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MEETING ABSTRACTS (PUBLISHED)

Meeting abstracts:

Cardiovascular Pharmacology and Endocrinology

A1.1

A novel role for adenosine kinase (ADK) in cardiac autophagy

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Background: Proper control of autophagy is important for cardiomyocyte homeostasis and adaptation to stress, as both excessive and insufficient autophagy have been implicated in heart failure development. LC3 is a ubiquitin-like (UBL) protein important for autophagosome formation and processing. Lipidation of LC3-I (conjugation to phosphatidylethanolamine; LC3-II) is vital for its role(s) in autophagy. LC3 lipidation is mediated by the sequential actions of an E1-activating enzyme, ATG7, an E2-conjugating enzyme, ATG3, and an E3-like ligase composed of ATG12–ATG5. While significant progress has been made in understanding how metabolic stress induces formation of autophagosomes, the physiological signals that restrain basal autophagy, particularly at the initial steps of LC3 activation and lipidation, are undefined. Adenosine exerts numerous protective effects in the cardiovascular system through stimulation of adenosine receptors, but its role in cardiac autophagy is not clear. The main route of myocardial adenosine removal is through intracellular phosphorylation and recycling into the adenine nucleotide pool by adenosine kinase (ADK). Here we used the ADK inhibitor, ABT-702, to investigate the role(s) of adenosine and ADK activity in cardiomyocyte autophagy.
Methods: To examine the *in vivo* role of ADK and adenosine signaling on cardiac autophagy, mice were injected i.p. with the ADK inhibitor, ABT-702 (10 mg/kg; 1–3 hrs) in the presence or absence of the adenosine receptor antagonist, theophylline (20 mg/kg). Body temperature was measured as an indication of globally increased interstitial adenosine. LC3-I, LC3-II, ATG7 (E1), ATG3 (E2), and ATG12–ATG5 (E3) proteins, as well as phospho-AMPK^{Thr172} and phospho-p70S6k^{Thr389} were measured by western blot. LC3 and ATG12 thioesters were examined by western blot under non-reducing conditions. For autophagic flux analysis, bafilomycin (3 µm/kg) was injected to inhibit lysosomal LC3 degradation. To examine the role of adenosine metabolism in cardiomyocyte LC3 lipidation and autophagic flux, cultured neonatal rat ventricular cardiomyocytes (NRVMs) were treated with adenosine (10 µM, 5 hrs) in the presence or absence of ABT-702 (0.3 µM) and/or bafilomycin (50 nM) and analyzed by western blot and immunofluorescence for LC3-II- and LC3-positive vesicles, respectively.
Results: ABT-702 increased cardiac LC3-II levels within 1 to 3 hours *in vivo*. The ABT-702-induced increase in LC3-II was not blocked by adenosine receptor antagonism with theophylline, indicating an adenosine receptor-independent mechanism. Co-administration of ABT-702 and bafilomycin further increased LC3-II formation, indicating that ADK inhibition increases LC3-II synthesis, rather than blocking its degradation. Conversely, treatment of NRVMs with adenosine inhibited LC3-II formation, and this effect was reversed by ABT-702 treatment, indicating that ADK metabolism of adenosine inhibits LC3 lipidation. *In vivo*, ABT-702 treatment increased formation of DTT-sensitive ATG12~ATG7 and LC3~ATG7 thioester complexes and increased ATG12–ATG5 conjugation prior to greater increases in LC3-II, indicating that ABT-702 stimulates ATG7 activity.
Discussion: These findings indicate that ADK metabolism of adenosine restrains LC3 lipidation and autophagy in the heart. Thus, inhibition of ADK with ABT-702 may provide a novel approach for increasing autophagy in protein aggregate related diseases of the heart and possibly other organs.
Acknowledgements: This research was supported by the Austrian Science Fund FWF (project P 31083-B34).

A1.2

Mechanism of sustained vascular smooth muscle relaxation by nitroglycerin

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Background: Biotransformation of nitroglycerin (glyceryltrinitrate, GTN) to nitric oxide (NO) is catalyzed by aldehyde dehydrogenase-2 (ALDH2). Since the active-site cysteine of ALDH2 is oxidized in the course of the reaction, thiols are required to sustain catalysis. While dithiothreitol (DTT) may perform this function *in vitro*, a similarly effective reductant has not been reported in cells. Within the context of ALDH2-catalyzed GTN biotransformation, the development of nitrate tolerance has been tentatively explained by irreversible turnover-dependent ALDH2 inactivation. However, the occurrence of this phenomenon in cells has not been investigated.
Methods: In the present study we expressed a recently developed fluorescent intracellular NO probe in vascular smooth muscle cells (VSMC) that also overexpress a double ALDH2 mutant (C301S/C303S). This mutant exhibits higher NO formation rates and shows a greater tendency towards irreversible inactivation than the wild-type enzyme. In these cells we determined the kinetics of formation and decay of GTN-derived NO and compared the results with GTN-induced relaxation of rat thoracic aortas.
Results: As expected, ALDH2 catalyzed sustained formation of NO in the presence of DTT, but only a short burst of NO, corresponding to a single turnover of ALDH2, in its absence. However, even without DTT the burst phase was followed by low nanomolar NO generation, suggesting slow regeneration of reduced ALDH2 by an endogenous reductant. In addition to the thiol-reversible oxidation of ALDH2, thiol-refractive, turnover-dependent inactivation was observed as well. Nevertheless, organ bath experiments with rat aortas showed that GTN caused longer-lasting relaxation than the NO donor diethylamine NONOate.
Discussion: Our results demonstrate that an endogenous reductant allows sustained generation of GTN-derived NO in the low nanomolar range that is sufficient for vascular relaxation. On a longer time scale, turnover-dependent irreversible inactivation of ALDH2 may render blood vessels tolerant to GTN. These results suggest that there may not be an efficient intracellular reductant of oxidized ALDH2 and that the inefficiency of ALDH2 reactivation may actually allow the enzyme to generate low but sufficient concentrations of NO over longer periods.
Acknowledgements: This work was supported by the Austrian Science Fund FWF (grant P24946).

A1.3

Regulatory interaction of cytoskeleton-associated proteins with soluble guanylyl cyclase in vascular smooth muscle

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Background: Nitric oxide (NO) and its target soluble guanylyl cyclase (sGC) play a central role in vessel homeostasis by initiating vasodilation via the NO/cGMP signaling pathway. In that regard sGC has become an important target in the therapy of cardiovascular diseases. Thus, compounds have been developed to pharmacologically modulate the enzyme. However, little is known about endogenous modulators of sGC. In a previous study we found an increase in maximal sGC activity *in vitro* caused by the presence of cytosolic preparations from porcine coronary arteries. The active principle, further referred to as sGC-activating factor (sGC-AF), could be enriched by ammonium sulfate fractionation of cytosols, indicating that it might be a protein. In the present study we isolated and identified the constituents of sGC-AF.
Methods: Column chromatography with an Äkta FPLC system utilizing hydrophobic interaction, ion exchange, and size exclusion (gel filtration), respectively, was used to isolate sGC-AF. SDS-PAGE and LC-MS/MS were conducted to identify the obtained proteins. sGC-AF activity was monitored by co-incubation of purified bovine lung sGC with the respective fractions and measurement of conversion of [α-^{32}P]GTP to [^{32}P]cGMP under stimulation with NO.
Results: The developed purification strategy yielded a protein mixture consisting of three major bands in SDS-PAGE. The respective 100, 70 and 40 kDa bands were identified as gelsolin, annexin A6, and actin, respectively. These proteins are all related to or part of the cytoskeleton/contractile elements of smooth muscle. Investigations addressing the interaction of sGC-AF with sGC revealed a heme-dependent mode of action. It was independent from the applied NO donor and was not linked to a redox process. In addition, the effect was not mimicked by bovine serum albumin or superoxide dismutase.
Discussion: The proteins obtained in the present study are all closely linked to the contractile apparatus of smooth muscle. While actin represents the basic framework of contractile elements, gelsolin and annexin A6 are involved in severing and membrane anchoring of actin, respectively. As the individual proteins were inactive it is presumed that sGC-AF is a protein complex rather than a single protein. The increase in activity caused by sGC-AF was similar to that observed with saturating concentrations of the sGC stimulator BAY 41-2272. This indicates that sGC-AF converts the NO-bound enzyme into a more active conformation. These results point towards an interaction of proteins of the contractile apparatus with sGC in a regulatory manner.
Acknowledgements: This work was supported by the Austrian Science Fund (P24946).

A1.4

Safety, tolerability, pharmacokinetics and pharmacodynamics of parenterally administered dutogliptin: a prospective dose-escalating trial

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Background: Animal studies suggest that inhibition of dipeptidyl peptidase 4 (DPP-4) may improve heart function and survival after myocardial infarction by increasing cardiac myocytes' regenerative capacity. Parenterally administered dutogliptin may provide continuous strong DPP-4 inhibition to translate these results into humans. This trial investigated the safety and tolerability as well as pharmacokinetics and pharmacodynamics (PK/PD) of parenterally administered dutogliptin after single and repeated doses.
Methods: In an open-label trial, volunteers received dutogliptin at increasing doses of 30–120 mg subcutaneously or 30 mg intravenously in the single-dose cohorts. Subjects in the multiple-dose

cohort received 60 mg, 90 mg or 120 mg dutogliptin subcutaneously once daily on 7 consecutive days.

Results: Forty healthy males were included in the trial. No related serious adverse events occurred. Out of the 153 related adverse events, 147 (96%) were mild local injection-site reactions, which did not require any medication. Subcutaneous bioavailability was approximately 100%. Multiple dose injection did not lead to accumulation of the study drug. All subjects receiving ≥ 60 mg dutogliptin yielded a maximum DPP-4 inhibition > 90%. The duration of DPP-4 inhibition over time increased in a dose-dependent manner and was highest in the 120 mg multiple-dose cohort, translating into 86% DPP-4 inhibition 24 hours after dosing.

Discussion: Parenteral dutogliptin had a good safety profile overall. Subcutaneous injection of dutogliptin resulted in approximately 100% bioavailability with peak plasma concentrations of 5,000 ng/ml after subcutaneous injection of 120 mg. Compared to 500 mg orally administered dutogliptin, this translates into a > 6-fold increase in maximal plasma levels [1]. The half-life of oral dutogliptin was 3-fold longer [1] than that after i.v. or s.c. dosing in the current trial. The apparently longer half-life after oral intake may probably be due to prolonged (but incomplete) resorption after oral intake. Subcutaneous injection of 120 mg dutogliptin reduced DPP-4 activity to below 6%, translating into > 85% DPP-4 inhibition over 24 hours. In comparison, currently available oral doses and formulations of gliptins are capable of reducing DPP-4 activity by 60–80% over 24 h [2]. However, besides its well-known function of improving glycemic control, DPP-4 activity influences several other pathways providing potential cardio- and renoprotective effects [3]. Notably, while low administration of the DPP-4 inhibitor vildagliptin could not yield benefits in cardiac function after infarction [4], 6 times higher doses, applied twice daily, indeed improved cardiac remodeling and renal function in a rat model of heart failure [5]. Yet, it is unclear, if and which degree of DPP-4 inhibition will reduce maladaptive cardiac remodeling in humans which may putatively translate into improved survival and quality of life. Clinical data to determine the required levels to facilitate beneficial cardiac remodeling post-infarction in humans are lacking. Large-scale clinical phase II/III trials involving patients after myocardial infarction are warranted to determine whether DPP-4 inhibition achieved by parenterally administered dutogliptin can prevent maladaptive remodeling.

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A1.5

Ca^{2+} *vs.* Na^{+}: How come Ca^{2+} ion channels are so specific?

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Background: Cations, such as H^{+}, Na^{+}, K^{+}, Ca^{2+} and Mg^{2+}, are essential for a variety of biological processes in every cell. Several of these processes depend on a discrimination between different ions with a very high accuracy. Ion channels are membrane proteins that allow ion flow through an otherwise ion-impermeable cell membrane upon a specific signal. These channels discriminate different ion types at their so called selectivity filter. Calcium and sodium channels share a rather similar selectivity filter, based on their amino acid sequence. However, calcium channels are highly selective towards calcium, with a ratio of 1000 : 1. This is intriguing, given the fact that the sodium concentration in the extracellular lumen is 70-fold higher than the calcium concentration and calcium and sodium ions are of approximately the same size. How this high selectivity can be achieved is a fundamental and long-standing question in this field.

Methods: With our first aim of studying the coordination sphere of different ions in the context of the selectivity filter, we limited the $Ca_V1.1$ structure [1] to its selectivity filter (four times TxExW) and parts of the P-helices for running the QM/MM MD simulations. Three systems were built: one with the Ca^{2+} ion, one with a Na^{+} and one with a K^{+} ion. The extension of the self-consistent-charge density-functional tight-binding method DFTB3 was used to treat the QM region in these initial studies (PM6 for Na^{+}) [2]. All the QM/MM MD simulations were run for 20 ps and only the ion was included in the QM region. This region was polarized by the environment within the electrostatic embedding scheme. The positions of all the alpha carbons were restrained with a harmonic constant (5 kcal mol^{-1} $Å^{-2}$) except for those residues that define the selectivity motif.

Results: The coordination sphere of Ca^{2+} remains similar to the cryo-EM model along the MD simulation: Glu292 and Glu1014 bind to the cation with one carboxylic oxygen and Glu614 binds with both oxygens. Four water molecules complete the first coordination shell (coordination number of 8). Two of these water molecules bridge the cation with Glu1323. In contrast, for K^{+} we observe significant changes in the coordination sphere. The coordination sphere is a distorted octahedron where the side chain of Glu1014 has moved apart from the monovalent ion. Additionally, one of the water molecules has been substituted by the backbone oxygen of Gly1322.

Discussion: This study gives mechanistic insights into Ca^{2+} *vs.* Na^{+} selectivity at the highly selective ion binding sites in a calcium channel and their water hydration shell throughout the conduction process.

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A1.6

12-Monoketocholate: a new perspective in metabolic syndrome treatments

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Background: Recent studies have revealed that bile acids (BAs) are not only facilitators of dietary lipid absorption but also important signaling molecules exerting multiple physiological functions. 12-Monoketocholate (12-MKC) is a stable semisynthetic bile acid salt with low toxicity. It has shown significant hypoglycemic activity in its own and a potential to enhance absorption of various active principles that are used in prevention and treatment of dislipidemia, diabetes mellitus and hypertension. This review summarizes recent analyses of 12-MKC as a potential therapeutic agent and development of novel 12-MKC-based therapeutics for treating disorders in metabolic syndrome.

Methods: The data of 12-MKC effects in metabolic syndrome have been provided from review and original scientific articles, published from 1999 to 2018. The research was performed using the following key words: bile acids, 12-MKC, diabetes, obesity, metabolism.

Results: A study conducted by Mikov *et al.* [1] indicated that after a nasal administration of 12-MKC alone in rats with type 1 diabetes, the glucose concentration was about 36% less than that obtained after subcutaneous insulin administration. The authors also confirmed that the oral administration of 12-MKC decreases the blood glucose level in diabetic rats. A recent study [2] has shown that the combination of 12-MKC and gliclazide exhibits even a better glycemic control in probiotic pretreated diabetic rats than 12-MKC alone. Overviewing numerous studies about 12-MKC as a potential adjuvant it has been derived that after oral administration in rats, 12-MKC has a promotory effect on the action of gliclazide, lovastatin, stevioside and enhances nasal permeation of insulin.

Discussion: 12-MKC has positive effects in metabolic syndrome, but the mechanisms remain poorly understood. Activation of farnesoid X receptor (FXR) and G protein-coupled bile acid receptor (TGR5) signaling pathways is one of the possible explanations. By activating FXR, BAs supress phosphoenolpyruvate carboxykinase (PEPCK), which is the rate-limiting enzyme of gluconeogenesis. In addition, enzymes such as glucose 6-phosphatase and fructose 1,6-bisphosphatase 1 which also participate in gluconeogenesis are shown to be repressed by BAs. The TGR5 signaling pathway stimulates energy expenditure in both brown adipose tissue as well as skeletal muscle. Suggested mechanisms of permeation enhancement involve 12-MKC effect on the efflux transporters in various tissues and the solubilization effect of this salt. Since 12-MKC is less hydrophobic and has a higher critical micellar concentration (CMC) than other bile salts, toxicity is minimised. Therefore, 12-MKC may serve as a potent therapeutic approach for the treatment of obesity, type 2 diabetes, and other components of the metabolic syndrome in humans.

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A1.7

Nitric oxide inhibits adipogenesis by *S*-nitrosation of CCAAT/enhancer-binding protein β

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Background: Within the last decades the prevalence of adipositas, obesity and associated diseases has been escalating world-wide highlighting the need for development of effective therapeutic concepts. 3T3-L1 adipocytes share many similarities with primary fat cells and represent a reliable *in vitro* model of adipogenesis. The aim of the present study was to investigate the effect of nitric oxide on adipocyte differentiation.

Methods: Adipogenesis was experimentally induced with a mixture of insulin, dexamethasone, and 3-isobutyl-1-methylxanthine in the absence and presence of increasing concentrations of *S*-nitrosoglutathione (GSNO) and diethylenetriamine NONOate (DETA/NO). After 7 days cells were harvested and analyzed for protein and triglyceride content as well as for mRNA and protein expression of early and late transcription factors and markers of terminal differentiation. *S*-Nitrosation and transcriptional activity of CCAAT/enhancer-binding protein β (C/EBPβ) were measured by biotin switch assay and dual luciferase reporter assays, respectively.

Results: GSNO exerted a prominent anti-adipogenic effect evident as reduced cellular triglyceride and protein content as well as decreased mRNA and protein expression of late transcription factors (*e.g.* peroxisome proliferator-activated receptor γ) and markers of terminal differentiation (*e.g.* leptin). By contrast, GSNO did not affect mRNA and protein expression of C/EBPβ, which represents a pivotal early transcription factor of adipogenesis. Differentiation was also inhibited by the NO donor DETA/NO. Biotin switch experiments showed significantly increased *S*-nitrosation of C/EBPβ variants liver-enriched transcriptional activator protein*, liver-enriched transcriptional activator protein, and liver-enriched inhibitory protein. Moreover, transcriptional activity of C/EBPβ was significantly reduced by the NO donor.

Discussion: Our data demonstrate that posttranslational *S*-nitrosative modification of C/EBPβ accounts for the anti-adipogenic effect of NO, suggesting that *S*-nitrosation represents an important physiological concept to control fat cell maturation.

Immunopharmacology and Infection

A2.1

Apolipoprotein A-IV potently suppresses eosinophil responsiveness *in vitro* and alleviates house dust mite-induced airway hyperreactivity in mice

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Background: Eosinophil accumulation orchestrated by allergic sensitization and T_h2-mediated immune response is a hallmark of allergic inflammation as observed in allergic rhinitis and severe asthma. Recent studies pointed out a crucial role for apolipoproteins in the pathogenesis of inflammatory diseases. However, the role of apolipoprotein A-IV (apoA-IV) in allergic inflammation has not been addressed thus far. Here, we explored the signaling mechanism and anti-inflammatory effects of apoA-IV on eosinophil effector function *in vitro* and *in vivo*.
Methods: *In vitro* studies included apoA-IV measurement in serum of healthy and allergic individuals, as well as migratory responsiveness, respiratory burst and calcium mobilization of human peripheral blood eosinophils. Allergen-driven airway inflammation was assessed in a mouse model of acute house dust mite (HDM)-induced asthma.
Results: ApoA-IV levels were significantly decreased in serum from allergic patients compared to healthy controls. Recombinant apoA-IV potently inhibited eosinophil responsiveness by means of shape change, integrin expression and chemotaxis. We were able to elucidate the underlying molecular mechanism, which was independent of ABCAI and SRBI binding but involved Rev-ErbA-α. Moreover, apoA-IV induced the PI3K/PDK1-dependent activation of PKA. Of note, systemic application of apoA-IV clearly prevented AHR and reduced the influx of inflammatory cells into the airways in a murine model of HDM-induced allergic asthma.
Discussion: ApoA-IV is an endogenous anti-inflammatory protein that potently suppresses eosinophil effector function. Here, we provide new insights into the molecular mechanisms underlying the apoA-IV-induced signaling in eosinophils. Our data indicate that exogenously added apoA-IV may represent a novel pharmacological approach for the treatment of allergic inflammation and other eosinophil-driven disorders.
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A2.2

Antioxidant and hepatoprotective activity of winter savory (*Satureja montana* L.) extract

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Background: The presence of secondary metabolites such as flavonoids, sterols, essential oils and tannins in the *Satureja* genus has various medical properties. Recent studies indicated potentially useful pharmacodynamic effects of *Satureja montana* L., such as antifungal and antibacterial activity, antioxidant, antidiabetic, antihyperlipidemic, as well as expectorant and vasodilatory effects. The aim of our study was to investigate the influence of Winter savory extract on biochemical parameters of liver and kidney function in serum, and antioxidant potential in rats exposed to oxidative stress using toxic doses of paracetamol.
Methods: The research was conducted on half-mature Wistar rats, divided into four groups of 6 animals each. The animals were pretreated orally for 7 days with Winter savory extract and saline followed by a toxic dose of paracetamol (600 mg/kg). Rats were sacrificed by cardiopunction, then blood and liver samples were taken to determine biochemical parameters of oxidative stress in serum and in the liver homogenate.
Results: The application of a toxic dose of paracetamol significantly increased the activity of liver transaminases in the serum compared to control ($p < 0.05$). Winter savory extract prevented the damage of liver tissue measured by the activity of oxidative stress enzymes, which was significantly higher in animals that were not pretreated with the extract before the toxic doses of paracetamol. Indicators of hepatic and kidney functions, as well as the concentration of oxidative stress enzymes, were significantly lower in animals that were pretreated with Winter savory extract compared with the group that received paracetamol alone.
Discussion: The results of this study showed that hepatotoxicity induced by a toxic dose of paracetamol was revealed by significant increase in activity of liver transaminases. The use of paracetamol toxic dose in the group pretreated with physiological saline led to a statistically significant increase in alkaline phosphatase (ALP) activity, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in rat serum compared to control. Activity of liver transaminases were lower in group of animals that were treated with Winter savory extract before paracetamol compared to animals that were treated with saline and paracetamol. Concentrations of malondialdehyde (MDA), catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) in rat serum were higher in the group of animals that were treated with toxic dose of paracetamol compared to animals pretreated with Winter savory extract before paracetamol. The results of our study are in accordance with the results of earlier studies using an *in vitro* model. The toxic dose of paracetamol leads to a significant disorder of biochemical parameters, liver and kidney function indicators and oxidative stress indicators. Pretreatment with Winter savory extract prior to the administration of the toxic dose of paracetamol improves biochemical and oxidative stress parameters. Improvement of antioxidant properties could be explained by the presence of different phenolic and terpenic compounds.
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Neuropharmacology and Neurosciences

A3.1

Mad2, a novel player in clathrin-mediated endocytosis, interacts with monoamine transporters

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Background: Monoamine transporters conduct the reuptake of serotonin (SERT), dopamine (DAT) and norepinephrine (NET) following neurotransmission. Surface levels and subcellular localization of

transporter proteins can be regulated by clathrin-mediated endocytosis (CME), during which cargo proteins internalize as part of surface-derived membrane vesicles. This process requires adaptor protein 2 (AP-2), which links cytosolic domains of cargo proteins to a cage of clathrin proteins. This leads to the formation of intracellular clathrin-coated vesicles. It is currently unknown how monoamine transporters connect to clathrin, as their intracellular domains do not provide any known interaction motif for AP-2 binding. Nevertheless, the carboxy-terminus of the transporter was shown to play a central role for its internalization [1]. Recent work shows that the insulin receptor (IR) interacts with AP-2 via a heterodimer of two mitotic spindle assembly checkpoint (SAC) proteins: Mad2 and Bubr1 [2]. A classical Mad2-interacting motif (MIM) in the IR C-terminus is crucial for AP-2 recruitment. Inspection of monoamine transporter C-termini reveals putative MIMs, similar to those found in other Mad2-interacting proteins (IR; CDC20; Mad1). Considering this similarity and the acknowledged but opaque role of the transporter C-terminus for endocytosis, it is reasonable to hypothesize that Mad2 initiates clathrin-mediated endocytosis of neurotransmitter transporters.

Methods: A combination of biochemical methods (GST pulldown, co-immunoprecipitation) was used to study a putative SERT–Mad2 interaction. Consequences of Mad2 depletion on SERT surface expression and subcellular localization were investigated using siRNA-mediated knock-down.

Results: The conducted experiments clearly show an interaction between Mad2 and monoamine transporters at the cellular surface. This interaction is dependent on the Mad2-interacting motif in the transporter C-terminus. Interestingly, the cognate transporter GAT1 misses Mad2 interaction. Mad2 depletion in YFP-SERT-expressing cells causes significant increase of SERT surface expression and differential SERT glycosylation. Furthermore, Mad2 knock-down depletes intracellular membrane compartments from YFP-SERT, indicating disturbed endocytosis.

Discussion: These results suggest a role for Mad2 during endocytosis of monoamine transporters. Since Mad2 shows marked expression in the brain, it is plausible to assume that the investigated interaction also occurs in a native biological system. Hence, this work could provide an answer to the puzzling question of the interplay between the monoamine transporter C-terminus and the endocytic machinery.

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A3.2

para-Substituted methcathinones as selective and unselective inhibitors of human dopamine and serotonin transporter

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Background: Methcathinone (MCAT) is a compound belonging to the class of cathinones and it is targeting monoamine transporters including DAT and SERT. Despite the importance of DAT and SERT as drug targets in several neurological disorders, the key factors underlying the selectivity profile of their inhibitors is still poorly understood. Recent findings from rat synaptosomes suggested that increasing the volume of the *para* substituent of MCAT results in a swap of the selectivity between human DAT and SERT [1]. Docking studies hint towards Ser149 in DAT and Ala169 in SERT as key residues involved in the difference of activity between DAT and SERT [2]. The aims of the present biochemical and pharmacological study are to understand (i) which chemical properties (***e.g.*** volume, polarity or lipophilicity) of the *para* substituent influence the selectivity profile of MCAT between DAT and SERT, and (ii) whether Ser149 in DAT and Ala169 in SERT can be experimentally verified as key residues.

Methods: We combined *in silico*-driven synthesis, mutagenesis, radiotracer flux assays and electrophysiology in HEK 293 cells expressing the human DAT and SERT wild type and respective mutants.

Results: We found that only MCAT and CF_3-MCAT showed high selectivity: 200-fold for DAT/SERT and 25-fold for SERT/DAT, respectively. This suggests that the high selectivity achieved is determined rather by specific features of these compounds than by the volume of the *para* substituent. Accordingly, we were not able to find any correlationship between the selectivity profile of the tested four MCATs with either volume, polarity and lipophilicity parameters. In addition, we have tested the *para*-substituted methcathinones in the swapping mutations DAT Ser149Ala and SERT Ala169Ser, and in line with our hypothesis these mutations did not revert the selectivity profile found in the wild-type transporters.

Discussion: Our findings provide insights on the introduction of the CF_3 group in *para* position of methcathinone showing that (i) this modification is sufficient to turn a DAT-selective agent into a SERT-selective agent, and that (ii) this effect is not dependent on the volume of the *para* substituent but on specific chemical features of the fluorine atoms which may influence the on- and off-rate of the MCAT moiety on DAT and SERT. The present study may be useful for developing new therapeutic approaches for different neurological disorders, such as depression or post-traumatic stress disorder, or for the development of new PET tracers.

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A3.3

Exploiting non-classical pharmacology of monoamine transporters to address multiple disorders associated with transporter conformational states

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Background: Monoamine transporters (MATs) encompass transporters for serotonin (5-HT), dopamine (DA) and norepinephrine (NE) that are expressed in cognate monoaminergic neurons. They shuttle monoamines from the synaptic cleft back into nerve terminals effectively terminating synaptic transmission and ensuring vesicular replenishment. Perturbed MAT functioning leads to advent of several neurological disorders. Most MAT mutations lead to ER-retained transporters and subsequent loss of function. Such mutations in DAT cause an infantile form of parkinsonism/dystonia. MATs also have a rich pharmacology binding to a plethora of exogenous ligands that act as either non-transportable inhibitors (*e.g.* cocaine) or transportable substrates (*e.g.* amphetamines). These drugs increase extracellular monoamine levels by inhibiting MAT function. While MAT ligands have been used as medications for certain psychiatric disorders, most are abused as recreational psychostimulants often leading to addictive disorders. Recent discovery of ligands that display atypical pharmacology at MATs has been a subject of intense research for treatment against addiction. These include atypical inhibitors and partial substrates whose mechanisms of action are unknown, but are assumed to stabilize certain transporter conformational states distinct from those on binding of cocaine and standard amphetamines. We hypothesize stabilization of these unique conformational states also confer atypical MAT ligands the ability to rescue ER-retained MAT mutants.
Methods: We used electrophysiological recordings to probe MAT conformational states stabilised by PAL-1045, a naphthyl propan-2-amine that was shown earlier to possess partial efficacy in inducing neurotransmitter efflux through SERT and DAT when compared to standard amphetamines. Using whole-cell patch clamping of HEK 293 cells stably expressing hSERT, we compared current profiles, under constant voltage, elicited by PAL-1045 in comparison to those elicited by 5-HT and a standard amphetamine *para*-chloroamphetamine (pCA). Binding kinetics of these substrates was also determined using electrophysiological means, and these rates were compared to those determined by standard radiotracer assays. Pharmacochaperoning abilities of PAL-1045 were tested on a SERT mutant (SERT-PG^{601,602}AA) using a combined immunoblotting, functional uptake assays and confocal microscopy approach.
Results: Under physiological conditions, PAL-1045 induced currents that showed a bell-shaped concentration–response curve over a range of 0.3–30 µM as opposed to Michaelis-Menten current profiles for 5-HT and pCA. This is indicative of internal PAL accumulation on membrane diffusion and high-affinity binding to inward-open state of SERT. Binding kinetics determined by electrophysiological recordings to outward-open SERT were in excellent agreement with those determined by radiotracer assays. Both readouts indicated low nM affinities for PAL-1045 governed by poor dissociation rates. 5-HT and pCA, on the other hand, show µM affinities. Owing to high-affinity binding to multiple conformational SERT states, PAL-1045 restored surface expression and transporter activity of non-functional folding-deficient mutant SERT-PG^{601,602}AA.
Discussion: The present study aims to unify anti-addictive properties of certain drugs with their previously unknown pharmacochaperoning capabilities. This hypothesis is currently being extended to rescuing of all documented DAT variants associated with infantile parkinsonism/dystonia expressed in heterologous systems with >50 atypical DAT ligands.
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A3.4

Binding behavior of different benzodiazepine ligands implies the use of more than one binding pose in their interaction with GABA$_A$ receptors

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Background: GABA$_A$ receptors are ligand-gated chloride channels, one of the major inhibitory receptors in the central nervous system. GABA$_A$-Rs are heteropentamers made up from 19 known subunits and are targets for many clinically important drugs. Among them are the family of the widely used benzodiazepines (BZs), which bind to GABA$_A$-Rs at the α+/γ2− interface. Understanding the particular molecular interaction of BZs and their GABA$_A$-R binding site is of crucial importance in developing new BZ ligands. It is assumed that all BZs interact with the receptor alike in a “common” binding mode, whereby three were suggested (CBM I, CBM II and CBM III). However, it is still argued in literature which is the most probable one. Some docking studies suggest CBM I, while others support CBM II. Previous docking studies in our lab suggested that a chiral methyl group, (position 3 of the 7-membered diazepine ring) could be used as a clinical reporter. Accordingly, we performed new computational modelling, which confirmed that ligands having a methyl group both in the (*R*)- as well as in the (*S*)-conformation can bind in binding pose I. If the drug, however, binds in binding pose II, only (*S*)-isomers will be able to bind, since (*R*)-isomers are sterically hindered. The aim of the current study was to test this hypothesis and provide experimental evidence in favor of one or the other binding mode.
Methods: Several sterosisomeric drugs from three different structural BZ classes, namely diazepam-, imidazobenzodiazepine- and triazolam-derivatives were investigated. We used [^{3}H]flunitrazepam displacement as well as two-electrode voltage-clamp electrophysiology in recombinantly expressed GABA$_A$-R subtypes containing α1β3γ2, α2β3γ2, α3β3γ2 and α5β3γ2 to determine ligand binding and functional activity of the three BZ classes.
Results: Interestingly, both imidazobenzodiazepine (*S*)- and (*R*)-isomers exhibited comparable binding affinities while the other two classes displayed a dramatic difference in binding affinities. Thereby, the (*R*)-isomers showed complete loss of binding ability whereas the (*S*)-isomers remained active.
Discussion: As predicted from our computational modeling, our experimental data indeed could provide insight into the nature of the interaction of different BZ with their specific GABA$_A$ receptor binding site. Surprisingly, the tested ligands did not behave in an identical

manner. According to our results, we could conclude that different chemically related benzodiazepine ligands tend to interact via different binding modes rather than using a common one.

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A3.5

N-Acetylcysteine reverses the anxiogenic effects of cisplatin in rats

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Background: Since the numerous side effects of cisplatin treatment seem to be accompanied with increased oxidative stress, the aim of this study was to estimate the possible beneficial effects of *N*-acetylcysteine (NAC) supplementation along with cisplatin administration in order to prevent the behavioral adverse effects of cisplatin.
Methods: Thirty-two male Wistar albino rats (250–300 g, 3 months old) were randomly divided in four equal groups: control, cisplatin (treated with single intraperitoneal injection of cisplatin, 5 mg/kg), NAC (single intraperitoneal injection of *N*-acetylcysteine, 500 mg/kg) and cisplatin plus NAC (simultaneous administration of cisplatin and NAC, 5 and 500 mg/kg, respectively) group. The behavioral testing was performed 5 days following the treatment by means of open field (OF) and elevated plus maze (EPM) tests.
Results: The anxiogenic effect of cisplatin was manifested through decrease in the cumulative duration and frequency to center zone in OF (60%, $p < 0.01$), as well as by reduction of cumulative duration and frequency to open arms in EPM test (70% and 65%, respectively; $p < 0.01$). Cisplatin administration also reduced the locomotor activity in both tests, expressed as total distance moved (45%; $p < 0.01$). The diminished exploratory activity following cisplatin treatment was manifested by means of decline in the number of rearings (65%; $p < 0.01$) in the OF test and the total exploratory activity episodes in the EPM test (60%; $p < 0.01$). Although the administration of NAC alone did not affect estimated behavioral parameters, simultaneous administration of NAC with cisplatin resulted in the attenuation of cisplatin-induced anxiety patterns. NAC increased the cumulative duration and frequency to center zone in OF (80% and 120%, respectively; $p < 0.01$), as well as the cumulative duration and frequency to open arms in the EPM test (200% and 135%, respectively; $p < 0.01$) compared to the cisplatin group. Also, administration of NAC along with cisplatin reversed the cisplatin-induced decrease in exploratory and locomotor activity to the control values.
Discussion: The results obtained in this study strongly suggest anxiogenic effects of cisplatin administration. However, the anxiogenic effect of cisplatin treatment was significantly attenuated by simultaneous application of NAC.
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A3.6

Elucidating the functional role of extracellular loop 4 in the transport cycle of the serotonin transporter

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Background: The serotonin transporter (SERT), a member of the solute carrier 6 (SLC6) family, is a monoamine transporter that mediates the reuptake of serotonin (5-HT) from the extracellular space. Thus, SERT is the key player in the termination of serotonergic signalling and is involved in the replenishment of synaptic 5-HT stores. SERT is an integral membrane protein comprising 12 transmembrane segments that are linked by 6 extracellular (EL) and 5 intracellular loops (IL). Studies on the functional role of structural motifs mostly rely on site-directed mutagenesis. Structural knockdown by mutagenesis, however, often results in a loss of function as a consequence of impaired protein folding and plasma membrane trafficking. Hence, a tool is required that allows to address the functional role of a structural motif of interest while protein folding and trafficking remains unaffected.
Methods: Whole-cell patch-clamp experiments were performed in human SERT (hSERT) expressing HEK 293 cells. Fast exchange of the bath solution (exchange rate ~100 ms) was ensured by a pressure-driven application device (Octaflow). 5-HT-induced currents were recorded in hSERT-expressing cells in the absence and presence of anti-SERT-EL4 antibody. In addition, ligand-binding (5-HT and anti-SERT-EL4) to SERT was assessed by capacitance measurements.
Results: Binding of anti-SERT-EL4 antibody to SERT reduced the membrane capacitance and blocked the 5-HT transport in a dose-dependent manner. The association and dissociation rate constants (k_{on} and k_{off}) of the antibody were calculated as 3.055 ± 0.631 $M^{-1}s^{-1} \cdot 10^7$ and 1.352 ± 0.082 s^{-1}, respectively.
Discussion: Our data indicate that employing antibodies as tools is a promising approach to study structural motifs by electrophysiological techniques. Blockade of the 5-HT transport upon the restriction of EL4 movement in SERT by antibody binding shows that EL4 has a role in the conformational changes on SERT required for 5-HT transport. Further experiments are planned by producing Fabs from anti-SERT-EL4 antibody to eliminate dimerization possibility.
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A3.7

4-Phenylbutyrate corrects folding-deficient creatine transporter-1 variants associated with the creatine deficiency syndrome

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Background: The human creatine transporter 1 (hCRT-1, SLC6A8) is a member of the sodium-dependent neurotransmitter transporter family. Creatine transporter deficiency (CTD) has been associated

with a number of disorders, ranging from epilepsy to mental retardation, autism, development delay, behavior problems, motor dysfunction to gastrointestinal symptoms. Diseases arising from misfolding of other proteins belonging to the SLC6 protein family have been reported in the literature; *e.g.* mutations in the dopamine transporter (DAT) cause infantile parkinsonism/dystonia. In CRT-1, sixteen mutations have been linked to the creatine deficiency syndrome in people [1]. One of these variants is a conservative mutation G132V, associated with severe mental retardation in children. Interestingly, the mutation of an equivalent glycine residue in *Drosophila melanogaster* DAT (G108Q-dDAT) leads to a sleepless phenotype in flies [2]. In the present study we examined the molecular basis of CTD-associated mutations and the pharmacological means by which these can be functionally recouped.

Methods: We generated 16 CTD-causing mutations in hCRT-1 by site-directed mutagenesis (Quickchange, Stratagene). All mutants were pharmacologically characterised by performing [^{3}H]creatine assays, as well by confocal microscopy and biochemical techniques.

Results: By creatine transport activity assays, we showed that most CTD mutants have less than 5% of the activity of the wild-type hCRT-1. Confocal microscopy experiments showed that the mutants are retained in the endoplasmic reticulum (ER) compartment, in a complex with an endogenous folding sensor calnexin, whereas the wild-type transporter reaches the cell surface. Using immunoblotting of detergent lysates, the mature fully-glycosylated band was only present in the wild-type hCRT-1 samples. Upon treatment with 4-phenylbutyrate, the surface expression and uptake activity of several mutants was restored.

Discussion: Treatment with the chemical chaperone 4-phenylbutyrate ought to be an effective therapy for some CTD patients. This work is clinically relevant and ought to grant promising therapeutic options eagerly awaited for by CTD patients worldwide.

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Oncology and Toxicology

A4.1

Deglycosylation of the cytokine co-receptor gp130 reveals a rapid protein turnover, which gives rise to biased signalling

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Background: The signal transducer glycoprotein 130 (gp130) is a common co-receptor for cytokines of the interleukin (IL)-6 family and is heavily *N*-glycosylated. The protein gp130 is expressed ubiquitously, including in cancer cells. Of particular interest, circulating IL-6 levels are elevated in late-stage melanoma patients and predict unfavourable outcome. Interestingly, the cholesterol-lowering drugs, the statins, trigger apoptosis in metastatic melanoma cells, while primary melanoma cells from the radial growth phase are virtually insensitive. Moreover, statins affect the glycosylation machinery in the endoplasmic reticulum by a reduction of endogenous dolichol levels. However, it is unclear whether the glycosylation status of gp130 is altered under these conditions and whether signal transduction is impaired.

Methods: Human melanoma cell lines reflecting early radial growth phase (WM35, WM278, WM793b) and advanced metastatic stages (A375 and 518a2) were investigated for expression of gp130. Glycosylation was probed by PNGase treatment of cell lysates, tunicamycin or simvastatin. Signalling was monitored for phosphorylation of STAT3 and ERK1/2. Treatments were analysed for multiple comparison with ANOVA and *post hoc* Dunnett test for statistical significance at a p value of < 0.05.

Results: The inhibition of *N*-linked glycosylation by tunicamycin resulted in an accelerated migration of gp130 in SDS-PAGE, shifting the 135 kDa band to an apparent molecular mass of 95–100 kDa. In order to confirm deglycosylation, PNGase treatment resulted in a similar pattern of gp130. Deglycosylation by tunicamycin was equally distributed to all melanoma cells, whereas simvastatin-induced deglycosylation was virtually absent in cells from the early disease stage. The kinetics of the deglycosylation revealed a rapid decline of the 135 kDa band with a half-life of 2.5 hours, implicating rapid turnover of this protein. Accordingly, a complete loss of basal STAT3 phosphorylation was observed with tunicamycin, while simvastatin prevented activation only in metastatic melanoma cells. Complementary to these observations, activation of ERK1/2 was significantly augmented in simvastatin-treated metastatic melanoma cells in the presence of IL-6, while primary melanoma cells were hardly affected.

Discussion: In conclusion, simvastatin gives rise to biased signalling via the IL-6 receptor subunit gp130 in a glycosylation-dependent manner.

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A4.2

Unraveling the role of CDK8 in triple-negative breast cancer metastasis

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Background: Cyclin-dependent kinase 8 (CDK8) and its closely related paralog CDK19 are serine/threonine kinases, which are involved in the regulation of transcriptional processes. In a recent study, CDK8 was identified as potential therapeutic target in estrogen receptor (ER)-positive breast cancer cells, as inhibition of CDK8 by chemical compounds or genetic knockdown impaired growth and progression of the breast cancer type *in vitro* and *in vivo*. The role of CDK8 in triple-negative breast cancer (TNBC) cells, however, has not been evaluated so far.

Methods: To meet the issue, murine triple-negative E0771 breast cancer cells were transduced with a vector containing CDK8-specific shRNA or a control vector and examined for proliferation and survival by FACS analysis. *In vivo*, cell lines were orthotopically injected into the mammary glands of immunocompromised NOD scid gamma (NSG) mice. In a second set of experiments cells were injected intravenously and examined for accumulation in the lungs.

Results: Analysis of proliferation and survival revealed no difference between control and CDK8 knockdown E0771 cell lines. In accordance, primary tumor growth of orthotopically transplanted E0771 control and CDK8 knockdown cells did not differ. Notably, mice injected intravenously with E0771 cells harboring a CDK8 knockdown had significant less lung metastasis than E0771 control cells. The finding correlated with a significant downregulation of Snail, Slug and Twist mRNA in CDK8 knockdown cells, indicating impaired epithelial–mesenchymal transition (EMT).
Discussion: Our experiments point to CDK8 to play a crucial role in regulation of EMT, a step important for cells to gain migratory properties and subsequently initiate metastasis. An orthotopic model of breast cancer metastasis is set up to address this step. To further study the therapeutic potential we are currently investigating the underlying mechanisms.
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A4.3

STAT1-deficient mice develop a B-cell malignancy reminiscent of JAK1/2-inhibition-associated B-cell lymhomas in MPN patients

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Background: The highly conserved JAK/STAT signaling pathway regulates proliferation, differentiation, apoptosis and immune responses. Several malignancies are associated with constitutive activation of STAT family members. Activating mutations in STAT3 drive the development of diffuse large B-cell lymphomas. The STAT3 counter-player STAT1 is generally considered a tumour suppressor. We observed that the loss of STAT1 provokes spontaneous haematopoietic tumours in mice.
Methods: Spontaneous hematopoietic tumours were analysed by FACS. The co-existence of a myeloid hyperplasia (MH) and a malignant B-cell disease was assessed via transplantations, myeloid cell depletion and all-*trans* retinoic acid (ATRA) treatment *in vivo*. High-purity sorting of individual haematopoietic cell lineages followed by transplantations were performed to identify the leukemia-initiating cell. Clonality of B cells was assessed by Southern blotting and PCRs for DJ rearrangements. Expression of STAT1-dependent target genes as well as general hallmark genes for B-cell lymphomas were analysed by qPCR. Two independent human patient cohorts were monitored for co-occurrence of myeloproliferative neoplasms (MPN) and B-cell lymphoma. Transcriptional profiles of human and murine patients were compared via RNA sequencing.
Results: STAT1-deficient mice develop an MH, which initially masks a malignant B-cell disease. Upon transplantation, malignant B cells arise and cause a fatal disease. The malignant B cells can be maintained *in vitro*. Transcriptional profiling reveals an up-regulation of *c-Myc*, *Bcl-2*, *SpiB*, *Mef2B*, *Card11* and *Cd274* (*PD-L1*) and down-regulation of *Socs-1*, *Cdkn2a*, *B2m* and *Prdm1*—alterations found in aggressive human B-cell lymphoma. The malignant B cells are already present in the *Stat1*$^{-/-}$ mouse during MPN. Elimination of the myeloid pool via ATRA freed these malignant B cells and allowed them to expand. We observed a similar switch from MPN to aggressive B-cell lymphoma in a subset of human patients upon inhibition of Janus kinase 1/2 (JAK1/2). The inhibition of JAK1/2 eliminates myeloid cells, but appeared to cause a fatal aggressive B-cell lymphoma later on. To identify the global frequency of this adverse effect, 626 MPN patients (557 with conventional, 59 with JAK1/2 inhibitor treatment) from Vienna and 929 (872 *vs.* 57) from Paris were monitored. In the cohort of 626 patients, B-cell lymphomas evolved in 5.8% upon JAK1/2 inhibition compared to 0.36% with conventional treatment (16-fold increased risk). A similar increase was observed in the independent cohort of 929 MPN patients. Comparison of transcriptional profiles identified 213 genes with overlapping expression patterns in murine and human patients. As in MH^{+} *Stat1*$^{-/-}$ mice, a significant proportion of MPN patients who developed an aggressive B-cell lymphoma harboured clonal B cells, which already existed during MPN.
Discussion: We conclude that JAK/STAT1 pathway inhibition in MPN is associated with an elevated frequency of aggressive B-cell lymphomas. Detection of a pre-existing B-cell clone will identify individuals at risk.
Acknowledgements: Austrian Science Fund (FWF-SFB 28, FWF-SFB 6101, 6103, 6106, 6107, P24295-B23, P27248-B28, P27132-B20), OeNB (P15936), WWTF Precision Medicine Program LS16-034.

A4.4

12-Oxo-chenodeoxycholic acid potentiates doxorubicin-induced oxidative stress through Nrf2 axis in breast adenocarcinoma cells

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Background: As a transcription factor, nuclear factor E2-like factor 2 (Nrf2) controls the expression of genes encoding cytoprotective proteins, including antioxidant enzymes counteracting oxidative and electrophilic stress to maintain redox homeostasis. Aberrant activetion of Nrf2 in malignant cells promotes high expression of cytoprotective proteins, which can decrease the efficacy of antineoplastic agents used for chemotherapy. The aim of this study was to analyse the expression of *NRF2* gene as well as antioxidative system genes in a human breast adenocarcinoma cell line (MCF-7) treated with doxorubicin and the bile acid 12-oxo-chenodeoxycholic acid (12-monoketocholic acid, 12MKC).
Methods: The MCF-7 cell line was maintained in required microenvironmental conditions until confluence was reached. Cells were afterwards treated with 0.25 µM of doxorubicin (D group) or co-treated with 0.25 µM doxorubicin and 25 µM 12MKC (DM group). Following 24 h of incubation, cells were collected, RNA was isolated and transcribed into cDNA. The expression of the genes for Nrf2 (*NRF2*), superoxide dismutase (*SOD*), catalase (*CAT*), and β-actin (*ACTB*) as a housekeeping gene, was determined using RT-qPCR. Gene expression was analysed using comparative $2^{-\Delta\Delta C_T}$ method and statistical analysis was performed using Anova and Tukey's post-hoc test.
Results: Compared to untreated group of cells, treatment of MCF-7 cells reduced expression of *NRF2* both in the D and in the DM group: 2.74 ± 0.57 ($p < 0.001$) and 1.74 ± 0.59 ($p = 0.014$), respectively. Expression of *SOD* was also repressed in the D and in the DM group: 3.68 ± 0.78 ($p < 0.001$) and 1.11 ± 0.37 ($p < 0.001$), respectively. On the other hand, the expression of *CAT* was induced in the D group 1.50 ± 0.34-fold ($p > 0.05$), whereas supressed in the DM group 1.15 ± 0.39-fold ($p > 0.05$), compared to control.
Discussion: Oncogene-induced mutations with gain of function of Nrf2 promote both ROS detoxification and tumorigenesis, whereas suppression of Nrf2 in neoplastic cells and alters redox homeostasis of malignant cells. Through suppression of *NRF2*, *SOD* and *CAT* genes, 12MKC exerts potential to impinge cellular antioxidative defence at the transcriptional level in MCF-7 cells treated with doxorubicin, with potential favourable effects in terms of therapeutic outcome.
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A4.5

Carvotacetones from *Sphaeranthus africanus* with anti-proliferative activity against several cancer cell lines

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Background: *Sphaeranthus africanus* L. (Asteraceae) has been used in traditional medicine in Vietnam to alleviate swelling and as a sedative. Pressed juice from fresh leaves of *Sphaeranthus africanus* have been used for mouth and throat washes to treat sore throat. The decoction is also used as antitussive and expectorant. The pounded leaves are applied externally to relieve pain and swelling [1]. Chemical investigations of *S. africanus* are scarce. Until now, only two compounds and one mixture of carvotacetone derivatives, chrysosplenol D, squalene, spinasterol, and stigmasterol were reported for this plant [2]. There is no literature dealing with cytotoxic activities of the plant.
Methods: The air-dried and milled leaves and stems of *S. africanus* were percolated with 96% ethanol at room temperature. The crude extract was then partitioned sequentially with *n*-hexane, dichloromethane, ethyl acetate and *n*-butanol. The dichloromethane extracts (SA-DCM) exhibited activity against CCRF-CEM cells. Bioassay-guided fractionation of SA-DCM was performed, and all isolates (**1**–**5**) were evaluated for their anti-proliferative activity against CCRF-CEM, MDA-MB-231, U-251, HCT-116 cancer cells and non-tumorigenic HEK 293 cells.
Results: Five carvotacetone derivatives, including two known: 3-angeloyloxy-5-angeloyloxy-7-hydroxycarvotacetone (**1**), 3-angeloyloxy-5-[2″,3″-epoxy-2″-methyl-butanoyloxy]-7-hydroxycarvotacetone (**2**), along with three new compounds: 3-angeloyloxy-5-[3″-chloro-2″-hydroxy-2″-methyl-butanoyloxy]-7-hydroxycarvotacetone (**3**), 3-tigloyloxy-5-angeloyloxy-7-hydroxycarvotacetone (**4**), and 3-angeloyloxy-5-hydroxy-7-hydroxycarvotacetone (**5**), were isolated from the aerial parts of *S. africanus* collected in Vietnam. Bioassay-guided fractionation was monitored by the anti-proliferative activity on CCRC-CEM human cancer cells. The structures of the compounds were determined on the basis of NMR and mass-spectroscopic data. Activities were evaluated *in vitro* against four human cancer cell lines (CCRC-CEM, MDA-MB-231, U-251, HCT-116). All compounds exhibited significant anti-proliferative activity against all four cell lines. CCRF-CEM was most sensitive to the compounds, with IC_{50} values ranging from 0.6 to 1.5 µM. Compounds **3** and **4** possessed the highest activity, with IC_{50} values in the four cell lines ranging from 0.6 to 2.9 µM and 1.3 to 2.5 µM, respectively. These compounds also showed inhibitory activity towards HEK 293 human embryonic kidney cells, with IC_{50} values ranging from 2.5 to 5.5 µM.
Discussion: This is the first time that anti-proliferative activity of *Sphaeranthus africanus* extracts and constituents have been reported, and constituents **1**–**5** are the most cytotoxic carvotacetone derivatives reported so far. Our results have shown that leukemia cells reacted more sensitive to these compounds than non-tumorigenic cells. Therefore, carvotacetones may be interesting lead compounds in leukemia research.
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Pharmacokinetics and Pharmacoepidemiology

A5.1

Pharmacokinetics and pharmacodynamics of echinocandins in ascites

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Background: The echinocandin antifungals anidulafungin (ANI) and micafungin (MICA) are recommended for treatment of candidemia. Their efficacy in peritonitis and thoracic empyema caused by *Candida* is less clear. Target-site concentrations, however, might be crucial for eradication of pathogens. Therefore, we refined an established method [1] for quantification of ANI and MICA in ascites and in pleural effusion in order to assess target-site pharmacokinetics. In addition, pharmacodynamics of ANI and MICA in ascites were assessed by *in vitro* simulation of fungal growth.

Methods: ANI and MICA were measured by high-pressure liquid chromatography (HPLC) and UV detection. Sample preparation was performed by protein precipitation with acetonitrile (ACN) and supernatant purification by solid phase extraction (SPE). Quantification was validated according to the bioanalytical method validation guidelines. Simulation of *C. glabrata* and *C. albicans* growth was performed in RPMI media and ascites spiked with ANI or MICA over a period of 144 h at 37°C. Numbers of fungal colony-forming units (CFU) were counted and assessed, considering the duplicity and the dilution.

Results: The lower limit of quantification (LLOQ) could be reduced from 0.1 to 0.05 µg/ml for ANI and MICA in ascites. Intra- and interday variability and reproducibility was within the required range (<15%). Accuracy of linearity was within 85–115%. Extraction recovery could be doubled for ANI in ascites. Simulation of fungal growth in RPMI and ascites showed differences for *C. glabrata* and *C. albicans* proliferation. In untreated ascites, an increase in CFU by 10^2 within 144 h was observed for *C. albicans*, whereas no significant proliferation was seen for *C. glabrata*. An ANI concentration of 1 mg/l in ascites caused complete eradication of *C. albicans*. However, neither ANI nor MICA affected CFU numbers of *C. glabrata* in ascites.

Discussion: Implementation of SPE and a modified ACN precipitation technique lead to significant improvement in LLOQ and extraction rates for ANI and MIC. Due to higher accuracy and reproducibility, this method appears to be suitable for quantification of ANI and MICA in pharmacokinetic studies, particularly, when low echinocandin concentrations are anticipated. Results of fungal growth simulations suggest that antifungal efficacy in ascites may depend on the *Candida* species and on the applied echinocandin.

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A5.2

Soft-tissue pharmacokinetics of ceftolozane/tazobactam: room for dose optimization?

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Background: Ceftolozane/tazobactam (CEF/TAZ) is a novel antibiotic to treat multi-resistant Gram-negative infections including soft-tissue infections. Available information of pharmacokinetics (PK) of CEF/TAZ in soft tissue and plasma protein binding is fragmentary.

Methods: We investigated single and repeated dose PK of CEF/TAZ in plasma, muscle and subcutis of eight healthy volunteers receiving 1.5 g CEF/TAZ as 1 h intravenous infusion every 8 hours. CEF/TAZ concentrations in muscle and subcutis were measured by microdialysis. Plasma protein binding was determined by ultrafiltration.

Results: Single and repeated dose concentration-time profiles of CEF/TAZ in investigated compartments are shown in Fig. 1. Mean plasma protein binding was 6.3% and 8.0% for CEF and TAZ, respectively. Taking plasma protein binding into account, unbound tissue/plasma AUC_{last} ratios after repeated dose were approximately 0.9 for both muscle and subcutis. Between single and repeated dose

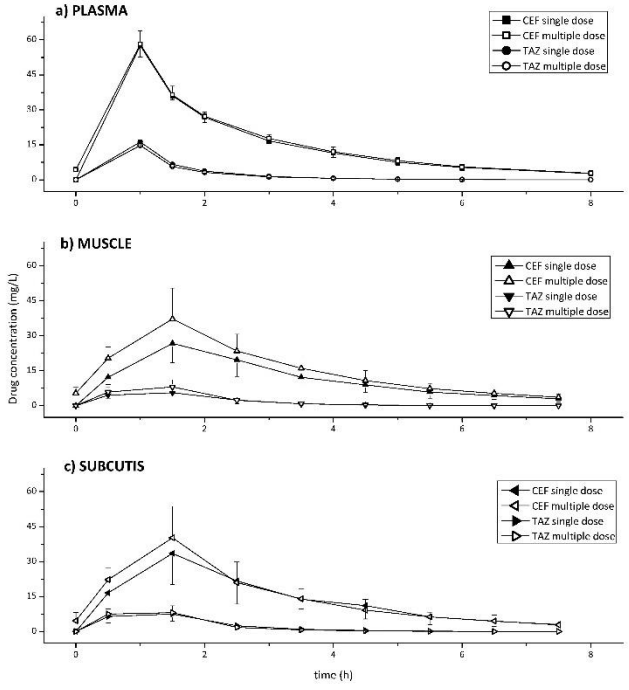

Figure 1: Concentration–time profiles of ceftolozane (CEF) and tazobactam (TAZ) in (**a**) plasma, (**b**) muscle and (**c**) subcutis in healthy volunteers following single or multiple intravenous doses of 1.5 g CEF/TAZ (means ± standard deviation, $n = 8$).

no appreciable accumulation occurred in plasma and subcutis. However, both CEF and TAZ showed pronounced accumulation in muscle after repeated dose, with an increase in mean AUC_{last} of 33% and 23% compared to single dose, respectively. Using the Enterobacteriaceae breakpoint of 1 mg/l, time above minimal inhibitory concentration (MIC) for unbound plasma CEF ($fT > MIC$) after repeated dose was 100%. For TAZ, the currently discussed PK/PD

index time above a threshold concentration ($T > C_T$, where C_T is calculated as 0.5 × MIC of ceftolozane) was 52.2%, which is markedly below the value of 75.7% associated with 1-log killing according to literature [1].

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A5.3

A link between antibiotic resistance and antibiotic consumption in Greece, Serbia and Norway

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Background: Increasing human antimicrobial consumption is widely considered highly influential, with agricultural use, environmental pollution, clonal and horizontal spread and long-term persistence also contributory. Recent evidence comes from meta-analyses that report positive associations between antimicrobial consumption and the development of resistance at both population and individual levels. The aim of this study was to measure the consumption of antimicrobial drugs in Greece, Serbia and Norway from 2012 to 2015 and to evaluate the relationship between consumption and resistance of selected bacterial strains in these countries.

Methods: This was a retrospective, observational, cross-sectional, population-based study of routinely collected data for consumption of antimicrobial drugs (from 2012 to 2015) and national antimicrobial resistance rates in Greece, Serbia and Norway (2014). The correlation (Pearson's *r*) between antimicrobial consumption values for the antibiotic class specific to the resistant strain and the rates of resistance in that strain was assessed.

Results: The results for year 2015 have shown that Norway is the country with the lowest consumption of antimicrobial drugs, followed by Serbia and finally Greece. Regarding trends in antibiotics use it can be noticed that both Greece and Serbia have increasing rates of consumption in the years 2012–2015, whereas Norway has a decreasing tendency. From the analysis of antibacterials subgroups it can be seen that β-lactam/penicillins are the drugs of choice in all three countries. Furthermore, the results from this study confirm the relationship between community antimicrobial consumption and serious resistant infections in patients.

Discussion: This study has emphasized the strength of relationship between community consumption rates and antimicrobial resistance rates in *E. coli*, *Acinetobacter* spp. and MRSA. As antibacterial resistance is a multifactorial problem, a multisectoral effort is needed to control it. The key areas of community recommendations for the control of bacterial resistance are surveillance of antibiotic consumption, awareness and understanding of antimicrobial resistance, optimizing antibiotic use, education, prevention and, finally, investment in new medicines and diagnostic tools.

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A5.4

Patient opinion and knowledge on drug use: a pilot study

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Background: Although more and more drugs become available every day, success of pharmacotherapy can be insufficient. Some of the reasons certainly are poor compliance and adherence due to inadequate patient–doctor communication which results in inadequate use of drugs and insufficient knowledge about drugs used. Also, there is a growing trend of self-medication. The aim of this research was to determine which groups of drugs are most frequently distributed in both public and private pharmacies, as well as to determine opinion and knowledge of the patients about drugs they use.

Methods: Research was done during a 1-month period in 5 private and 5 public pharmacies in Novi Sad, Serbia. Participants were interviewed about treatment, doses, dosing intervals, side effects and possible interactions. Patients were interviewed just after drug distribution.

Results: The total number of patients interviewed was 1,894 (956 in public and 938 in private pharmacies). The majority of patients were with prescribed treatment (89% in public, and 72% in private pharmacies). The structure of drugs distributed in both private and public pharmacies was very similar. Most frequently distributed groups of drugs were: ATC group C: drugs for treatment of cardiovascular disorders (~40%); ATC group N: drugs for treatment of nervous system disorders (~22%); ATC group A: drugs for treatment of alimentary tract disorders (~11%). Knowledge on route of administration, prescribed doses and dosing interval seems to be satisfactory. The majority of patients (88% in public, and 84% in private pharmacies) were properly informed on prescribed doses and dosing intervals. The main obstacle in proper drug use in patients is insufficient knowledge about side effects and possible drug interactions. A very small number of patients (approximately 25% of patients in both public and private pharmacies were informed on possible side effects) while an even lower number was informed on possible interactions (less than 10%).

Discussion: According to the results of the study it is obvious that little attention is paid on informing patients about side effects and drug interactions. A certain number of patients will be discouraged to continue treatment when facing unfamiliar side effects. Inadequate drug combinations, and drug–food interactions can lead to pharmacodynamic and pharmacokinetic interactions leading to more frequent side effects and insufficient therapeutic response.

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A5.5

Prevalence and predictors of self-medication among medical and pharmacy students

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Background: Self-medication is considered common among prospective health care professionals. Attitudes towards conventional and complementary medicine may affect their future pharmacotherapy practice. The aim of this research was to determine attitudes and prevalence of self-medication among population of first and final year medicine and pharmacy students.
Methods: Research was performed as a cross-sectional study at the Faculty of Medicine, University of Novi Sad, and included 192 first and last year students of medicine and pharmacy. Students filled out a demographic and self-medication questionnaire created for the purpose of this research.
Results: Self-medication was reported by 81.3% students. The most frequently self-prescribed medications were conventional drugs. Independent risk factors for self-medication identified in the logistic regression analysis were last year of studies (OR 7.29, 95%-C.I.: 2.28–22.90), living alone (OR 3.46, 95%-C.I.: 1.44–8.34) and consumption of cigarettes (OR 8.55, 95%-C.I.: 1.05–69.38). Last year students had more confidence in conventional medicine compared to herbal drugs, and had better knowledge about safety and risks of co-administration of herbal and conventional drugs.
Discussion: Results are in accordance with the study concucted at the University in Ljubljana, as well as studies in other countries, where students of the final year were more inclined towards self-medication, probably due to better knowledge acquired through studies and higher degree of confidence. However, this practice is not risk-free. Self-medication may result in irrational drug use, delayed seeking of medical advice, and increased side effects. Self-medication is an important issue among the population of medical students, especially among final year students. No difference in attitudes and behavior was found in relation to study program.
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A5.6

Factors associated with refill adherence to antidiabetic medication in patients with type 2 diabetes

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Background: Despite the presence of effective antidiabetic drug therapy, the problem of suboptimal adherence to pharmacotherapy is particularly common among patients with type 2 diabetes mellitus (T2DM). The aim of this study was to evaluate the association of medication refill adherence with socio-demographic and clinical characteristics of patients with T2DM to determine whether such factors could guide intervention strategie.
Methods: We conducted a retrospective chart review of 323 patients T2DM attending the primary health care center of the Foča municipality in eastern Bosnia and Herzegovina and measured adherence to treatment with antidiabetics. Refill adherence was determined from repeat prescriptions. Satisfactory refill adherence was defined as the percentage of the patients with refills covering ≥ 90% of the prescribed treatment between 1 January 2015 and 31 December 2015.
Results: The majority of patients were treated with oral therapy (84.2%). A total of 282 patients (87.3%) had satisfactory refill adherence. Age (older patients), educational level (patients with secondary school), employment (retired patients) and duration of T2DM (patients with longer duration of T2DM) were associated with higher adherence. Gender, maritual status, BMI, FBG, smoking, copayment, type of antidiabetic therapy, number of medicines and frequency of dosing were not associated with adherence.
Discussion: In the examined population, medication refill adherence was associated with age, educational level, employment and duration of diabetes, although these factors explained only a small amount of adherence variability. Although ingestion adherence is the goal, refill adherence is a necessary condition for ingestion adherence. To enhance adherence, physicians need better predictors to target their efforts to patients most in need of attention in eastern part of BiH. Prescription claims data could serve this purpose.
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A5.7

Preliminary study on gliclazide–probiotic bacteria interactions in *in vitro* conditions

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Background: Recently, great attention has been paid to the implication of gut microflora composition in interindividual differences in drug metabolism and therapeutic response [1]. Gliclazide belongs to the sulfonylurea family of insulin secretagogues and is characterized by large interindividual differences in the therapeutic response. The origin of these variations is not fully understood and may be the consequence of different gut microflora profiles between patients. Therefore, the aim of this study was to make preliminary assumptions of gut microflora influence on interindividual differences in gliclazide response based on *in vitro* assessment of gliclazide transport and biotransformation in probiotic bacteria.
Methods: Samples of gliclazide with probiotic bacteria were incubated for 24 hours at 37°C. After adequate sample preparation, intracellular and extracellular concentrations of gliclazide were determined at seven time points by high-performance liquid chromatography. Gliclazide biotransformation and potential metabolic products formed by enzymatic activity of probiotic bacteria were examined by appropriate software packages.
Results: During the twenty-four-hour incubation with probiotic bacteria, at all time points, statistically significantly lower concentrations of gliclazide in extracellular content were observed compared to controls. Accordingly, concentrations of gliclazide increased in probiotic cells over time. After 24 hours the total concentration of gliclazide, as the sum of intracellular and extracellular content, reached about 70% of the concentration from the beginning of the experiment (from 209.16 ± 6.26 µg/ml to 131.21 ± 1.17 µg/ml, $p < 0.01$). Potential metabolic pathways of gliclazide biotransformation by enzymatic

activity of probiotic bacteria involve reactions of hydrolysis and hydroxylations.

Discussion: Considering the fact that the total amount of gliclazide significantly decreased after the incubation period, it is assumed that one part of gliclazide is transformed to its metabolic products. It can be concluded that there are important interactions between gliclazide and probiotic bacteria, both at the level of active and passive transport into the cells, and at the level of drug biotransformation by enzymatic activity of probiotic bacteria. The effect of these interactions on the final therapeutic response of gliclazide should be further studied and confirmed in *in vivo* conditions.

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Drug Discovery

A6.1

Cyclotides as novel inhibitors of human prolyl oligopeptidase

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Background: Cyclotides are plant-derived mini-proteins. Cyclotides have been discovered in various flowering plant families in particular plants of the violet (Violaceae) and coffee (Rubiaceae) family [1]. Their characteristic circular cystine-knot motif confers them structural stability. They are expressed as natural peptide libraries essentially with great molecular diversity and hence these peptides are interesting starting points for drug discovery. For instance, native circular cystine-knot peptides are potent and selective inhibitors of serine-type proteases.

Methods: Here we present the discovery of the first cyclotide from the tropical plant *Psychotria solitudinum* as a specific inhibitor of the human prolyl oligopeptidase (POP) using a bioassay-guided fractionation approach combined with target-based pharmacology. Additionally, we biochemically and pharmacologically characterize the inhibition of other proline-specific endo- and exopeptidases for the reported and novel identified cyclotide inhibitors.

Results: Plant extracts of four species of the *Psychotria* and one *Viola* species were characterized for inhibition of human POP *in vitro* at concentrations of 100–400 µg/ml. The most promising *P. solitudinum* extract submitted to a pharmacology-guided isolation resulted in the novel cyclotide psysol 2 (IC_{50}: ~ 25 µM) as the most abundant compound in this plant peptide library. The molecular structure and amino acid sequence of psysol 2 was characterized by manual *de novo* sequencing using tandem mass spectrometry. The specificity for POP inhibition was determined by comparison of the inhibitory activity towards other serine proteases, namely trypsin and chymotrypsin, which both appeared unaffected by psysol 2 up to 100 µM. Preliminary structure–activity studies suggested that proline residues might by important for the observed POP inhibition since kalata B1, a cyclotide with high sequence homology to psysol 2, also inhibited POP activity with an IC_{50} of 5.6 µM [2].

Discussion: The enzyme POP is well known for its role in memory and learning processes, and it is currently being considered as a promising therapeutic target for cognitive deficits and neurodegenerative diseases, such as schizophrenia and Parkinson's disease. In the context of discovery and development of future POP inhibitors for therapeutic applications, cyclotides may be suitable candidates considering that small-molecule POP inhibitors fail to provide enough selectivity for the enzyme class of post-prolyl-cleaving endopeptidases.

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Training and Education

A7.1

Medical students' knowledge and attitudes regarding medical cannabis

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Background: The beneficial medical properties of cannabis have been widely recognized for the treatment of a variety of diseases; however, future doctors in Serbia are receiving scant information about this topic through formal education. The aim of this study was to explore if clinical teaching and previous use of marijuana influence students' knowledge regarding therapeutic use and potential abuse of medical cannabis.

Methods: An anonymous questionnaire was administered to a random sample of 350 students of third, fifth and sixth year of integrated academic studies in medicine.

Results: Out of 350, the survey was completed by 316 students (response rate 90.3%). Approximately two-thirds (67.7%) of students were female, while approximately a third (105; 33.2%) of students reported lifetime use of marijuana. Students who had previously used marijuana were more familiar with both therapeutic and side effects ($p < 0.001$) of cannabis, as well as with potential cannabis abuse. Previous marijuana users were more familiar with the current legalization process in Serbia ($p = 0.006$) and a significantly larger number of them thought that the use of cannabis for therapeutic purposes should be legalized in Serbia ($p = 0.006$). The year of study did not affect the knowledge about the therapeutic and adverse effects of cannabis, nor the students' attitudes toward abuse and legislation.

Discussion: Students who had previously used marijuana significantly more frequently stated that they were familiar with possible therapeutic effects of medical cannabis and its side effects,

which is in line with earlier studies conducted in Serbia and USA. No difference was observed between the year of the study with respect to therapeutic and adverse effects, implying that clinical teaching and hospital training during the fifth and sixth years of the study had no influence on students' knowledge with respect to medical cannabis. Students who have never used marijuana were more familiar with possible cannabis abuse. Likewise, an earlier study showed that greater belief in medical benefits and lower belief in medical risks were strongly associated with history of cannabis use. A significantly greater number of previous users believed that the use of cannabis for therapeutic purposes should be legalized in Serbia, which is also in agreement with other, similar studies. Students' knowledge correlated with previous marijuana consumption, as previous marijuana users were more knowledgeable about therapeutic and side effects, while students who never consumed marijuana were more aware of possible abuse. Year of the study had not significant influence both on the knowledge about medical cannabis and attitude toward its legislation. However, introduction of clinical teaching based on up-to-date research and clinical applications of medical cannabis is necessary in Serbia.

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