properties of the whole extract in spin trapping experiments using DEPMPO and a hydroxyl radical generating Fenton system. In addition, formation of a mixture of free radicals was observed under alkaline conditions suggesting the involvement of phenolic compounds. We were not able to identify the radicals involved; however, the polyphenolic compound gallic acid was identified by HPLC as a possible main active ingredient.

Conclusions: Our preliminary results show antioxidant properties of aqueous ASMq solutions. Free radicals are only detectable under alkaline conditions. Only the hydrophobic parts of ASMq inhibit cell proliferation.
A15
Molecular engineering of the TRPC3 pore structure identifies Ca\(^{2+}\) permeation through TRPC3 channels as a key determinant of cardiac calcineurin/NFAT signaling
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BMC Pharmacology 2010, 10(Suppl 1):A15

Background: TRPC channels have been identified as key players in cardiac remodeling and as crucial upstream components of NFAT signaling. The linkage between non-selective TRPC conductances and calcineurin/NFAT signaling may involve either direct TRC-mediated Ca\(^{2+}\) entry or indirect mechanisms involving crosstalk with other cardiac Ca\(^{2+}\) transport systems.

Methods: The pore structure of TRPC3 was analyzed by site-directed mutagenesis guided by a molecular modeling approach combined with patch-clamp measurements in the HEK293 expression system. TRPC3-mediated Ca\(^{2+}\) entry as well as NFAT translocation was investigated by fluorescence microscopy using Fura-2 and expression of a GFP-NFAT fusion protein in HEK293 as well as in HL1 cells.

Results: Elimination of Ca\(^{2+}\) permeation through TRPC3 abrogated its ability to trigger NFAT translocation in both HEK293 cells and in HL1 atrial myocytes. Wild-type TRPC3 was found capable of initiating NFAT translocation in atrial myocytes by a small, homogenous elevation of cytoplasmic Ca\(^{2+}\) that was independent of voltage-gated Ca\(^{2+}\)2 channels. By contrast, a Ca\(^{2+}\) impermeant TRPC3 mutant strongly promoted endothelin-induced Ca\(^{2+}\) signals in HL1 cells via enhanced activity of Ca\(^{2+}\)2 channels without concomitant NFAT translocation.

Conclusions: Our results demonstrate two strictly separated Ca\(^{2+}\) signaling functions of cardiac TRPC3 channels as well as a tight and efficient link between TRPC3-mediated Ca\(^{2+}\) permeation and calcineurin/NFAT signaling.

A16
Interaction of manganese with striatal dopamine turnover in human alpha-synuclein transgenic mice
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BMC Pharmacology 2010, 10(Suppl 1):A16

Background: It is thought that the interaction of genetic and environmental factors is an important risk factor for Parkinson’s disease (PD). α-Synuclein (α-syn) is a protein of special interest in PD because mutations in α-syn (A30T or A45E or A46K) lead to PD. Manganese (Mn) is a heavy metal known to cause parkinsonian symptoms. Therefore, we investigate the effect of manganese (Mn) on human α-syn expressing mice.

Materials and methods: C57/B16 mice expressing either human α-syn or the A30T/A30P doubly mutated human α-syn under the tyrosine hydroxylase promoter and nontransgenic sister mice were exposed at the age of 4 months to either MnCl\(_2\) (1%) enriched or control food. Locomotor activity was quantified every 2 months using automated activity chambers. Mice were sacrificed at the age of 7 or 20 months. Tyrosine hydroxylase positive cells in the substantia nigra pars compacta were quantified in a blinded manner. Neurochemical analysis of neurotransmitters and amino acids was performed in the striatum using high performance liquid chromatography.

Results: Mobility was increased by Mn, no significant difference due to the transgenes could be found. Striatal Mn content was significantly increased about threefold. Quantification of dopaminergic cells in the substantia nigra pars compacta showed a significant cell loss in aged mice (~10%) but no effect of Mn or transgenes (3-way ANOVA with factors gene, Mn and age). In 7 months old mice, neurochemical analysis showed interactions between transgene and Mn exposure for the ratio homovanillic acid : dopamine as well as aspartate (2-way ANOVA with factors gene and Mn). These values were increased in human α-syn expressing compared to non-transgenic mice which were control-fed (17 and 11%, respectively). There was no increase when animals obtained Mn-enriched food. Contrary, mutated α-syn-expressing mice showed an increase compared to non-transgenic and human α-syn-expressing mice only when they obtained Mn-enriched food. Analysis of the same parameters in the 20 months old mice did not reveal any significant changes.

Conclusions: Under our experimental conditions, Mn and α-syn, wild-type and doubly mutated, did not induce signs of neurodegeneration, neither separately nor in interaction. However, Mn interferes with the dopamine system through human α-syn: manganese exposure decreased DA turnover in the striatum of mice expressing human α-syn wild-type. This effect was lost by the two parkinsonism inducing mutations.

Acknowledgements: Supported by the Medical-Scientific fund of the Mayor of Vienna #2578 to C.P.

A17
Analogological target for antiplatelet therapy: the prostaglandin E\(_2\) receptor EP\(_4\)
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BMC Pharmacology 2010, 10(Suppl 1):A17

Background: Acute myocardial infarction is one of the leading causes of death in the world which is caused by coronary artery thrombosis. Platelets play a central role in cardiovascular thrombosis. Platelet aggregation caused due to a ruptured atherosclerotic plaque could eventually lead to vascular occlusion. Another important component of vascular diseases is inflammation. During inflammation, prostanoids (PG) like PGI\(_2\), PGE\(_2\) and PGG\(_2\) are released which are also involved in thrombosis. Lower concentrations of PGE\(_2\) enhance platelet aggregation whereas higher concentrations inhibit aggregation. PGE\(_2\) acts via 4 receptors: EP\(_1\), EP\(_2\), EP\(_3\) and EP\(_4\) (G\(_i\) signalling). The role of the EP\(_4\) receptor in enhancing platelet activation and aggregation has been looked at in detail but the role of the EP\(_4\) receptor is largely unknown. We were interested in how this receptor modulates platelet aggregation and what are the signalling mechanisms involved in this process.

Methods: Platelet aggregation assays were performed ex vivo using a platelet aggregation analyser (Aggregometer II). Blood from healthy human donors was used to obtain platelet-rich plasma. Aggregation was induced using ADP or collagen. Different agonists and antagonists were added to investigate their effects on platelet aggregation. Ca\(^{2+}\) flux changes caused by addition of agonists were also examined using a fluorescent Ca\(^{2+}\) dye (Fluo-3) by flow cytometry. Expression of the EP\(_4\) receptor on the surface of platelets was established using indirect flow cytometry whereas expression of CD62P, PAC1 and CD41 was examined using direct flow cytometry. In vitro thrombus formation was assessed by flowing whole blood on collagen-coated Cellix biochips at ~30 dyne/cm\(^2\) using the Mirus nanopump.

Results: We observed that human platelets express EP\(_4\) receptors. A selective EP\(_4\) agonist potently inhibited the platelet aggregation as induced by ADP or collagen. This effect could be completely reversed by using an EP\(_4\) antagonist, but not by PGI\(_2\), PGG\(_2\) or TXA\(_2\) receptor antagonists. Moreover, an EP\(_4\) antagonist enhanced the PGE\(_2\)-induced stimulation of platelet aggregation, indicating a potent anti-aggregatory activity of the EP\(_4\) receptors. Interestingly, the inhibitory effect of the EP\(_4\) agonist was brought about by protein kinase C but
not adenylyl cyclase, accompanied by attenuated Ca$^2+$ flux, decreased activation of glycoprotein IIb/IIIa and down-regulation of P-selectin. Most importantly, in vitro thrombus formation was effectively reduced by the EP$_4$ agonist and this effect was reversed using the EP$_4$ antagonist.

Conclusions: These findings indicate that the EP$_4$ receptor is a potential biological drug target in anti-platelet therapy.

A18
The role of the carboxyl terminus in folding of the serotonin transporter
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BMC Pharmacology 2010, 10(Suppl 1):A18

Background: The serotonin transporter (SERT) is a member of the SLC6 family of solute carriers. SERT plays a crucial role in synaptic neurotransmission by retrieving released serotonin. The intracellular carboxyl terminus of various neurotransmitter transporters has been shown to be important for the correct delivery of SLC6 family members to the cell surface. A previous study showed that deleting the C-terminus of SERT impaired transporter activity and compromised its delivery to the plasma membrane [1], but this study did not offer any mechanistic explanation for these effects. The hypothesis of the current project has been that the C-terminus of SERT is required for folding and trafficking of the serotonin transporter.

Materials and methods: We employed several approaches to examine the given hypothesis, including (1) serial truncations of the carboxyl terminus, (2) scanning the C-terminus of SERTs for motifs that are required for expression of functional transporter, (3) localization by confocal laser scanning microscopy, biochemical characterization binding studies, uptake studies, (4) bacterial expression of SERT with a C-termally fused GFP tag [2], (5) communoprecipitation of the misfolded mutant with calnexin [3], and (6) test for possible chemical and pharmacological chaperone effects of SERT.

Results: Here we studied the importance of the C-terminus in trafficking and folding of human SERT. Serial truncations followed by mutagenesis identified sequence spots (PC641,602, RI607–609) within the C-terminus relevant for export of SERT from the endoplasmic reticulum (ER), R607,608 is homologous to the RL-motif that in other SLC6 family members provides a docking site for the COPII protein complex Sec24D. The primary defect resulting from mutation at PG607,608 and R607,608 was impaired folding; because mutated transporters failed to bind the inhibitor [3H]imidipramine. In contrast, when retained in the ER (e.g. by dominant negative Sar1) the wild-type transporter bound [3H]imipramine with an affinity comparable to surface-expressed transporter. SERT-R607,608AA and SERT-RI607–609AAA were partially rescued by treatment of cells with the nonspecific chemical chaperone DMSO or the specific pharmacocopherone A20 (which binds to the inward-facing conformation of SERT) but not by other classes of ligands (inhibitors, substrates, amphetamines).

Conclusions: These observations (i) demonstrate a hitherto unappreciated role of the C-terminus in the folding of SERT, (ii) indicate that the folding trajectory proceeds via an inward-facing intermediate and (iii) suggest a model where the RI-motif plays a crucial role in preventing premature Sec24-recruitment and export of incorrectly folded transporters.

References

A19
Calmodulin kinase II regulates amphetamine-induced reverse transport in the dopamine transporter: implications for the importance of the dopamine transporter in Angelman syndrome
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BMC Pharmacology 2010, 10(Suppl 1):A19

Background: The dopamine transporter (DAT) mediates dopamine (DA) reuptake once DA gets released into the synaptic cleft; thereby, the DAT regulates DA content available for synaptic transmission. Psychostimulants like amphetamines can induce the reverse operation and induce outward transport, thereby increasing extracellular dopamine concentrations. Increases of DA in the synaptic cleft are associated with psychosis and drug addiction. Influx and efflux of substrate via the DAT are thought to be asymmetrical and were shown to possess consensus sites for the regulation by intracellular kinases. It was demonstrated that the loss of N-terminal serines ablates amphetamine-induced reverse transport in the DAT and that Ca$^{2+}$/calmodulin-dependent protein kinase IIa (CaMkIIa) can physically bind the DAT C-terminus and phosphorylate N-terminal serines. Pharmacological inhibition of CaMkIIa with KN93 dramatically reduces amphetamine-induced efflux in both cells stably transfected with the human DAT and in rat striatal slices. Here we show that amphetamine-induced DAT-mediated efflux is reduced in CamKIIa mutants and in a mouse model of Angelman syndrome (AS).

Methods: Mouse striatal synaptosomes preloaded with [3H]MPP$^+$, a substrate for the DAT, were superfused using a superfusion system. [3H]MPP$^+$ release was induced by the addition of 3 μM D-amphetamine. Superfusates were collected and quantified using a scintillation counter. Immunoblots and [3H]CFT binding experiments were used to assess DAT protein expression levels. Uptake assays were performed in striatal synaptosomes using [3H]MPP$^+$ as substrate. For non-specific uptake we used the DAT-inhibitor mazindol.

Results: Both pharmacological inhibition of CamKIIa or genetic ablation of CamKIIa function (in CamKIIa knock-out and AS mice) reduces amphetamine-induced reverse transport in the DAT. As CamKIIa is one of the brain’s most abundant proteins involved in a plethora of regulatory processes it is not possible to pharmacologically target it in human AS patients. However, the DAT would be a possible target also for these patients and it might be promising to further investigate potential DAT influencing medications to treat Angelman syndrome.

Conclusions: Pharmacologic inhibition of CamKIIa as well as genetic knock-out or activity-downregulation of CamKIIa reduce amphetamine-induced reverse transport in the DAT.

A20
α-N-Heterocyclic thiosemicarbazones induce ER stress-mediated CHOP activation
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Background: Blocking of DNA synthesis through inhibition of ribonucleotide reductase has been proposed to be the main mechanism of anti-neoplastic action for α-N-heterocyclic thiosemicarbazones. Currently the best-studied agent of this class of compounds is triapine (3-amino-2-carboxydeoxythiosemicarbazone), which has been tested in several phase I and II clinical trials. We synthesized triapine (HL)2 and the corresponding terminally N4-dimethylated derivative, 3-aminoopyridine-2-carbaldehyde...
N-N-dimethylthiosemicarbazone (HL1). Previously, we have shown that dimethylation of the terminal amino group leads to significant amplification of the activity in cytotoxicity assays. Previously, we also discovered intrinsic fluorescence properties for both compounds. Here we present a study of intracellular distribution of the compounds and a possible new mechanism of action for α-N-heterocyclic thiosemicarbazones by induction of endoplasmic reticulum (ER) stress.

Methods and results: Fluorescence microscopy was performed on living SW480 cells (colon carcinoma) treated with HL1 and HL2. Microscopy images show a strong affinity to the nuclear membrane and to cytosolic structures. Co-localization studies revealed both agents are associated with structures of ER and mitochondria and co-staining images suggest an involvement of ER in its mechanism of action. SW480 cells were treated for 15 h with the compounds in micromolar concentrations and immunoblotting analyses were performed, resulting in high protein levels of the ER stress-mediated C/EBP homologous protein (CHOP). CHOP is known to be transcriptional activated when functions of the ER are severely impaired and is associated with mitochondria mediated apoptosis pathway. The cytotoxic potencies of HL1 and HL2 were determined in SW480 (colon carcinoma) and 41M (ovarian carcinoma) cells by means of the colorimetric MITT assay. The IC50 value of triapine (HL1) is 0.35 ± 0.02 µM in SW480 cells and 0.45 ± 0.03 µM in 41M cell line. HL2 showed IC50 values of 0.33 ± 0.02 µM in SW480 and 0.21 ± 0.13 µM in 41M cells, respectively. Comparing triapine with its N1-dimethylated derivative, a 1.6–2.1-fold higher activity was observed.

Conclusions: Our results suggest that α-N-heterocyclic thiosemicarbazones induce ER stress-mediated CHOP activation and subsequent apoptosis signaling, which is a novel mechanism of action for this class of compounds. Further investigations will help to clarify in detail the role of ER stress induction in the mode of action.

A21 The high-affinity binding site for tricyclic antidepressants resides in the outer vestibule of the serotonin transporter
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BMC Pharmacology 2010, 10(Suppl 1):a21

Background: The structure of the bacterial leucine transporter LeuTα was used as a model for mammalian Na+/Cl−-dependent transporters, in particular the serotonin transporter (SERT). The crystal structure of LeuTα liganded to tricyclic antidepressants predicts simultaneous binding of inhibitor and substrate. This is incompatible with the mutually competitive inhibition of substrates and inhibitors of SERT.

Methods: We explored the binding modes of tricyclic antidepressants by homology modeling and docking studies. Two approaches were used subsequently to differentiate between three clusters of potential docking poses: (i) a diagnostic SERT<sup>S5F</sup> mutation, which greatly reduced the affinity for [3H]imipramine but did not affect substrate binding, and (ii) competition binding experiments in the presence and absence of carbacholamine (i.e. a tricyclic imipramine analog with a short side chain that competes with [3H]imipramine binding to SERT).

Results: Binding of releasers (para-chloroamphetamine, methylene-dioxy-methamphetamine/ecstasy) and of carbacholamine were mutually exclusive, but Dixon plots generated in the presence of carbacholamine yielded intersecting lines for serotonin, MPP<sup>+</sup>, paroxetine and ibogaine.

Conclusions: These observations are consistent with a model, where (i) the tricyclic ring is docked into the outer vestibule and the dimethylamino propyl side chain points to the substrate binding site, (ii) binding of amphetamines creates a structural change in the inner and outer vestibule that precludes docking of the tricyclic ring, (iii) simultaneous binding of ibogaine (which binds to the inward-facing conformation) and of carbacholamine is indicative of a second binding site in the inner vestibule, consistent with the pseudo-symmetric fold of monoamine transporters. This may be the second low-affinity binding site for antidepressants.

A22 4-Methylthioamphetamine (4-MTA) induces mitochondrial-dependent apoptosis in SH-SYSY cells independently of dopamine and noradrenaline transporters
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BMC Pharmacology 2010, 10(Suppl 1):a22

Background: 3,4-Methylenedioxymethamphetamine (MDMA or ‘ecstasy’) tablets are frequently contaminated by 4-MTA (‘flatliner’), an amphetamine derivative which is known to induce severe human intoxication and even death. Although an equipotent inducer of SERT-dependent 5-HT release in vivo, 4-MTA does not induce MDMA-like serotoninergic neurotoxicity in rats. Instead, 4-MTA users typically report unpleasant sympathomimetic effects such as tachycardia, tremors, stomach cramps, headache and sweating following ingestion. Here, for the first time we investigate the cytotoxic potency of 4-MTA in a catecholaminergic model in which to examine the molecular mechanisms of 4-MTA and MDMA-induced cytotoxicity in vitro. Cell viability was determined using the MTT assay and validated using flow cytometry via PI exclusion. ROS production, mitochondrial membrane potential (MMP), apoptosis and the cell cycle were examined via flow cytometry using DA, JC-1, annexin V/PI and PI respectively. The level of intracellular calcium was determined ratiometrically by confocal microscopy using two visible wavelength Ca<sup>2+</sup>-sensitive dyes, Flu-3 and Fura Red.

Results: 4-MTA was significantly more cytotoxic than MDMA at 24 h, demonstrating an EC<sub>50</sub> of 0.60 mM in contrast to 2.01 mM for MDMA. In addition, the combination of MDMA and 4-MTA at low concentrations significantly increased cytotoxicity compared to that of each drug alone. 4-MTA-induced cell death was reduced by the anti-oxidant N-acetyl-L-cysteine (NAC) but not by the non-selective monoamine transport inhibitor indatraline, indicating that monoamine transport is not a requirement of 4-MTA-induced cytotoxicity. Drug-induced cell death was pre-empted by rapid intracellular Ca<sup>2+</sup> influx, mitochondrial membrane depolarization (MMD), ROS production and caspase 9 activation. MDMA and 4-MTA also induced phosphatidylserine exposure and caspase-dependent DNA fragmentation at 24 h indicative of cell death via apoptosis.

Conclusions: Although both MDMA and 4-MTA induced apoptosis via the mitochondrial death pathway, 4-MTA does so at more physiologically relevant concentrations and may therefore be a potent synergistic adjunct when mixed with MDMA.

Acknowledgements: This work was supported by the Health Research Board (HRB), Ireland.

A23 A molecular switch between the outer and the inner vestibules of the voltage-gated Na<sup>+</sup> channel
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BMC Pharmacology 2010, 10(Suppl 1):a23

Background: Voltage-gated ion channels are transmembrane proteins that undergo complex conformational changes during their gating transitions. Both functional and structural data from K<sup>+</sup> channels suggest that extracellular and intracellular parts of the pore communicate with each other via a trajectory of interacting amino acids. No crystal structures are available for voltage-gated Na<sup>+</sup> channels but functional data suggest a similar intramolecular
communication involving the inner and outer vestibules. However, the mechanism of such communication is unknown. Here, we report that amino acid I1575 in the middle of transmembrane segment 6 of domain IV (DIV-56) in the rNa1.4 channel may act as molecular switch allowing for interaction between outer and inner vestibule.

Methods and results: Cysteine scanning mutagenesis of the internal part of DIV-56 revealed that only mutations at site 1575 rescued the channel from a unique kinetic state (‘ultra-slow inactivation’, Iun) produced by the mutation K1237E in the selectivity filter. The fact that mutations at site 1575 modulate Iun produced by K1237E strongly suggests an interaction between these sites. To elucidate how Iun is generated, we ‘forced’ large organic cations through the channel, which resulted in a significant linear correlation between the increase in the amplitude of recovery from Iun and the diameter of the permeating cations. Additionally, we show that a permeation pathway for the permanently charged lidocaine analogue QX222, opened by mutations at site 1575, is closed by the addition of K1237E to these I1575 mutants.

Conclusions: These results support the notion that an increase in the size of the selectivity filter produced by the mutation K1237E gives rise to an interaction of E1237 with I1575 of the adjacent S6 segment thereby generating IUS.

Acknowledgements: This work was supported by grants P21006-B11, P17509-B11, P13961-B05 from the FWF.

A25
Altered sodium channel function in dystrophic/utrophin-deficient cardiomyocytes
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BMC Pharmacology 2010, 10(Suppl 1):A25

Background: Duchenne muscular dystrophy (DMD), caused by mutations in the dystrophin gene, is an inherited disease characterized by progressive muscle weakness and degeneration. Besides the relatively well-described skeletal muscle degenerative processes, DMD and some other muscular dystrophy types are also associated with cardiovascular complications including cardiomyopathy and cardiac arrhythmias. The current understanding of the patho-mechanisms underlying these cardiovascular complications is still very limited, but recent research points to a contribution of dysfunctional ion channels in dystrophic cardiomyocytes.

Materials and methods: By using the whole cell patch-clamp technique, the functional properties of voltage-gated sodium channels were studied in cardiomyocytes derived from normal and dystrophic mice. In addition, a computer model was used to simulate the effects of altered sodium channel properties on the cardiac action potential. Besides the most common mouse model for human DMD, the dystrophin-deficient mdx mouse, we also used mice additionally carrying a mutation in the utrophin gene. The mdx-utr double mutant mouse exhibits a more severe cardiac disease phenotype than the mdx mouse, and is thought to represent a more suitable animal model for human DMD.

Results: We found that dystrophic cardiomyocytes show a reduced sodium current density compared to wild-type cardiomyocytes. In addition, extra utrophin deficiency significantly shifted both the sodium channel activation and inactivation curve to more depolarised potentials, which was not observed in only dystrophin-deficient mdx cardiomyocytes. Computer modelling revealed that the described sodium channel impairments in dystrophic cardiomyocytes suffice to affect the action potential.

Conclusions: Sodium channel dysfunction may perturb electrical impulse propagation in the dystrophic heart, and thus contribute to cardiac complications associated with the muscular dystrophies.

Acknowledgements: Supported by the Austrian Science Fund (FWF, P19352-B11 and P21006-B11).

A26
The interactome of the A2A adenosine receptor in vitro and in vivo
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Background: The A2A adenosine receptor is a prototypical G protein-coupled receptor. It is expressed in a wide variety of cells including as different types as neurons, platelets, cells of the immune system and muscle. The cell-specific expression of the A2A adenosine receptor is controlled by at least three different upstream non-coding exons and their corresponding promoters. Compared to other G protein-coupled receptors the A2A receptor holds an unusually long intracellular carboxy terminus, which consists of 122 amino acids. This C-terminus turned out to be the docking site for other proteins. Using a yeast-2-hybrid screen we have previously identified proteins interacting with the C-terminus including ARNO/cytohesin-2, SAP102 and USP4.

Materials and methods: To verify these interactions in vivo and to identify new interacting proteins of the A2A adenosine receptor we chose
a two-step proteomics approach: we first expressed tagged receptors in HEK293 fibroblasts using various TAP (tandem affinity purification)-tag variants; the differently tagged receptors were analyzed for expression, localization and their pharmacological properties (ligand binding and cAMP accumulation) to identify tags suitable to further analyze the receptor's interactome. These tagged receptors were then used to optimize the purification and to make the first initial screens using 2D-nano-LC-MS/MS approach.

Results and conclusions: We could identify two tags suitable for further analysis of the A2A adenosine receptor interactome. One of the tags kept the receptor to a large extent in the endoplasmatic reticulum, while the second tag allowed surface expression. Pharmacological properties of the receptors were comparable to untagged versions of the receptor. LC-MS/MS analysis of the purified ER trapped version of the receptor revealed proteins putatively involved in the folding of the receptor like heat shock proteins. The A2A adenosine receptor expressed at the cell surface will be used in the in vivo approach. To perform this we generate a transgenic mouse expressing the TAP-tagged version of the A2A adenosine receptor under the control of its endogenous promoters (homologous knock-in). This will allow us to examine tissue and developmental specific interaction partners of the A2A adenosine receptor.

A27 Conformation of membrane-inserted P-glycoprotein

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BMC Pharmacology 2010, 10(Suppl 1):A27

Background: The human genome codes for 49 members of the large ABC protein family. Most of them are membrane transporters. The first structures of exporters have been solved in recent years. Although a remarkable achievement, some uncertainty remained with respect to the physiological conformation of the transporter, which seems not to be fully compatible with all biochemical evidence. During crystallization, the transporter is extracted from its native membrane environment and solubilized by surfactants. This procedure might have influenced the transporters' conformation and lead to the structures observed in the crystals. We focus on the multidrug resistance transporter P-glycoprotein, which prevents xenotoxic compounds from entering the body and from penetrating into the brain, and confers resistance to chemotherapy.

Methods: We used homology modelling and MD simulations to identify the equilibrium conformation of the membrane inserted transporter.

Results: An initial P-glycoprotein model was built using the Sav1866 template [1]. The model was in compliance with most experimental data; discrepancies were observed in the central pore, where biochemical evidence suggested a smaller distance between the two sides of the wing-like helical bundle of the transmembrane region. Bending the structure allowed us to create a model that showed a consistently better agreement with biochemical data [2]. MD simulations were used to probe for the equilibrium conformation in the membrane environment. Simulations were initiated from both the Sav1866-based and from the bend model. Analysis of the two trajectories revealed a tendency to converge to a common conformation, as the simulation started from the wing-like structure showed a motion that brought the two wings closer.

Conclusions: Our results therefore indicate that the membrane environment does have an effect on the equilibrium conformation and that this conformation differs from the one observed in the crystal structure of the bacterial homolog Sav1866.

References

A28 Selective serotonin reuptake inhibitor-induced cell death: in search of a mechanism
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Background: Selective serotonin reuptake inhibitors (SSRIs) have been observed to drive programmed cell death in Burkitt lymphoma cells. Accordingly, they were proposed as a new modality in the treatment of Burkitt lymphoma. However, they induce apoptosis with little if any appreciable selectivity. Actually, the selectivity appears to be so low that SSRIs can also kill protozoa (such as Trichomonas vaginalis). Acetylated versions of SSRIs, which do not inhibit serotonin reuptake, kill cells in concentration ranges comparable to those of their non-acetylated original versions. Thus, the obvious SSRI target, SERT, is not required for cell killing.

Methods: For cell viability assays, HEK293 cells and T. vaginalis were incubated with SSRIs or related compounds and subsequently analyzed for propidium iodide-positive cells using flow cytometry. Caspase activation was measured in cell lysates as fluorescence intensity using cleavable fluorogenic substrates of caspases 3/7, 4, 8 and 9, respectively. Activation of autophagy and endocytosis was evaluated by immunocytochemistry and by expression of fluorescent labelled markers in HEK293 cells visualized by confocal microscopy.

Results and conclusions: By testing various SSRIs and tricyclic antidepressants, we found a significant correlation in their ability to kill HEK293 cells and T. vaginalis. In search of a mechanism of SSRI-induced cell killing, we found that (i) SSRIs cause activation of caspases 3/7, 4, 8 and 9. This is blocked by z-vad-fmk (caspase inhibitor used as internal control) and by the vacuolar ATPase inhibitor bafilomycin A1, but not by salubrinal, eyarestatin I and bongkrekic acid; (ii) paroxetine and compounds identified by a chemical library screen (REM14 and REM25) lead to accumulation of vacuoles within cells; paroxetine-induced vacuole formation is inhibited by bafilomycin A1; (iii) paroxetine-induced vacuoles stain for Rab4 and Rab7 (markers for early and late endosomes), but not for LCB3 (a marker for autophagosomes). The structure-activity profile for SSRIs and related compounds will be verified by extracting the pharmacophore and by chemical library screens. The additional goal is to identify compounds that block the action of SSRIs and to thereby define their site of actions.

Acknowledgements: This work is supported by PLACEBO of the GEN-AU programme of the Austrian Federal Ministry of Science and Research.

A29 The pulmonary microvascular endothelial barrier function is controlled by the PGE2–EP4 signaling axis
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BMC Pharmacology 2010, 10(Suppl 1):A29

Background: Endothelial cells, like gate-keepers of the vascular bed, can actively protect against the inflammatory process. Prostaglandin E2 (PGE2) could be one of the mediators that can promote the barrier function of endothelial cells. PGE2 exerts its cellular effects by binding to four different E-prostanoid receptors (EP1–4) that belong to the family of G protein-coupled receptors. This project aimed at characterizing the barrier-protective properties of PGE2 and especially EP4 receptor on human pulmonary microvascular endothelial cells (HMVEC-L).

Methods: The endothelial barrier properties were analyzed by measurements of transendothelial electrical resistance (TEER) by Endothel
and by ECIS (Electric Cell substrate Impedance Sensing) devices. Morphological studies were performed with immunofluorescence microscopy. Different protein expressions were detected by flow cytometry. Leukocyte diapedesis was studied by performing transendothelial migration assays of neutrophils and eosinophils.

Results: We found that the EP1 receptor is expressed on HMVEC-L.
PGE2 and the selective EP1 receptor agonist (ONO-AE1-329) prevented the barrier-disrupting effect of thrombin on the endothelial monolayer, as it was visualized by VE-cadherin staining. Selective blocking of EP4 receptors (EP4 antagonist ONO-AE3-208) inhibited the protective effect of PGE2 on endothelial monolayers. PGE2 and the EP4 agonist enhanced the regeneration of electrically wounded endothelial monolayers. The specificity of EP4 receptor involvement was proven by using the EP4 receptor antagonist and selective agonist for EP3 and EP4 receptors. PGE2 and the EP4 receptor agonist attenuated the TNF-α-induced up-regulation of E-selectin. Surprisingly, this effect was not affected by an adenylyl cyclase inhibitor, but inhibition of PKC activity reversed the effect of the EP4 receptor agonist and PGE2 at reducing the E-selectin expression. In the cell interaction assays, thrombin or TNF-α increased the permeability of endothelial monolayers which also enhanced the transmigration of neutrophils and eosinophils, respectively. These effects were prevented by the selective activation of EP3 receptors.

Conclusions: Our data support the hypothesis that endothelial cells, as gatekeepers of the vessel wall can actively participate in the inflammatory process. We have shown that PGE2, via activating EP4 receptors, enhances the barrier function of the endothelium by protecting the endothelial adherent junctional network and preventing leukocyte diapedesis. Therefore, EP4 agonists might be promising new therapeutic tools in treating inflammatory diseases.

A30 Tracking the Aa3 adenosine receptor
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BMC Pharmacology 2010, 10(Suppl 1):A30

Background: The Aa3 adenosine receptor has become a drug target in the treatment of Parkinson’s disease, psychotic behavior and dementia. In addition, targeted deletion of this receptor in mice leads to hypertension, increased platelet aggregation, male aggressiveness and decreased susceptibility to ischemic brain damage. The potential clinical relevance of this receptor is obvious. The Aa3 adenosine receptor, a prototypical GPCR, is known to signal via restricted collision coupling with Gαi. In addition, it is able to stimulate MAP kinase/ERK in a Gαi-dependent way but dependent on the lipid microenvironment of the membrane. Hence, we characterized the mobility and the targeting of the Aa3 receptor in nerve cells.

Methods: Receptor mobility was measured using fluorescence recovery after photobleaching (FRAP). A fluorophore-tagged version of the Aa3 receptor expressed in the cell membrane was bleached using an intense laser beam and the lateral diffusion rate of the receptor was determined. We also implemented the method of single molecule tracking, which allows for the observation of movements of single receptors in real spatial and temporal resolution.

Results: We introduced a palmitoylation site in the proximal part of the C-terminus of the Aa3 receptor; this led to the loss of restricted collision coupling of the receptor to its G protein. We also deleted a DVELL motif in the distal part of the C-terminus, which disrupted the interaction of the receptor with a "synaptic associated protein" (SAP102). The mobility of these mutants has been compared with wild-type Aa3 receptors in different compartments of hippocampal neurons.

Conclusions: The signaling properties of the Aa3 adenosine receptor depend on its localization within several membrane compartments. Targeting to specific compartments depends on the interaction with "accessory proteins".

A31 Luminescence resonance energy transfer-based intramolecular distance measurements in leucine transporter from Aquifex aeolicus
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BMC Pharmacology 2010, 10(Suppl 1):A31

Background: Solute carrier 6 (SLC6) membrane proteins are integral membrane proteins and of particular pharmacological interest because they are targets of many clinically important drugs. These SLC6 proteins play crucial roles ranging from nutrient uptake to neurotransmitter clearance. A leucine transporter LeuTaa3 from Aquifex aeolicus has been recognized as a bacterial orthologue of mammalian SLC6 family proteins. LeuTaa3 has been crystallized and its structure was resolved to high resolution. With respect to its kinship to other SLC6 transporters, though with low sequence identity (~20–25%), there are crucial regions in transmembrane segments 3, 5, 6 and 8 where conservation reaches ~50%. For this reason LeuTaa3 provides a good structural paradigm to study homology models of SLC6 family members and learn more about the structure/function relationship in mammalian transporters.

Methods and results: In order to test proposed models, we initiated a study to measure intramolecular distance changes associated with the dynamic process of substrate transport. We employed luminescence resonance energy transfer (LRET) to measure the changes in intramolecular distances. For LRET-based measurements we have introduced LBT (lanthanide binding tags) to accommodate terbium, as the donor element, along with cysteines, where acceptor fluorophores are attached, at selected positions in LeuTaa3. After expression and purification of these mutants, we obtained the first distances at atomic resolution.

Conclusions: Taken together our LRET measurements can help us to validate or propose a dynamic substrate transport model for LeuTaa3. Our future plan focusses on the establishment of functionality assays for screening of functional LeuTaa3 mutants along with their LRET measurements.

A32 Endogenous dynorphin in emotional control and stress response revisited
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BMC Pharmacology 2010, 10(Suppl 1):A32

Background: We recently demonstrated a clearly anxiolytic phenotype of dynorphin-deficient (dynKO) mice on the C57bl/6N background. However, other groups observed a less prominent and partially paradigm-dependent anxiogenic phenotype or even anxiogenic phenotype of other dynKO mice. Therefore we backcrossed our dynKO mice onto the balb/c background and evaluated their anxiety-related behaviour.

Methods: In this study, we investigated anxiety and stress-related behaviour of germ-line prodynorphin knockout (dynKO) mice. Behavioural data were complemented by measurement of corticosterone serum levels.

Results: Male dynKO mice exhibited about 2-fold ambulation in the open field center and intermediate areas. DynKO mice showed also longer distance travelled (2-fold) and more time spent on open arms of the elevated plus maze test. Significantly higher numbers of mice entering the open lit area in the light-dark test were observed in dynKO as compared to wild-type mice. As observed on the C57bl/6N background, only minor changes were observed in the stress-coping abilities measured in the tail suspension and forced swim tests. A reduction of basal corticosterone levels was observed in dyn-KO mice.
Conclusions: Taken together our data support the anxiogenic effects of endogenous dynorphin as observed on the C57Bl/6N background. However, the phenotype is less clear on the balb/c background.

Acknowledgements: This project was supported by the Austrian Science Fund (P21017).

A33
Neuropeptide Y in the basolateral amygdala modulates the acquisition of conditioned fear
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BMC Pharmacology 2010, 10(Suppl 1):A33

Background: Neuropeptide Y (NPY), a highly conserved 36 amino acid peptide, is widely distributed in the central nervous system. Besides its functions in various metabolic processes NPY has attracted considerable attention in modulating emotional-affective behavior. NPY exerts a pronounced anxiolytic effect most likely mediated by Y1 receptors, whereas stimulation of predominantly pre-synaptic Y2 receptors results in increased anxiety. The role of NPY in the processing of fear, however, is still not conclusive. The current study aims to elucidate the role of NPY in Pavlovian fear conditioning, a simple form of associative learning.

Methods: NPY KO mice as well as KO mice for the different NPY receptors (Y1, Y2 and Y4) were subjected to a delay fear-conditioning paradigm (5 tone/shock pairings), followed by an extinction session 24 h later (40 tone alone presentations).

Results: NPY KO mice revealed faster acquisition and excessive expression of conditioned fear. Baseline freezing was increased on retention/extension day and the ability to distinguish an explicitly paired tone from an unpaired tone was limited, both indications for a generalization of conditioned fear. Moreover, NPY KO mice displayed a pronounced deficit in the extinction of fear memory. Within session, extinction as well as extinction recall were significantly impaired in NPY KO mice. Expression of NPY by an AAV-vector in the basolateral amygdala (BLA) partly ameliorated deficits seen in NPY KO mice. Y2 KO mice showed increased acquisition and delayed extinction, whereas no obvious phenotype was seen in Y2 KO mice. Y2 KO mice exhibited a significant deficit in fear extinction.

Conclusions: Our data indicate that NPY in the BLA exerts a protective role in the acquisition of fear memories. In addition, it facilitates extinction of conditioned fear. Experiments performed in Y receptor KO mice suggest a prominent role of the Y1 receptor in acquisition and extinction of conditioned fear, whereas Y4 receptors seem to be involved in extinction learning.

A34
Fear learning triggers structural changes at GABAergic synapses in the basal amygdala
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BMC Pharmacology 2010, 10(Suppl 1):A34

Background: In Pavlovian fear conditioning, in which initially a conditioned stimulus (CS) is repeatedly paired with an aversive stimulus (US), animals learn to associate CS with US, hence attaching emotional significance to sensory stimuli. Conditioned fear can be suppressed when the CS is repeatedly presented alone, a phenomenon known as fear extinction. Previous studies suggest that the mechanisms underlying fear conditioning involve inhibitory neurotransmission through GABA receptors. GABA_A receptors are generally composed of two α, two β and one γ subunits. Sixteen GABA_A receptor subunits have been identified in mammals, which indicates that these receptors may have multiple biophysical and pharmacological properties. Recent work showed that fear conditioning induces a dramatic downregulation of benzodiazepine binding sites and transcripts for gephyrin and some GABA_A receptor subunits in the basal nucleus of the amygdala (BA), which were restored to control levels after fear extinction.

Methods: In this work, we analysed by means of the freeze-fracture replica immunolabelling technique (SDFS-FRL) the synaptic and extrasynaptic content of the GABA_A-2 subunit in the BA of C57Bl/6 mice that underwent fear conditioning as well as extinction. Using this technique, we could also measure the full synaptic area of GABAergic synapses. Immunogold particles for GABA_A subunits tend to concentrate in clusters of intramembrane particles (IMP) on the protoplasmic face of the plasma membrane, indicating that labelled IMP clusters represent GABAergic synapses.

Results: The average size of GABAergic synapses in control mice was 0.030 ± 0.019 µm² (n = 223). The mean synaptic size (0.040 ± 0.025 µm², n = 198) in fear conditioned animals was significantly (p < 0.0001, one-way ANOVA) increased, whereas in mice that underwent extinction it returned to values similar to those of controls (0.032 ± 0.021 µm², n = 154). The distribution frequency of the area of GABAergic synapses was also found to be significantly different between fear conditioned and control mice (p < 0.01). The labelling density for the GABA_A-2 subunit was significantly lower in both synaptic (p < 0.05, Kruskal-Wallis) and extrasynaptic (p < 0.05, Kruskal-Wallis) areas in fear conditioned animals. However, the decrease in synaptic density appears to be a consequence of the increased synaptic area.

Conclusions: These results suggest that in normal animals fear conditioning produces an enlargement of GABAergic synapses maintaining the number of GABA_A receptors substantially unaltered.

A35
Short-chain methotrexate polyglutamate MTPXG2 as outcome parameter in rheumatoid arthritis
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BMC Pharmacology 2010, 10(Suppl 1):A35

Background: Due to its positive benefit-risk ratio, methotrexate (MTX) is a first-line therapy in the treatment of rheumatoid arthritis (RA). Although MTX is very effective, the large interpatient variability in drug response is a major drawback in clinical practice. Erythrocyte methotrexate polyglutamates (MTPXG1–7) — intracellular storage products of methotrexate — are supposed to correlate with clinical efficacy. In particular, long-chain polyglutamates (MTPXG4–5) are strongly retained in the cells and are therefore discussed to be potential markers for clinical response in rheumatoid arthritis. We hypothesized that concentrations of methotrexate polyglutamates could correlate with clinical response parameters in rheumatoid arthritis.

Methods: A randomized, double-blinded controlled clinical trial including nineteen patients was performed according to GCP (Good Clinical Practice) guidelines and ethical principles that have their origin in the Declaration of Helsinki. Patients received a dose of 25 mg methotrexate per week administered orally. Laboratory testing and pharmacokinetics were performed at week 1, week 5, week 10 and at the end of study in week 16. The primary outcome parameter for clinical evaluation was DAS-28 (Disease Activity Score in 28 joints). DAS-28 calculation incorporates clinical parameters (ESR, swollen and tender joints) as well as subjective parameters (VAS pain). For measurement of methotrexate polyglutamates in erythrocytes, we used an HPLC (High Pressure Liquid Chromatography) method with post-column photodiode array followed by fluorimetric detection.

Results: Correlation of pharmacokinetics and clinical parameters using Spearman’s correlation coefficient showed a statistically significant
Conclusion: In particular, MTXPG2 seems to be an indicator for clinical response and may serve as a marker for drug monitoring. These seminal data should stimulate further trials to define the potential role of measuring erythrocyte concentrations of MTX for clinical prediction of MTX response.

A36

Teicoplanin pharmacokinetics during albumin dialysis

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BMC Pharmacology 2010, 10(Suppl 1):A36

Background: Albumin dialysis (AD) is a therapeutic option in severe cholestatic liver failure. However, it can significantly enhance drug elimination. Pharmacokinetic data on antimicrobial agents administered under this clinical condition are very sparse. Teicoplanin (TP) is a large glycopeptide, containing six compounds with a molecular weight between 1,500 and 1,900 Da. TP has a similar spectrum of activity and similar efficacy compared to vancomycin. It is active against a variety of bacteria, such as Streptococcus, Staphylococcus aureus including methicillin-resistant S. aureus (MRSA), Enterococcus faecalis and E. faecium. TP is eliminated by the kidneys with a terminal half-life of 80–180 h. Its protein binding is ~90%.

Methods: TP plasma concentrations were measured in a 30-year-old critically ill patient (body weight 50 kg), who was treated with albumin dialysis because of severe cholestatic liver failure with refractory purpura and obtained TP because of recurrent gram-positive sepsis. Albumin dialysis was performed with the molecular absorbent recirculating system (MARS). Two separate cycles of AD were analysed.

Results: After a 1,200 mg loading dose, doses of 1,200 and 1,000 mg on day 2 and 3, respectively, were administered during 2 cycles of AD for achieving TP trough levels above 10 µg/ml, which are recommended for most infections. During the first and the second AD cycle, the TP peak concentrations amounted to 98.7 and 99.8 µg/ml, the trough concentrations 27.0 and 15.7 µg/ml, the half-life was 4.6 and 6.4 hours, the apparent volume of distribution 0.28 and 0.32 L/kg and the TP clearance 43 and 35 mL/h/kg, respectively. TP levels were about 40% reduced post- vs. prefilter. Within 8 hours of AD, the TP serum concentration decreased by about 75%. The decline in serum levels is similar to that observed during continuous veno-venous hemofiltration.

Conclusions: Despite a considerable elimination of TP by AD therapeutic serum levels could be maintained during the entire treatment by administered high doses — exceeding the recommended doses — and close drug monitoring.

A37

Endotoxin-induced microparticle formation is subject to tolerance development in humans

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BMC Pharmacology 2010, 10(Suppl 1):A37

Background: Microparticles (MP) are small membrane vesicles expressing tissue factor and staining positive for annexin V which are released from different cell types during cell activation and apoptosis. MP are considered to promote coagulation and impair vascular function. Circulating concentrations of MP are elevated in patients with systemic inflammatory conditions.

Methods: Formation of MP was studied in 8 healthy male subjects exposed to intravenous E. coli endotoxin (LPS; 2 ng/kg body weight) who were re-challenged after 17 ± 11 days. In addition, intravenous vitamin C (25 g) or placebo was administered 3.5 hours after LPS on alternate trial days in random order. Venous blood samples were taken before, 3 h and 6 h after LPS administration. MP were detected using FACS analysis. Leukocyte count and body temperature were measured as positive controls. All results are expressed in medians in the unit 106 MP/μL plasma.

Results: MP formation was increased 3 h and 6 h after LPS administration. However, this effect was only demonstrable after the first LPS challenge with an increase of MP from baseline 596 units [range 399–1704 units] by a factor of 1.05 at 3 h and 3.87 at 6 h after LPS, and abolished after the second LPS exposure with a decrease from 874 units [430–1404 units] by a factor of 1.17 at 3 h and 1.25 at 6 h. This increase was independent of vitamin C administration. In contrast, reactivity of body temperature and leukocytosis 3 h and 6 h after LPS was similar between the study periods.

Conclusions: Our results demonstrate rapid and marked formation of MP in healthy men after systemic LPS challenge. The development of tolerance to LPS is observed in the absence of mitigated systemic inflammatory responses. This suggests that MP formation follows different pathways than those of inflammatory mechanisms.

A38

Does VEGF polymorphism play a role in the treatment success with VEGF inhibitors in patients with CNV?

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BMC Pharmacology 2010, 10(Suppl 1):A38

Background: Along with risk factors like smoking, hypertension, atherosclerosis and low choroidal blood flow, genetic susceptibility is a primary contributor to the development and progression of wet age-related macular degeneration (AMD). Vascular endothelial growth factor (VEGF) is a central angiogenic regulator and there is general agreement now that it is one of the most important triggers for the progression of neovascular AMD. In the present study we tested the hypothesis that VEGF gene polymorphisms play a role in the treatment success with VEGF inhibitors in patients with CNV.

Methods: One-hundred-sixty-two eyes of 143 patients with neovascular AMD who were scheduled for their first treatment with intravitreally administered ranibizumab were included in this trial. All patients were aged over 50 years and had angiographically verified neovascular AMD. Blood from the finger pad was collected on blood cards for genotyping for the VEGF polymorphisms rs1413711, rs3025039, rs2010963, rs833061, rs699947, rs3024997 and rs1005230. At each follow up visit, visual acuity was reassessed and an ophthalmic examination was carried out. The number of retreatments as well as the visual acuity outcome was analyzed in dependence of the VEGF polymorphisms.

Results: The included patients were reinjected with ranibizumab 2 to 19 times, resulting in a total treatment period of 42 to 1182 days. Neither the number of retreatments nor the visual acuity outcome was associated with any of the studied haplotypes.

Conclusions: The success of anti VEGF treatment is not dependent on VEGF gene polymorphisms.
A39

Antimicrobial activity of antibiotics in urine under different physiological conditions

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BMC Pharmacology 2010, 10(Suppl 1):A39

Background: In vitro activity of antimicrobials is commonly assessed in well-characterized growth media like Mueller-Hinton broth (MHB); however, such media hardly reflect in vivo conditions. We set out to compare activity of selected, mostly novel, antibiotics in MHB and urine, an important compartment for bacterial infections, at different conditions.

Methods: Urine obtained from healthy volunteers was pooled and sterile filtered. Microdilution tests were performed with Escherichia coli ATCC 25922 in MHB and human urine. The pH of urine was adjusted to values ranging from 5 to 8. For simulating different glucose levels in urine of diabetic patients, urine was adjusted to glucose levels of 100 and 1000 mg/dL. Bacterial growth in different media was investigated by growth curves.

Results obtained from MIC testing were confirmed by use of bacterial killing curves. Klebsiella oxytoca ATCC 700234 was used to investigate transferability of finding to other strains. Each experiment was performed 5 times.

Results: Growth of bacteria was similar for MHB and adjusted urine. Mean ratios (fold changes) of MICs for E. coli at the 6 different conditions simulated in urine compared to MIC-values in standard MHB are presented. Results were confirmed by time-killing curves and were concurrent for E. coli and K. oxytoca. Fosfomycin activity in urine was in unison higher than in MHB adjusted by glucose-6-phosphate.

Conclusions: Strong impact on activity was observed at low pH values for fluoroquinolones, trimethoprim, amikacin and tigecycline. Remarkably, all these antimicrobials act intracellularly while impact of pH on cell membrane-active antibiotics like β-lactams and colistin was much lower. Impairment of diffusion into the bacterial cell due to modification of the electric charge of the drug seems likely.

A40

The influence of lutein supplementation on macular pigment optical density in patients with dry AMD compared with healthy control subjects

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BMC Pharmacology 2010, 10(Suppl 1):A40

Background: Lutein and zeaxanthin are carotenoids that are selectively taken up into the macula of the eye, where they may protect against development of age-related macular degeneration (AMD). Past studies have reported an age-dependent decline of macular pigment optical density (MPOD) in patients with AMD. In the present study we investigated the age-dependence of MPOD and melatonin using spectral fundus reflectance. In addition, we hypothesized that patients with AMD have a reduced MPOD as compared to healthy controls.

Methods: A total of 82 healthy subjects and 96 patients with AMD were included in this study. The healthy control subjects showed a wide range of ages (mean 51.6 years, range 21–79 years). Patients with AMD were significantly older (mean 71.2 years, range 21–80 years). Spectral fundus reflectance of the fovea was measured in a 2.3° detection field with a custom-built fundus reflectometer. Calculation of MP and melanin optical density was based on a previously published fundus reflectance model [1].

Results: No age dependence of MPOD (r = −0.15) or melanin optical density (r = 0.08) was found in the healthy control group. Patients with AMD, however, showed a reduced MPOD (0.35 ± 0.12) as compared to the healthy control group (0.39 ± 0.12, p = 0.013). No significant difference in melanin optical density was observed between the two groups (AMD: 1.05 ± 0.24; healthy controls: 1.03 ± 0.24, p = 0.58).

Conclusions: A variety of studies showing a decline in MPOD with age are based on heterochromatic flickerphotometry data. By contrast, most data indicating no change in MPOD with age are based on the use of objective technology such as fundus reflectance or autofluorescence. The present study confirms the latter studies. In addition, the data of the present study indicate that patients with AMD have reduced MPOD, but not melanin optical density. This supports the hypothesis that reduced macular pigment is associated with AMD.


A41

BEZ235 impairs gastric cancer growth by inhibition of PI3K/mTOR in vitro and in vivo

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BMC Pharmacology 2010, 10(Suppl 1):A41

Background: Gastric cancer at advanced stage of disease is a major health problem and the prognosis with the current therapeutic treatment strategies remains poor. PI3K/mTOR pathway mutations, especially PTEN, PI3K3C and AKT mutations and p56 overexpression, are found frequently in gastric cancer patients and are linked with poor outcome. Thus, we evaluated the dual PI3K and mTOR inhibitor BEZ235 against gastric cancer in vitro and in vivo.

Materials and methods: Three gastric cancer cell lines (N87, MKN28 and MKN45) were treated with BEZ235 (20–80 nM) and assessed for cell viability, cell death and cell cycle distribution. PI3K/mTOR protein target modulation was measured by Western blotting. For in vivo studies athymic nude mice were inoculated with N87 or MKN45 cells bilaterally and treated daily with 20 or 40 mg/kg BEZ235. Tumor [18F]fluorothymidine (FLT) uptake was measured via small animal PET.

Results: In vitro, treatment of gastric cancer cells with 20–80 nM BEZ235 decreased cell growth in a dose-dependent manner in all cell lines tested (up to ~70%). This anti-proliferative activity was linked with a G1 cell cycle arrest (up to 75%). No significant induction of apoptosis by BEZ235 was observed. On the molecular level, BEZ235 led to a decrease of phosphorylation of AKT and S6 protein. In vivo, treatment with 20 and 40 mg/kg BEZ235 resulted in a significant anti-tumor effect in a N87 gastric cancer xenograft mouse model. BEZ235 therapy had no anti-tumor effect on MKN45 xenografts despite similar potent PI3K/mTOR target inhibition by BEZ235 in both xenograft models. However, expression of the proliferation marker thymidinymidine kinase 1 correlated with sensitivity to BEZ235 in vivo. In line, [18F]FLT uptake was significantly reduced only in the BEZ235-sensitive tumor xenograft model as measured by small animal PET.

Conclusions: In conclusion, our study shows that dual PI3K/mTOR inhibition by BEZ235 is a valuable target for gastric cancer therapy but is tumor model-dependent. Correlative studies with implementation of non-invasive imaging tools such as [18F]FLT PET might be a novel and promising strategy for optimizing clinical testing of dual PI3K/mTOR inhibitors.

A42

Vertical inhibition of the mTORC1/mTORC2/PI3K pathway shows synergistic effects against melanoma in vitro and in vivo

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BMC Pharmacology 2010, 10(Suppl 1):A42

Background: The phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway has been shown to be involved in the development of melanoma. PI-103 is a novel kinase inhibitor blocking PI3K class IA and mTOR complex 1 and 2. Here, we studied the effect of
targeting the PI3K/mTORC1/mTORC2 pathway by PI-103 and rapamycin in melanoma cells and a melanoma mouse model.

Materials and methods: Human melanoma cell lines (518A2, 607B, A375, Mel-Juso, SKMel-28) were treated with PI-103 and assessed for cell viability, apoptosis and cell cycle distribution. PI3K/mTOR protein target modulation was measured by Western-blotting. For siRNA experiments, cells were transfected with 50 nM/μL Silencer Select siRNA against PI3Kα (p110α catalytic subunit of PI3K). For in vivo studies athymic nude mice were inoculated with 518A2 cells and treated daily with PI-103 (20 mg/kg/d) and sirolimus (1 mg/kg/d). Paraffin-embedded xenograft sections were stained for p-AKT (Ser473) and p-S6 (Ser240–244).

Results: Dual targeting of PI3K and mTOR by PI-103 induced apoptosis and cell cycle arrest, and inhibited viability of melanoma cells in vitro. Combined treatment with PI-103 and the prototypic mTORC1 inhibitor rapamycin led to synergistic suppression of AKT and ribosomal S6 protein phosphorylation and to induction of apoptosis. In vivo, PI-103 and rapamycin displayed only modest single agent activity but the combination significantly reduced tumor growth compared to both single agents.

Conclusions: Taken together, our study underscores the importance of the PI3K/mTORC1/mTORC2 pathway in melanoma and demonstrates that rational combination of compounds that lead to an optimal blockade of a critical pathway ("vertical inhibition") may provide an effective strategy for future treatment of melanoma.

Thrombus formation is not pH-dependent
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BMC Pharmacology 2010, 10(Suppl 1):A43

Background: Experimental data have suggested that gastric acid impairs clot formation and platelet aggregation, and stimulates fibrinolysis. Thus, changing gastric pH from pH 3 to pH 7 may ameliorate acute gastrointestinal bleedings. The aim of this study was to evaluate the impact of pH on clot formation and stability in an ex vivo thrombus model.

Materials and methods: Venous blood from healthy subjects was perfused over denuded porcine aorta at 5 mL/min at a shear rate of 212 sec⁻¹ for thrombus formation. To evaluate the impact of pH changes, a co-perfusion with different acidic solutions was performed. Clot stability was evaluated by incubation of the isolated thrombus with pH solutions or gastric juice for up to four hours. The fibrinolytic activity was assessed by measuring D-dimer concentrations in the supernatant media. Thrombus size was evaluated by measurement of D-dimer concentration of the plasmin-degraded thrombus.

Results: Co-perfusion with different pH solutions did not affect thrombus size compared to control conditions. Clot incubation at pH 3, 5 or 7, for up to 4 h did not increase supernatant D-dimer concentrations. Incubation with gastric juice at pH 5 for 4 h significantly elevated supernatant D-dimer compared to pH 3 and pH 7 (70 ng/mL ± 0.00 vs. 353 ± 4.90 vs. 0.14 ± 0.12 mg/dL).

Conclusions: Clot formation is unaltered by concomitant changes in pH at low shear rate. Likewise, there is trivial if any effect of pH or gastric juice on thrombus stability, indicating that acute pharmacological modulation of gastric pH might yield only little impact on thrombus formed on gastrointestinal ulcers.

Inhibition of tissue factor pathway inhibitor (TFPI) by ARC19499 improves clotting of hemophiliac blood
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BMC Pharmacology 2010, 10(Suppl 1):A45

Background: Recently, differences in development of bacterial resistance were observed for macrolides with short and long elimination half life. Although one might speculate that this observation is to be explained by prolonged sub-inhibitory concentrations at the site of bacterial dwelling, the pharmacokinetic (PK) confirmation of this theory is missing. Thus we set out to compare PK of azithromycin and erythromycin in plasma, polymorphonuclear leukocytes (PML) and interstitial space fluid (ISF) of infected soft tissues.

Methods: Azithromycin (500 mg q.d.) or erythromycin (500 mg q.d.) treatment for three days was randomly administered to 6 healthy volunteers each. Macrolide concentrations were determined on day 1 and day 3 (full PK profile) and on day 5 and 10 (sparse sampling) by tandem mass spectrometry. Concentrations in ISF of muscle and subcutis were determined by microdialysis.

Results: During active treatment concentrations in plasma and ISF were higher for erythromycin than for azithromycin, whereas in white blood cells they were comparable. Except for azithromycin plasma concentrations and erythromycin PML concentration significant accumulation was observed for all compartments between single dose and day 3. For erythromycin concentrations were not detectable at day 10 for all compartments. In contrast, azithromycin mean concentrations at day 10 were 8 ± 0 ng/mL in plasma, 9 ± 3 ng/mL in muscle, 4 ± 2 ng/mL in subcutis and 14,217 ± 2,810 ng/mL in PML.

Conclusions: MIC50 of azithromycin for typical pathogens of the respiratory tract or soft tissue infections are in the range of 125–4,000 ng/mL. The present study confirms that for azithromycin 7 days...
after end of treatment sub-inhibitory concentrations are present, thereby strongly supporting the hypothesis of increased development of resistance due to PK reasons. Higher concentrations in PML than in plasma or ISF indicate that slow back-distribution from cells into extracellular space is the underlying PK mechanism.

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**Small-animal PET evaluation of [11C]MC113 as a PET tracer for P-glycoprotein**

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**BMC Pharmacology 2010, 10(Suppl 1):A46**

**Background:** The radiolabelled inhibitor of the multidrug efflux transporter P-glycoprotein (P-gp) [11C]Elacridar was developed as a positron emission tomography (PET) tracer to assess expression levels of P-gp at the blood-brain barrier (BBB) [1]. [11C]Elacridar was shown to interact specifically with P-gp at the rodent BBB, but its brain PET signal was very low, which was possibly caused by transport of [11C]Elacridar by P-gp [1]. In an attempt to get a better understanding of the required properties of an effective P-gp PET tracer we evaluated [11C]-labelled MC113, a structural analogue of elacridar, which was characterised as an unambiguous non-transported P-gp inhibitor and which possesses lower molecular weight, lower lipophilicity and higher potency for P-gp inhibition than elacridar (EC50 for inhibition of [1H]vinblastine transport in Caco-2 cell monolayers: 0.6 μM vs. 2.0 μM for elacridar) [2].

**Methods:** Female wild-type (n = 3) and Mdr1a/b−/− (n = 2) mice (Taconic Inc., USA) underwent paired PET scans with [11C]MC113 using a microPET Focus220 scanner (Siemens, Medical Solutions, USA). A baseline scan (150 min), during which the P-gp inhibitor tariquidar (15 mg/kg) was administered i.v. at 60 min after radiotracer injection, was followed by a second 60-min scan at 2 h after administration of tariquidar. Whole-brain time-activity curves were calculated using the image analysis software Amide.

**Results:** [11C]MC113 was evaluated using an identical set-up which we had previously used for [11C]Elacridar and which employed a combination of chemical and genetic knockout of P-gp [1]. [11C]MC113 had a 3 times higher peak brain activity uptake than [11C]Elacridar, but otherwise behaved identically to [11C]Elacridar, in that brain activity uptake was higher in Mdr1a/b−/− than in wild-type mice and that inhibitor administration increased brain activity uptake in wild-type mice. However, the observed effects were smaller for [11C]MC113 than for [11C]Elacridar.

**Conclusions:** Our data suggest that [11C]MC113 interacts with P-gp at the murine blood-brain barrier, but as for [11C]Elacridar its in vivo behaviour points to transport by P-gp. The higher brain activity uptake of [11C]MC113 might be an advantage over [11C]Elacridar for PET imaging of P-gp.

**Acknowledgements:** The research leading to these results has received funding from the European Community’s 7th Framework Program under grant agreement no. 201380 (Euripides) and from the Austrian Science Fund (FWF) project “Transmembrane Transporters in Health and Disease” (SFB F35).

**References**


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**Dose-response assessment of tariquidar for inhibition of P-glycoprotein at the human blood-brain barrier using (R)-[11C]Cverapamil PET**

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**BMC Pharmacology 2010, 10(Suppl 1):A47**

**Background:** Positional emission tomography (PET) with the radiolabelled substrate of the multidrug efflux transporter P-glycoprotein (P-gp) [(R)-11C]Cverapamil (VMPI) can be used to assess P-gp function at the blood-brain barrier (BBB). We have shown in rats that performing PET scans after half-maximum inhibition of P-gp with the third-generation P-gp inhibitor tariquidar (TQD) is more sensitive for detecting regional differences in cerebral P-gp function than VMPI baseline scans [1]. In order to translate this concept to humans a detailed understanding of the dose-response relationship of TQD for inhibition of P-gp at the human BBB is required.

**Materials and methods:** Healthy male subjects (n = 3 per dose group) underwent VMPI PET scans and arterial blood sampling at 1 h after infusion of TQD at doses of 3, 4, 6 and 8 mg per kg body weight. Brain uptake of radioactivity was quantified as the ratio of the area under the time-activity curve (AUC) in whole brain and in arterial plasma (AUCbrain/AUCplasma). Radiometabolites of VMPI in plasma were assessed with a previously described solid-phase extraction protocol [2]. Data were pooled with data from a previous pilot study in 5 healthy male subjects, who underwent paired VMPI PET scans before and after administration of 2 mg/kg TQD [2].

**Results:** TQD was well tolerated in all but 1 subject, who belonged to the 8 mg/kg dose group. In this subject the observed effects were smaller for [(R)-11C]Cverapamil PET. In J Nucl Med Mol Imaging 2010, 37:942-953.


**References**


Heterogeneous penetration of cefpirome and moxifloxacin into abscesses after simultaneous administration in humans

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BMC Pharmacology 2010, Volume 10 Suppl 1
http://www.biomedcentral.com/1471-2210/10?issue=S1

Background: Abscesses are often successfully treated with antimicrobial agents when drainage is not feasible, but appropriate data on antibiotic abscess penetration in humans are missing. This study aimed at evaluating and comparing pharmacokinetics of cefpirome and moxifloxacin in the same abscesses to evaluate their eligibility for this indication.

Methods: After simultaneous administration of 2 g cefpirome and 400 mg moxifloxacin to patients drug levels were measured in plasma over 8 h, and in differently located abscesses (n = 12) at incision.

Results: At incision performed 158 ± 112 min after administration, cefpirome concentrations in abscess ranged from below the limit of quantification to 47 mg/L (8.4 ± 14.1 mg/L), and moxifloxacin concentrations ranged from below the limit of quantification to 9.2 mg/L (1.9 ± 3.4 mg/L). Relative to plasma, abscess concentrations of moxifloxacin were significantly higher than of cefpirome (p = 0.037). Inhibitory concentrations of both antibiotics reported for abscess-relevant bacterial species were reached in several, but not in all abscess observations. Antibiotic abscess penetration could not be adequately explained considering covariates such as pH of abscess fluid, or the ratio of surface area to volume of abscesses, linked to plasma pharmacokinetics.

Conclusions: Cefpirome and moxifloxacin were detectable in most abscesses after a single dose and might be eligible if conservative treatment is required. However, antibiotic abscess penetration was highly variable and unpredictable, and clinicians should anticipate insufficient drug levels in some cases.