ABSTRACTS

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PHARMACOLOGY, BIOLOGY AND MEDICAL SCIENCES

METFORMIN TRANSPORT ACROSS THE RAT PLACENTA IS MEDIATED BY COOPERATIVE ACTIVITY OF ORGANIC CATION TRANSPORTER 3 (OCT3/SLC22A3) AND MULTIDRUG AND TOXIN EXTRUSION 1 (MATE1/SLC47A1)

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Metformin has been considered in pregnant women with gestational diabetes mellitus as an alternative to insulin. However, the recommendation for the use of metformin in pregnancy was introduced without proper knowledge of its transplacental passage and effects on fetus. In our previous studies we described expression, localization and function of Organic Cation Transporter 3 (OCT3) and Multidrug and Toxin Extrusion 1 (MATE1) transporters in the rat placenta; since metformin is a substrate of these transporters, in this study we hypothesized the role of OCT3 and MATE1 transporters in the placental passage of metformin. In open-circuit perfusion setup we observed concentration-dependent clearance of metformin across the placenta in both maternal-to-fetal and fetal-to-maternal directions; furthermore, at low metformin concentrations, transplacental clearance in fetal-to-maternal direction was higher than that in maternal-to-fetal direction. Using closed-circuit perfusion setup, we observed a steady decrease in fetal metformin concentrations indicating the ability of metformin to cross the placenta from fetal to maternal compartment even against its concentration gradient. At high metformin concentration, this asymmetry was annulled. Assuming this asymmetry was caused by placental solute carrier transporters, we further used a common OCT3 and MATE1 inhibitor, 1-methyl-4-phenylpyridinium iodide (MPP^+), which can, at a concentration of 1000 μM fully inhibit placental OCT3/MATE1 pathway. We observed significant inhibitory effect of MPP^+ on the transplacental passage of metformin indicating the involvement of OCT3/MATE1 vectorial pathway in

passage of metformin from the fetal to maternal circulation. Additionally, we employed pH values from 6.5 to 8.5 on the maternal side of the placenta to study the involvement of MATE1 in elimination of metformin from trophoblast cell to the maternal compartment; with increasing pH on the maternal side of the placenta, the transport of the metformin from fetus to mother decreased significantly, confirming metformin elimination from trophoblast cells by MATE1. In conclusion, our findings suggest involvement of OCT3 and MATE1 transporters in the transplacental transfer of metformin across the rat placenta.

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THE LIGAND COMPETITIVE REACTIONS ON RECEPTOR CAN BE DETERMINED USING A REAL-TIME RADIOIMMUNOASSAY

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The competitive assays enable to determine the binding characteristic of new prepared ligands. The ligand characterization is the necessary first step for further study of a tested ligand behavior. This mentioned approach is applied in newly introduced radiopharmaceuticals too. However, the traditional methods in ligand binding characterization are time and material (especially for chemicals) demanding. To accelerate the binding characterization of a ligand and to safe the expanses the novel technique has been developed with the employment of an automatic radioimmunoassay. The technique uses the already previously described instrument LigandTracer, which allows real time detection of cell receptor associated radioactivity.

The study included the real time detection of the competition between radioiodinated natural ligand and unlabeled natural and non-natural ligands on the binding domain of targeted epidermal growth factor receptor (EGFR). The study was performed *in vitro* with the employment of cancer cell lines expressing EGFR in high density.

The results of measurement carried out using the automatic radioimmunoassay revealed the binding ability of employed unlabeled ligands in the competition with a natural ligand. The pre-bound natural ligand was displaced with either low concentrations or high concentrations of the competitors or was not. On the basis of obtained results, the novel automatic technique has proved its capability to be skillful method for the labeled ligand versus unlabeled ligand competition analysis. Moreover, this technique exceeds traditional methods of ligand binding characterization thanks to its measurement simplicity and low cost runs. The disadvantage of this technique lies in the first acquisition costs.

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PHARMACOKINETIC INTERACTIONS OF CYCLIN-DEPENDENT KINASE INHIBITORS WITH P-GLYCOPROTEIN *IN VITRO*

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Inhibition of cyclin-dependent kinases has become a novel approach in cancer treatment over the last decades. Although much attention has been devoted to the pharmacodynamic properties of cyclin-dependent kinase inhibitors, the pharmacokinetic aspects have not been evaluated in detail to date. The aim of this project was to investigate potential interactions between five cyclin-dependent kinase inhibitors and P-glycoprotein using *in vitro* accumulation methods in MDCKII-MDR1 and MDCKII parent cell lines. Established P-glycoprotein substrates, Hoechst 33342 and daunorubicin were applied in these experimental setups. Purvalanol A showed the highest inhibitory potency in both methods, followed by roscovitine, olomoucine II, flavopiridol and SNS-032 with slight differences between methods used. Moreover, we investigated substrate affinities of olomoucine II and purvalanol A towards P-glycoprotein. Measuring the ATPase activity, we determined that olomoucine II is a P-glycoprotein substrate. Purvalanol A can be classified as a non-substrate as it changed the ATPase activity insignificantly. Both these findings were confirmed by transport assays using monolayers of MDCKII cells stably expressing ABCB1. Based on our results, it is reasonable to expect a considerable impact of the studied cyclin-dependent kinase inhibitors on both pharmacodynamic and pharmacokinetic behavior of other simultaneously administered drugs. In addition, we confirmed that purvalanol A, roscovitine and olomoucine II can synergistically potentiate cytostatic effect of daunorubicin, a P-glycoprotein substrate, in MDCKII-MDR1, HCT-8 and HepG2 cell lines. At the same time, we brought evidence that this synergism is, at least partly, caused by pharmacokinetic interactions on P-glycoprotein.

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STUDY OF INTERACTION OF ANTIRETROVIRAL DRUGS WITH ABC EFFLUX TRANSPORTERS USING *IN VITRO* CELL CULTURE MODEL

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Several ATP-binding cassette (ABC) and solute ligand carrier (SLC) transporters have been found to affect body disposition of antiretroviral drugs thereby affecting concentration

of these compounds in the site of human immunodeficiency virus replication. In our study we aimed to investigate whether tenofovir, tenofovir disoproxil fumarate (tenofovir DF), emtricitabine, lamivudine, and zidovudine are substrates of P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2), multidrug resistance-associated protein 2 (ABCC2), organic cation transporter 1 (OCT1) or multidrug and toxin extrusion protein 1 (MATE1). We performed *in vitro* transport assays using monolayers of polarized MDCKII cells stably overexpressing the transporter of interest (ABCB1, ABCG2, ABCC2, OCT1 and MATE1), which were cultivated on semipermeable membranes. Based on our results, tenofovir is not transported by ABCB1, ABCG2 or ABCC2. In contrast, its orally used prodrug, tenofovir DF, was identified to be a substrate of ABCG2 and ABCB1. Zidovudine was also shown to be transported by ABCG2 and, moreover, interaction between tenofovir DF and zidovudine on ABCG2 was revealed. Emtricitabine and lamivudine are not substrates of any of the tested transporters including OCT1 and MATE1. In conclusion our study suggests that bioavailability and cellular uptake of tenofovir DF can be hampered by ABCB1 and also by ABCG2 transport proteins. Moreover, drug-drug interactions can appear on both ABC transporters resulting in higher (toxic) or subtherapeutic concentrations of the antiretrovirals, which could lead to pharmacotherapy failure.

The study was supported by PRVOUK P40 "Drug Development and Study".

OXIDATIVE DAMAGE IN PATIENTS WITH NON-SMALL CELL LUNG CANCER TREATED WITH PLATINUM DERIVATES

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Chemotherapy has been the mainstay of treatment for advanced Non-small cell lung cancer. The most commonly used is a combination of drugs inclusive platinum derivatives (cisplatin, carboplatin). There is growing evidence suggesting that the cytotoxic activity of cisplatin is also closely associated with increased generation of reactive oxygen species (ROS). Interaction of ROS with cellular components may result in damage to biomolecules including DNA. Therefore, it is possible that oxidatively damaged DNA, which arises as a result of chemotherapy with cisplatin, may be involved in the therapeutic effect of the drug. In our study, we investigated the level of oxidative DNA damage caused by cisplatin chemotherapy in patients during the entire chemotherapy.

For the evaluation of DNA damage were used Comet assay test and ELISA. In the case of comet assay was evaluated the amount of oxidized pyrimidines in DNA of periphery lymphocytes. In the case of ELISA was evaluated the level of 8-hydroxy-2-

deoxyguanosine (8-OHdG) in urine. Sampling was performed before chemotherapy, in the middle of chemotherapy and after chemotherapy.

In comet assay results we can see, that the level of oxidative DNA damage (oxidized pyrimidines) increases in the course of whole chemotherapy (16.14: 20.41: 22.34% Tail DNA), but there is no statistically significant difference between measurements. Results of 8-OHdG measurement in urine by ELISA were similar to Comet assay results. The level of 8-OHdG increases in the course of whole chemotherapy (13.69: 14.37: 14.97 ng/mmol) but there is also no statistically significance difference between mentioned measurements.

As described above, we have not found any significance differences between each measurement performed by both tests, but we can see trend for increasing oxidative damage between measurements. We found out in our measurement high individual variability (especially in the comet assay measurement) and this can reflect individual differences in metabolism and repair capacity.

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IL-1 RECEPTOR BLOCKADE ALLEVIATES ENDOTOXIN-MEDIATED IMPAIRMENT OF RENAL DRUG EXCRETORY FUNCTIONS IN RATS

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Acute renal failure (AKF) is a serious complication of sepsis, which significantly worse the prognosis of patient. The major mechanism underlying the development of AKI is the activation of NF-κB pathway by endotoxins (LPS) released from bacterial cell wall and subsequent massive production of nitric oxide (NO) and cytokines such as TNF-α, IL-1β. In this study, we therefore tested hypothesis if two potent anti-inflammatory agents, dexamethasone and anakinra, an IL-1 receptor antagonist, may prevent impairment of renal functions during endotoxemia in rats. Special focus was paid on the mechanisms responsible for drug excretion. Rats were divided into four groups: saline controls, LPS-treated, and dexamethasone or anakinra (both in a dose of 10 mg/kg)

pre-treated endotoxemic rats. Untreated LPS administered rats developed within 10 h typical symptoms of AKF characterized by reduced glomerular filtration rate, microalbuminuria, glomerular and tubular histological damage, increased fractional excretion of sodium, and decreased tubular secretion of azithromycin, the prototype substrate for multidrug transporters. Administration of either immunosuppressant was able to prevent all these symptoms and even to restore the AZT secretory clearance to control values. Interestingly this effect was related to up-regulation of organic anion transporters 1–3, but not to multidrug transporters Mdr1 and Mrp2, which were induced by LPS and paradoxically down-regulated by the drugs. In addition, both agents were equally able to alleviate the signs of developing intrahepatic cholestasis. Comparison of both agents revealed more complex action of dexamethasone – besides reduction of plasma cytokines seen after both agents, dexamethasone also markedly reduced plasma levels of NO as a result of reduced iNOS expression in the kidneys and liver. In summary, our data points toward major role of IL-1β for the development of AKF during endotoxemia. IL-1 receptor blockade as well as dexamethasone administration was able to mitigate renal injury imposed by LPS and demonstrated favorable effect on the mechanism important for renal drug elimination.

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PRECISION-CUT RAT KIDNEY SLICES AS A METHOD FOR PHARMACOLOGICAL STUDIES

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The study was aimed at introducing of a new pharmacological/toxicological method useful for study distribution and elimination of drugs *in vitro*. We have introduced in the methodical apparatus of our laboratory the method rat kidney slices. In the first step of our experiments, we standardized the technique for slicing fresh kidneys obtained from the animals with use of Krumdieck Tissue Slicer. The following step was evaluation of precision-cut rat kidney slices viability. Consequently, an optimization of incubation conditions was carried out. As the final step, a preliminary accumulation study with radiolabeled substrate of OAT1 transporter was performed.

The viability of the kidney slices was assessed using two types of tests on cellular viability: the first determined damage of cellular membranes measuring lactate dehydrogenase or protease leakage, the second evaluated metabolic state of the kidney slices using a tetrazolium salt reduction. Thereafter, we evaluated the optimal method for lysis of the slices necessary to measure concentrations of experimental agents accumulated in the slices. The incubation of the renal slices was carried out at 37 °C in humid atmosphere of air and 5% CO_2.

Optimal viability of the rat kidney slices was observed up to 15 min of incubation. After this interval, the viability indicated by both types of the used tests was gradually decreased. As the best method for solvation of the slices was selected lysis by Solvable buffer (pH = 11). This method enables full and homogenous solution of the renal tissue. Finally, we demonstrated usefulness of the rat renal slices technique for accumulation studies using [^{3}H]tenofovir.

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SUNITINIB EFFECTS ON AORTIC ENDOTHELIUM IN NORMOTENSIVE AND HYPERTENSIVE RATS

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Sunitinib is a novel multi-targeted tyrosine kinase inhibitor with broad-spectrum activity on cancer cell proliferation and angiogenesis. The drug is approved for the treatment of renal cell carcinoma and gastrointestinal stromal tumor. Although sunitinib rank among biologically targeted anticancer drugs, significant adverse effects may occur during the treatment, including severe hypertension and cardiovascular toxicity. The healthy endothelium is currently recognized to be essential for the regulation of the vascular structure and function. Moreover, endothelial dysfunction is one of the main characteristics of chronic arterial hypertension. However, the impact of sunitinib treatment on arterial endothelium remains unsure. Therefore, the aim of the present study was to investigate whether chronic sunitinib treatment induces vascular toxicity and endothelial dysfunction in normotensive and hypertensive rats. For this purpose, the expression of various markers of inflammation, oxidative stress and endothelial dysfunction was evaluated in the aortic endothelium.

Inbred spontaneous hypertensive rats (SHR) and their normotensive (Wistar Kyoto, WKY) littermates were used in the study (n = 32). Sunitinib (10 mg/kg) was administered daily by gastric gavage for 8 weeks followed by 5 days wash out period and then treatment re-challenge lasting 2 and 8 weeks for WKY and SHR rats, respectively. Control groups received water in the same schedule. Samples of aorta were collected for immunohistochemical and stereological analysis.

Sunitinib treatment resulted in significant increase in aortic endothelial expression of PECAM-1, ICAM-1 and HO-1 in WKY rats. In addition, sunitinib induced a significant increase of PECAM-1, ICAM-1, eNOS, iNOS, ET-1 and HO-1 in SHR rats.

Our findings show that chronic sunitinib treatment induces the expression of markers of endothelial dysfunction. These changes could be involved in the treatment-induced

impairments of vascular function and may co-determine the development of arterial hypertension. The findings of this study deserve further investigation in order to establish mechanistic role in the development of vascular complications accompanying treatment with this modern anticancer drug.

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STUDY OF TRANSPORT OF TENOFOVIR AND TENOFOVIR DISOPROXIL FUMARATE ACROSS THE RAT TERM PLACENTA

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Tenofovir disoproxil fumarate (TDF) is an orally used prodrug of tenofovir (TFV), a nucleotide reverse transcriptase inhibitor that has high potency to suppress HIV infection. TDF-containing antiretroviral (ARV) regimens are recommended by WHO for prophylaxis of mother-to-child transmission of HIV in pregnant infected women. ARV prophylaxis also depends on sufficient transplacental passage of drugs from mother to fetus. To select optimal therapy it is of great importance to have detailed knowledge on drug transplacental kinetics including factors that may affect the transplacental pharmacokinetics, such as drug efflux ABC transporters, e.g. P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). The aim of this study was to describe transplacental transport of TDF and TFV employing the method of dually perfused rat term placenta. Using open-circuit perfusion system, transport of TDF or TFV was investigated in both fetal-to-maternal and maternal-to-fetal directions. At low TDF concentration (50 nM), significant asymmetry in transplacental clearance was observed in favor of fetal-to-maternal direction. Increasing TDF concentration to 500 μM annulled this asymmetry, indicating capacity-limited transport mechanism. Employing closed-circuit perfusion system, we confirmed the concentration-dependent saturable transport. Moreover, application of specific inhibitor of P-gp and BCRP (GF120908) resulted in significant suppression of TDF transport from fetal circulation to mother. In the case of TFV, we observed weak transplacental passage and no involvement of capacity-limited mechanism. In conclusion, our findings suggest an important role of P-gp and BCRP in transport of TDF but not TFV. In our model, both drugs seem to be inadequately transferred from maternal to fetal circulation to provide fetal protection.

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ENDOGLIN AND eNOS MIGHT PLAY A PROTECTIVE ROLE IN ENDOTHELIUM IN EARLY ATHEROGENESIS

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It has been demonstrated that endoglin (TGF-β receptor III, CD105) is able to regulate eNOS expression in vessels, and thus strongly affect function of endothelium. In addition, we previously showed increased expression of endoglin and eNOS in aortas with reduced atherosclerosis, suggesting that endoglin might be athero-protective and/or endothelium-protective marker. Moreover, it has been demonstrated that levels of soluble form of endoglin (measured in blood) are related to hypercholesterolemia and endothelial dysfunction.

In this study, we hypothesized whether endoglin and eNOS, together with other markers important for endothelial function, could be involved in different atherosclerosis susceptibility between C57BL/6J and C3H/HeJ mice.

C57BL/6J mice (n = 16) and C3H/HeJ mice (n = 16), both divided into two groups, sustained on either chow or cholesterol (1%) diet for 8 weeks. Biochemical analysis of blood cholesterol fractions, ELISA analysis of soluble endoglin and Western blot analysis of endoglin, TGF-βRII, eNOS/phospho-eNOS, ICAM-1 expressions in aorta were performed.

Atherogenic index (total cholesterol / HDL cholesterol ratio) was significantly raised in both strains after cholesterol diet, with stronger impact in C3H/HeJ mice. Surprisingly, cholesterol diet resulted in a dramatic induction of potential endothelium-protective markers – endoglin, TGF-βRII, eNOS/phospho-eNOS, and a significant reduction of pro-inflammatory ICAM-1 in C3H/HeJ mice (athero-protective strain). On the opposite, endothelium-protective markers were not affected and ICAM-1 expression was increased in C57BL/6J mice (athero-prone strain) after cholesterol diet.

We propose that endothelial expression of endoglin and eNOS might be involved in different atherosclerosis susceptibility between C57BL/6J and C3H/HeJ mouse strains, suggesting that endoglin might be an interesting target for affecting endothelial dysfunction.

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TRIENTINE AND 8-HYDROXYQUINOLINES SURPASS THE STANDARD DRUG D-PENICILLAMINE IN AFFINITY TO COPPER *IN VITRO*

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Copper is an essential trace element which forms an integral component of many enzymes. Since disorder of copper homeostasis at systemic or local level can be observed in various pathologies, the use of copper chelators may represent a promising therapeutic strategy.

This study was aimed at: 1) formation of an *in vitro* methodology for screening of copper chelators, and 2) detailed analysis of interaction of copper with clinically used D-penicillamine (D-PEN) and trientine; and compounds from 8-hydroxyquinoline group. EDTA was used for comparison as a standard chelator.

The methodology based on bathocuproinedisulfonic acid disodium salt (BCS) was used at four (patho)physiologically relevant pH conditions (4.5–7.5) and enabled both cuprous and cupric ions chelation assessment and cupric reduction assessment as well. In the case of potent chelators, the stoichiometry of the complex could be estimated too. From the tested compounds, trientine, clioquinol, chloroxine and EDTA were the most active copper chelators, followed by non-substituted 8-hydroxyquinoline. Unexpectedly, the less active compound was D-PEN. The activity of trientine, 8-hydroxyquinoline and D-PEN was substantially influenced by pH conditions. From all the tested substances, only D-PEN showed significant potential to reduce cupric ions which can have unfavourable consequences.

Conclusively, our assay employing BCS represents a rapid, simple and precise method for copper chelation measurement. In addition, lower binding affinity of D-PEN compared to trientine and 8-hydroxyquinolines was demonstrated (Fig. 1).

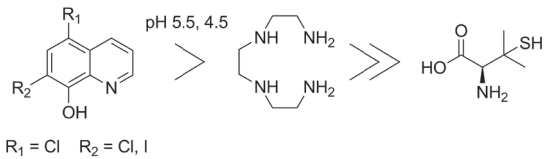

Fig. 1. A schematic depiction of copper chelating potency of the tested compounds.

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MITOGEN-ACTIVATED PROTEIN KINASE KINASE 1 AND 2 (MEK1 AND 2) INHIBITORS, UPREGULATES CYP3A SUBFAMILY MEMBERS GENE EXPRESSION IN HEPATOCELLULAR CARCINOMA CELLS

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The extracellular signal-regulated kinase (ERK) pathway (Ras-Raf-MEK-ERK) is pivotal in many biological processes. ERK cascade is involved in cell proliferation and survival, and constitutive activation of the ERK pathway is often found in human tumors. In the current study, we evaluated the effect of ERK pathway inhibition on the expression of the main liver xenobiotic-metabolizing enzyme cytochrome (CYP) P450. CYP genes are controlled in different ways and at multiple levels. The transcription of CYP3A subfamily members are regulated mainly by nuclear receptors such as pregnane X receptor (PXR), constitutive androstane receptor (CAR).

In the current work, we investigated the effect of ERK cascade inhibition on basal expression of CYP3A4 mRNA transactivation and CYP3A subfamily members mRNA expression in HepG2 cells using specific MEK1 and 2 inhibitors U0126, PD 0325901 or PD184352. Our results suggest that all MEK1/2 inhibitors induced mRNA of CYP3A4, 5, 7 in HepG2 cells. Consistently, U0126 activated CYP3A4 gene reporter construct in transient transfection assays. Further experiments revealed that only U0126 is a ligand of PXR but not PD 0325901 or PD184352. Finally we found that MEK1/2 inhibitors downregulated small heterodimer partner (SHP), the known repression factor of nuclear receptors.

We suppose that ERK pathway regulates CYP3A subfamily members expression through transcription regulation and primary mechanism involves SHP. These results show evidence that inhibition of ERK signaling in hepatocellular carcinoma cells may influence basal expression of main detoxification enzymes of CYP3A subfamily.

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INTERACTIONS OF ACYCLIC NUCLEOSIDE PHOSPHONATES WITH SELECTED RENAL SLC AND ABC TRANSPORTERS

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The nephrotoxicity of the acyclic nucleoside phosphonates (ANPs) with antiviral and antiproliferative activities is suggested to depend on the renal tubular transport. The aim of the study was to investigate *in vitro* whether typically in the kidney expressed SLC

transporters (hOAT1, hOCT2, hCNT2, hCNT3) and drug efflux transporters (ABCB1, ABCG2) may have a potential to interact with the selected antivirals. In addition, relationships between affinity of ANPs to the transporters and their cytotoxicity were assessed. The potency to interact with the transporters was compared to that of two clinically used ANPs, tenofovir and adefovir. To determine the contribution of receptor mediated endocytosis, we tested competition for uptake by ANPs and specific transporters ligands in the transiently transfected cells with appropriate cDNA of transporter. Several tested ANPs exhibited a significant inhibitory interaction with hOAT1. However, besides GS-9191, ANPs showed lower potency to interact with hOAT1 in comparison with adefovir and tenofovir. No significant interactions of the tested ANPs with hOCT2, hCNT2 and hCNT3 were observed. GS-9191 was found to inhibit active transport mediated both by ABCB1 and ABCG2, while PMEO-DAPy showed a potency to interact only with ABCB1. The other studied ANPs exhibited no significant inhibitory interaction with the studied efflux transporters. With exclusion of GS-9191, a relatively lipophilic compound, ANPs were more cytotoxic in cells overexpressing hOAT1. This could indicate that hOAT1-mediated intracellular accumulation is responsible for cytotoxic effect. In addition, this finding suggests that such type of ANPs is not only inhibitor but also substrate of hOAT1.

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EFFECTS OF ATORVASTATIN TREATMENT ON ENDOGLIN EXPRESSION IN TNF-ALPHA INDUCED INFLAMMATION IN HUVECs

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Endoglin (CD105, TGFβRIII) is strongly expressed in endothelial cells, affects the expression of eNOS and plays important role in atherosclerosis. In our previous papers, we demonstrated that atorvastatin treatment increases the aortic expression of endoglin and eNOS in mice aorta, decreases levels of soluble endoglin in blood and reduces atherosclerosis in aorta, suggesting its interesting role in atherogenesis. In this study, we wanted to elucidate whether statin induced eNOS expression depends on endoglin *in vitro* in human endothelial cells HUVEC.

HUVEC cells were exposed to TNFα (10 ng/ml) for 2, 4, 6 and 24h to mimic inflammatory conditions. Atorvastatin was added 24h before TNFα exposure, at a concentration of 3 μM and 5 μM, DMSO 0.1% (v/v) was used as control. Cells with siRNA of endoglin were prepared by using Amaxa HUVEC Nucleofector kit. The protein expression was determined by flow cytometry and Western blot analysis. Levels of soluble endoglin were detected by means of ELISA.

We showed that TNFα treatment for 24h significantly decreased endoglin and eNOS expression in HUVECs ($p < 0.001$; $n = 4$), together with significant increase of soluble endoglin

in medium ($p < 0.01$; $n = 4$). Pretreatment of atorvastatin at a concentration of 5 μM, for 4 and 6 and 24 hours intervals of TNFα exposure, significantly prevented decrease of endoglin expression ($p < 0.05$; $p < 0.01$; $n = 4$), in comparison with cells treated only by TNFα. Endoglin silencing reduced significantly the expression of endoglin and additionally expression of eNOS.

Inflammation in endothelial cells results in reduced expression of endoglin and eNOS in HUVECs, which could be prevented by atorvastatin. Moreover, atorvastatin induced eNOS expression depends on endoglin. Since endoglin and eNOS plays important role in various cardiovascular pathologies including atherosclerosis, hypertension, diabetes, preeclampsia and hereditary hemorrhagic telangiectasia, we propose that statin effects on tissue and soluble endoglin in these diseases should be evaluated in clinical studies.

The study was supported by grant from The Grant Agency of Charles University in Prague number 300811/C and grant SVV/2012/265003.

NEW TETRAZOLE BASED DERIVATIVES AS POTENCIAL ANTITUBERCULAR AGENTS

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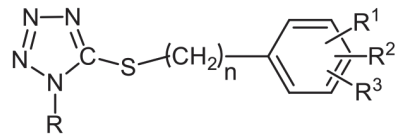
Tuberculosis (TB) is a disease featured as a chronic bacterial infection caused by *Mycobacteria tuberculosis*. They have infected two billion people and every year cause eight million new cases of tuberculosis and two million deaths. This problem is complicated by tuberculosis mycobacteria that are resistant to existing tuberculostatics. Due to the unusual structure and chemical composition of the mycobacterial cell wall, effective tuberculosis treatment is difficult, making many antibiotics ineffective and preventing the entry of drugs. The aim of this work is the design and synthesis of new improved candidates with antimycobacterial activity based on our previous reseach. We want to study the influence of electron properties of functional groups on aromatic ring as appropriate antimycobacterial activity for the pharmacophore and nature of side chain on tetrazole ring, which is probably responsible for its pharmacokinetics.



R= alkyl, aryl

R^1, R^2, R^3 = EWG, EDG, H

The study was supported by the European Social Fund and the state budget of the Czech Republic. Project no. CZ.1.07/2.3.00/30.0061.

SYNTHESIS OF NOVEL AROYLHYDRAZONE IRON CHELATORS

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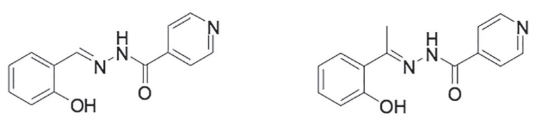
For years aroylhydrazone iron chelators have been studied for their cardioprotective effect. Aroylhydrazones are tridentate chelators binding free intracellular Fe ions and therefore protect cardiomyocytes from reactions leading to formation of free radicals where iron stands as a catalyst. SIH (salicylaldehyde isonicotinoylhydrazone) showed cardioprotective effect as well as advantageous pharmacokinetic properties. Nevertheless it suffers from low stability in plasma[1].

Our research group previously prepared a number of SIH analogs with increased stability and pronounced cardioprotective effect along with low toxicity[2]. Some of the analogs also possess a promising antiproliferative activity[3].

The goal of our current work is to synthesize new aroylhydrazones based on our previous results in order to enhance their cardioprotective and antiproliferative effects.

The current syntheses cover modifications of both ketone and hydrazide part of the molecule. Regarding the former, we aimed at ketones with nitro group in various positions on the phenyl ring and ketones with elongated or branched alkyl chain. For the evaluation of the influence of the hydrazide we prepared more lipophilic hydrazides and products with different nitrogen heterocycles. All final products will be subjected to studies of their chelation potential, stability, own toxicity, cardioprotective and antiproliferative activities.



SIH and selected analogs.

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SYNTHESIS OF 1-SUBSTITUTED 5-ALKYLSELANYL-1*H*-TETRAZOLES

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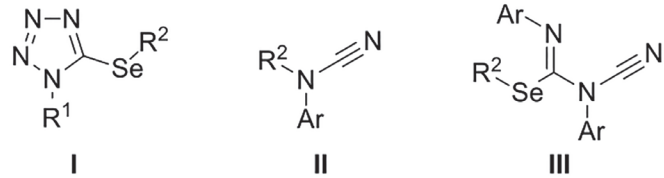
Selenium has long been considered as one of the most toxic elements. However, increasing attention is paid to selenium-containing compounds since discovery of essentiality of selenium for normal function of the immune system, for normal human development, growth and metabolism. Nowadays selected selenium-containing compounds are promising biologically active substances exhibiting antifungal, antimycobacterial, antiviral and anticancer properties.

In this work we focused on the synthesis of selenium analogues of 1-alkyl/aryl-5-alkylsulfanyl-1*H*-tetrazoles, which showed high antimycobacterial activities. The aim of this work was to find suitable methods for the preparation of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles and to evaluate their antitubercular as well as cytotoxic activity.

Analogously with widely used and efficient methods for the preparation of 1-substituted 5-alkylsulfanyl-1*H*-tetrazoles from isothiocyanates, alkyl/arylisoselenocyanates were used as a starting material in the synthesis of target 1-substituted 5-alkylselanyl-1*H*-tetrazoles.

We found that the reaction of corresponding isoselenocyanates with sodium azide in water or in various organic solvents mostly led to the precipitation of selenium powder and formation of various decomposition products.

One-pot reaction of alkyl/arylisoselenocyanates with sodium azide and alkylating agent in MeCN or THF in the presence of water increased the yields of 1-alkyl/aryl-5-alkylselanyl-1*H*-tetrazoles (**I**). 1-Alkyl derivatives were synthesized in good yields as the only products. In the case of 1-aryl derivatives, the target selanyltetrazoles **I** were obtained only in moderate yields due to the formation of *N*-alkyl-*N*-arylcyanamides (**II**) and/or (*Z*)-*Se*-alkyl-*N*-cyano-*N,N'*-diarylisoselenoureas (**III**) as the main products regardless of the method used.



R^1 = alkyl, aryl
R^2 = alkyl

1-Substituted 5-alkylselanyl-1*H*-tetrazoles (**I**) exhibited high antimycobacterial activities and no significant toxicities against isolated human hepatocytes and JEG-3 cells.

This project was supported by the Grant Agency of Charles University (Project no. 55610/2010) and by the Charles University in Prague (SVV 265 001).

CONFORMATIONAL STUDY OF 2-METHOXY-2'-HYDROXYTHIOBENZANILIDE DERIVATIVES

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Tuberculosis (TB) is one of the most dangerous and the most widespread infectious diseases. Therefore, there is still an urgent need for new antituberculotic drugs. Recently, we have shown that some 2-methoxy-2′-hydroxybenzanilides and their thioxo analogues have potential antimycobacterial properties[1]. Benzamides and thiobenzamides have been studied for last few years also for their stereochemical behaviour. Some of them have been found as chiral auxiliaries[2], stereochemical properties of other ones were used for construction of several oligomeric artificial structures with defined conformation[3] and finally, the conformations of benzamide derivatives have also provided the fashion how to transfer stereochemical information in the molecule[4].

During our study of antimycobacterial properties of thiobenzanilides it was revealed, that NMR spectra of all of them contained two sets of ^{1}H and ^{13}C resonance signals. By the deeper study of this phenomenon it was approved that these two sets of resonance signals belong to two conformers of 2-methoxy-2′-hydroxythiobenzanilides. In this contribution it will be presented the means and the results of conformational study of the CS-NH, Ar-CSNH and Ar-NHCS bonds of these derivatives. These results were obtained by chemical modification of above mentioned derivatives and by NMR spectrometry of these compounds.

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SYNTHESIS AND EVALUATION OF POTENTIAL INHIBITORS OF MYCOBACTERIAL ISOCITRATE LYASE

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The global burden of tuberculosis, its health and socio-economic impacts, the presence of drug-resistance and a threat of latent tuberculosis should serve as a strong impetus for the development of novel antimycobacterial agents, especially with an innovative mechanism of the action[1].

The identification of unique mycobacterial pathways and their targeting belongs to the contemporary approaches. Isocitrate lyase (ICL), an essential enzyme of glyoxylate shunt, splits isocitrate to succinate and glyoxylate. Disruption of *icl* gene attenuated bacterial persistence and virulence without affecting the growth during the acute phase of infection. Additionally, ICL is not blocked by conventional therapy, which is thought to be an important reason for the length of anti-tuberculosis therapy[1,2,3].

Recently, a review dealing with ICL inhibition has been published[2]. Our portfolio of compounds evaluated as potential ICL inhibitors is represented by previously published salicylanilides, their esters with benzoic, pyrazinoic, benzenesulfonic acids and *N*-acetyl-L-phenylalanine[1]. Latterly, we synthesized salicylanilide esters with 4-(trifluoromethyl) benzoic acid[4] and salicylanilide *N*,*N*-disubstituted carbamates. Derivatives of itaconic acid, 4-nitrophenol and 4-nitroaniline (mostly simple esters, amides and Schiff bases) represent other investigated chemical groups.

In general, salicylanilide derivatives act as moderate inhibitors of mycobacterial ICL at the concentration of 10 μmol/L. Among salicylanilides, 4-(trifluoromethyl)benzoates showed the most favorable inhibition properties (inhibition rates of 12–27%) with 2-[(3-bromophenyl)carbamoyl]-4-chlorophenyl 4-(trifluoromethyl)benzoate superiority[4]. From other compounds, 2-methylene-4-[(4-nitrophenyl)amino]-4-oxobutanoic acid showed the highest ICL inhibition – 55% rate at 10 μmol/L and 9% rate for the concentration of

1 μmol/L. Some of the compounds were comparable or superior to 3-nitropropionic acid, a known standard inhibitor.

All derivatives were evaluated against drug-sensitive *Mycobacterium tuberculosis* and atypical mycobacterial strains (*M. avium*, *M. kansasii*), while the most antimycobacterial active compounds were assayed additionally for their activity against multidrug-resistant tuberculous strains with different resistance profile. All of these derivatives exhibited activity against *M. tuberculosis* and atypical mycobacteria with MIC values ≥ 0.125 μmol/L.

As expected and as it arose from the physiological role of ICL, there is no direct correlation between ICL inhibition rates and MIC values.

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FURTHER DEVELOPMENT OF SYNTHESIS OF EXOCYLIC-DOUBLE-BOND-CONTAINING UNSATURATED SIX-MEMBERED HETEROCYCLES

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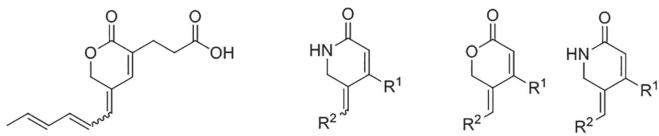
In this research, our primary motivation was to accomplish total synthesis of gelastatin A and B (**1**) because of their biological activity[1]. The synthesis should have proceeded in a way which would enable installation of various substituents on the heterocyclic core easily, so that we could modulate the activity itself. In this task we weren't successful. We, however, have found out, that six-membered lactams (**2**) of general structure shown below are much more stable than expected. Because very little of such compounds and their chemical behavior is known and because they could be the target of stereoselective

Michael additions, which would furnish highly branched lactones and lactams with defined stereochemistry, we pursued our research in terms of simplification the basic synthetic protocol. Breakthrough in this area should give us easy access to variable starting materials (**3**) for further experiments. We started our investigation towards lactones as their precursors are commercially available or easily accessible. So far, we have reached partial success and also observed some unexpected results.



1 **2** **3**

The study was generously supported by the Grant Agency of Charles University (No. 0255/2010) and Charles University in Prague (SVV-265-001).

SYNTHESIS OF POTENTIAL ORGANOCATALYSTS BASED ON ALCALOIDS VASICINE AND VASICINONE

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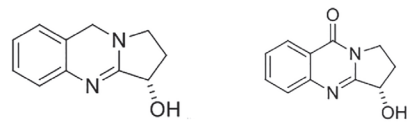
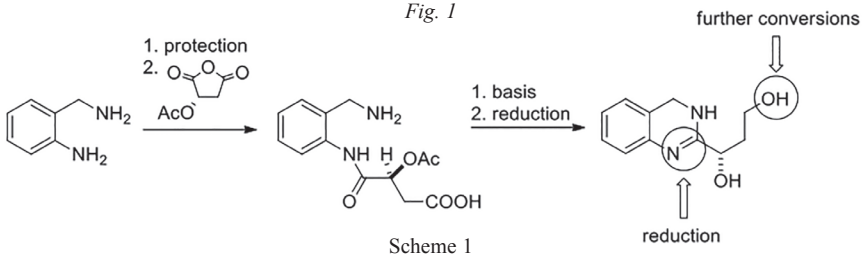
Series of substances derived from vasicine-type alkaloids (Fig. 1) was synthesized. These derivatives are recently being tested for their potential organocatalytic activity. Two synthetic pathways for introducing the chiral moiety were optimized using aminobenzylamine and α-hydroxy carboxylic acids (malic, lactic) and their derivatives (Scheme 1).



vasicine vasicinone

Fig. 1



Scheme 1

The study was supported by GA UK (No. 5671/2012), GA ČR (No. P207/10/2048) and Charles University Research (SVV-265-001).

SYNTHESIS OF POTENTIAL ANTITUBERCULOTICS BASED ON BENZYLSULFANYL HETEROAROMATES

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Tuberculosis (TBC) is a widespread infectious disease affecting about one third of world population. For example, in 2010, about 8.8 mil new cases of TBC were found and 1.7 mil people died. Another major problem is the presence of strains resistant to the conventional treatment. Therefore, the search for new antitubercular drugs, active also against these resistant strains, is highly important. Numerous studies dealing with the synthesis of new antitubercular drugs have been published to date. However, only one substance has been implemented to the clinical practice since 1963 (bedaquiline in 2013).

It was discovered that some substances containing benzylsulfanyl group bound to heteroaromates had the same or comparable antimycobacterial activity as standard isoniazide.

In this work we focused on the synthesis of antimycobacterially active compounds based on these benzylsulfanylheteroaromates. The modifications in the heterocycle part as well as in the linker between substituted phenyl group and heteroaromate have been carried out.

Antimycobacterial activities of these compounds as well as their toxicity against isolated human hepatocytes are being evaluated.

This project was supported by the Grant Agency of Charles University (Project no. 55610/2010) and by the Charles University in Prague (SVV 265 001).

DETERMINATION OF ABSOLUTE CONFIGURATION OF *NERRINE BOWDENII* AKALOID BY THE MODIFIED MOSHER'S METHOD

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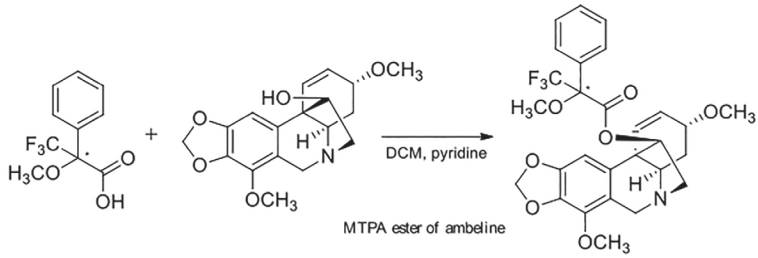
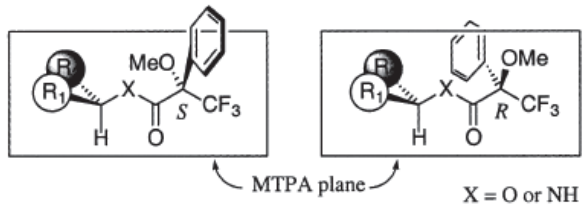
The Department of Pharmaceutical Botany and Ecology, Faculty of Pharmacy in Hradec Králové has isolated number of alkaloids from bulbs of *Nerrine Bowdenii* Watson (Amaryllidaceae). Most of them were analysed in Laboratory of structure and interactions (Department of Inorganic and Organic Chemistry). Structures of alkaloids were determined

by using multidimensional NMR spectroscopy, except the exact stereochemistry. Therefore, MTPA (2-methoxy-2-(trifluoromethyl)acetic acid) esters were prepared to yield pairs of diastereomeric derivatives.



The absolute conformation was assigned using the correlation model based on the *sp* conformation in which the C – CF_3 bond is *synperiplanar* to the C = O bond of the ester carbonyl group[1].



Analysis of the $\Delta\delta = \delta_S - \delta_R$ values obtained from the (R)- and (S)-MTPA esters led to the determination of the absolute configuration[2,3]. Stereochemistry of one of these unknown compounds was determined as ambeline[4].

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SYNTHESIS OF CERAMIDES TYPE A AND EO

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The primary function of the skin is to provide resistance of the body against water loss and penetration of exogenous substances, such as toxins or bacteria. The main skin barrier

is situated into stratum corneum, the top layer of the skin. It is composed of corneocytes (flat cells) and the lipidic matrix surrounding them. Lipids, filling the intercellular space of stratum corneum, are composed of equimolar mixture of ceramides, cholesterol and free fatty acids.

Twelve structure types of ceramides occur in the skin. These types are derived from 4 aminoalcohols – sphingosine (S), phytosphingosine (P), 6-hydroxysphingosine (H) and dihydrosphingosine (DS). Primary amino group of these aminoalcohols is acylated by non-substituted acid (N), α-hydroxy acid (A) or ω-hydroxy acid esterified with linoleic acid (EO).

The aim of my work is to prepare ceramides of the A and EO type, because these are not commercially available, and to study their barrier properties.

Synthesis of ceramides of type A started from lignoceric acid (tetracosanoic), which was α-brominated and the bromine substitued by hydroxyl. The racemic mixture of α-hydroxy acids was separated enzymaticaly using lipase. R-isomer was obtained this way, but not the S-isomer, which is natural.

In the future, we will try to oxidate racemic α-hydroxyacid to α-oxoacid and then reduce it, using chiral catalyst to obtain pure (S)-isomer. Resulting (S)-acid will react with sphingoid base to create ceramides of type A.

Synthesis of ceramides of type EO started from 16-bromohexadecanoic acid, which was converted into protected ω-hydroxy aldehyde by series of reactions. This was the first fragment for the Wittig reaction. The second fragment was obtained also from 16-bromohexadecanoic acid by converting it to phosphonium salt. After the Wittig reaction, the unsaturated product was methylated, hydrogenated and unprotected to obtain 32-hydroxydotriacontanoic acid. Currently we are optimizing the conditions of the hydrogenation of the double bond. Then, the obtained acid will react with linoleic acid to form an ester. After the reaction with sphingoid base, this ester will provide a ceramide type EO.

This work was supported by the Czech Science Foundation (207/11/0365) and by Charles University (SVV 265 001).

EFFECT OF SPHINGOMYELIN ON THE STRATUM CORNEUM MODEL MEMBRANES

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Stratum corneum (SC) is the outermost layer of the epidermis responsible for the barrier function of the skin. The cutaneous barrier prevents excessive water loss and entry of harmful substances. The SC consists of cornified cells embedded in the extracellular lipid matrix. Whereas the composition of lipid matrix (ceramides (CER) 40–50% (in weight), free fatty acids (FFA) 7–13%, cholesterol (CHOL) 20–33%, cholesterol esters 0–20% and cholesterol

sulphate (CHS) 0–7%) is well known, the structural organization of the SC lipids remains discussed. Recent research indicates that the altered SC lipids metabolism and increased cutaneous permeability play an important role in the skin disease pathophysiology. For example, reduced enzymatic activity of acid sphingomyelinase and decreased ratio of ceramides in SC have been detected at atopic dermatitis and Niemann-Pick disease. The acid sphingomyelinase breaks down one of the ceramide's precursor sphingomyelin (SM) to ceramide and phosphocholine. Moreover, the acid sphingomyelinase inhibitor-induced accumulation of SM has been found in murine SC. The aim of our study is to reveal, if does the increased fraction of SM contribute to the impaired cutaneous barrier function. We used a simplified lipid model to study the effect of SM on permeability, structure and phase behavior of SC. The equimolar mixture of CER (NS type), FFA and CHOL and 5% (in weight) of CHS represents a reference model of normal SC. To simulate the reduced sphingomyelinase activity, from 25 to 100% of CER were replaced with SM. The lipid mixtures were deposited on the polycarbonate filters and served as model membranes for permeation experiment using Franz type diffusion cells. Fourier transform infrared spectroscopy (FT IR) was employed to study the temperature dependent phase behavior and structural organization of SC model membranes. Preliminary results indicate that ceramide precursor sphingomyelin does not significantly increase the permeation through the model membranes. However, SM modulates the structural arrangement and the phase behavior of SC model membrane.

The study was supported by the operational programme ECOP, registration number CZ.1.07/2.3.00/30.0061, Increasing of the R&D capacity at Charles University through new positions for graduates of doctoral studies, Czech Republic.

CHAIN LENGTH – SPECIFIC PROPERTIES OF SKIN CERAMIDES

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Ceramides (Cer) are essential constituents of the skin barrier. Together with cholesterol and fatty acids, they fill the intercellular space of the upper skin layer (stratum corneum, SC). The decrease in content and changes in the composition of Cer are associated with diseases such as atopic dermatitis or psoriasis. Structure of Cer and their role in the skin diseases have been known for decades, but the importance of the individual structural features in their molecules is poorly understood.

Our long-term aim is to study the structural requirements for Cer function in a competent skin barrier to lay the design criteria for skin barrier repair agents, study the underlying mechanisms, and to identify any diagnostic or predictive parameters for the skin barrier function.

In this study we have focused on an importance of acyl chain length. Previously, we found that the short-chain Cer can not maintain the skin barrier function – they

increase the permeability of both the skin and model SC lipid membranes with maxima at C4–6 acyl. Now, we studied chain order and packing of multilamellar lipid systems by using infrared spectroscopy and X-ray diffraction and molecular organization by Langmuir monolayers and atomic force microscopy. We found that the native long-chain Cer membranes form highly ordered tightly packed continuous domains of Cer with fatty acids. To the contrary, membranes containing short-chain Cer formed mainly less ordered short Cer-rich domains that occupied more space and might provide a more permeable shortcut for exogenous substances.

Thus, the exceptionally long acyl chains found in human skin Cer are essential for their barrier function and should be included in the design of Cer analogs for a prospective treatment of skin barrier disorders.

The study was supported by the Czech Science Foundation (GACR 207/11/0365) and the Charles University in Prague (GAUK 652412 and SVV 265 001).

THE SIMULTANEOUS ANALYSIS OF A PRODRUG – BORONYL SALICYLALDEHYDE ISONICOTINOYL HYDRAZONE AND ITS ACTIVE FORM – SALICYLALDEHYDE ISONICOTINOYL HYDRAZONE IN BIOLOGICAL MATERIALS

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Salicylaldehyde isonicotinoyl hydrazone (SIH) is a biocompatible iron chelator with low toxicity and significant antioxidative and cardioprotective effects. Unfortunately, this compound suffers from a short biological half-life associated with the cleavage of the hydrazone bond. Boronyl salicylaldehyde isonicotinoyl hydrazone (BSIH) was synthesized as a pro-drug that is selectively bioactivated by hydrogen peroxide to SIH. This ability provides a possibility to better focus a pharmacological effect of this chelator and avoid adverse effects of the former compound based on an iron deprivation.

The aim of this study is to utilize HPLC-UV for investigation of stability of BSIH in plasma *in vitro* under physiologically relevant conditions and compare these results with the stability of parent SIH. The method was also used to study the compound bioactivation in cell culture media. The samples were analyzed on a HPLC column Zorbax Bonus-RP (150 × 3 mm, 3.5 μm) protected with a guard column. Mobile phase was composed of 0.02 M phosphate buffer (pH 6.0) and a mixture of methanol and acetonitrile (40:60, v/v), in a ratio of 60:40 (v/v).

Plasma samples were precipitated using methanol and the cell medium was simply diluted with the same solvent. Linearity of the developed method was proven within the range from 5 to 40 μg/ml and 10 to 50 μg/ml for cell medium and plasma, respectively. Precision and accuracy of the method were assessed only for plasma and reached acceptable values.

The stability experiment revealed that pro-chelator BSIH is significantly more stable in plasma *in vitro* (37 °C) when compared to SIH that could be beneficial to get more favorable pharmacokinetic profile. Activation of BSIH in cell medium was studied in a pilot experiment using a physiologically relevant concentration of hydrogen peroxide. Fifty and 100 μmol/l of BSIH was incubated (37 °C, dark) with 200 μmol/l of hydrogen peroxide and assessed within 24 hours. Only 15.9% (50 μmol/l) and 28.0% (100 μmol/l) of BSIH was detected in the end of the experiment.

In further experiments, the HPLC method developed in this study will be modified for UHPLC analysis, which could significantly shorten analysis time and improve resolution. Acquired results will be evaluated and the better method will be selected for a full validation according to EMA guidelines. Finally, the method will be used to study BSIH bioactivation in cell medium and plasma in details.

The study was supported by GAUK 367911 and SVV 265001.

SYNTHESIS OF NON-PERIPHERAL SUBSTITUTED PHTHALOCYANINES WITH ALKYLSULFANYL GROUPS

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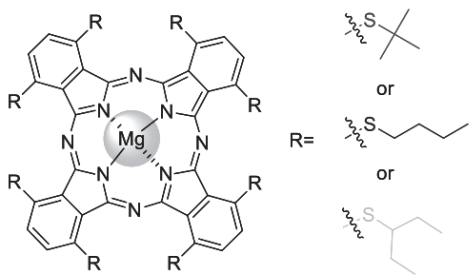
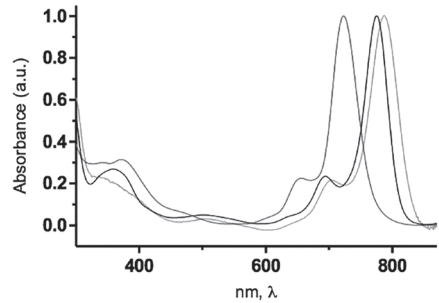
The first part of my work was focused on attempts to synthesize unsymmetrical Azaphthalocyanines (AzaPc) with senzoric properties via solid phase synthesis. At first we tried to prepare disubstituted pyrazinedicarbonile (as a precursor of AzaPc) bearing one tertiary amino group and one hydroxyl group. Presence of hydroxyl was necessary for binding to solid phase – Wang resin. The synthesis of unsymmetrical precursors was attempted by benzoin condensations. The syntheses were unsuccessful despite many attempts and the project was left.

The aim of next work was synthesis of phthalocyanines (Pc) substituted in non-peripheral position by alkylsulfanyl analogues (*tert*-butyl, *n*-butyl, pentan-3-yl) and comparison of their photophysical and photochemical properties. The synthesis of precursors for cyclotetramerization was the first step of this work. They were prepared by nucleophilic substitution of 3,6-ditosylphtalonitrile by appropriate alkylthiol. Cyclotetramerization of prepared precursors was accomplished by magnesium butoxide as an initiator of reaction. These Pcs were confirmed by spectral methods. We observed unusual hypsochromic shift that can be caused by torsion of the macrocycle in the UV-Vis spectrum of the magnesium Pc with *tert*-butylsulfanyl substitution[1]. The crystallographic analysis may bring the answer.





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MOLECULAR MODELING STUDY ON NOVEL ANTIMYCOBACTERIAL PYRAZINAMIDE DERIVATIVES

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Tuberculosis remains one of the world's leading infectious diseases not only in developing countries. *Mycobacterium tuberculosis* multidrug resistant strains and the prospect of nosocomial transition are currently highly problematic. These issues make the development of new, better-tolerated therapeutics even more important[1].

Pyrazinamide (PZA) is an important sterilizing antimycobacterial drug, which helps to shorten the duration of current standard treatment. The PZA's mechanism of action is not to this day satisfactorily explained. Pyrazinoic acid has been shown to be active moiety of PZA. It was proven to inhibit various functions at acid pH in *M. tuberculosis.* PZA is able to inhibit fatty acid synthases (FAS I and FAS II) contributing to the synthesis of mycobacterial cell wall. FAS I is involved in synthesis of short-chain mycolic acids, FAS II mediates long-chain mycolic acids synthesis. One of the crucial enzymes of FAS II is Enoyl-acyl carrier protein reductase (InhA; E.C.1.3.1.9). Several inhibitors of InhA are used as antimycobacterial treatment (isoniazid, triclosan)[2].

Molecular modeling study with series of 27 5-chloropyrazineanilides was performed in order to clarify possible mechanism of InhA inhibiton. AutoDock Vina 1.1.2, a new program for molecular docking and virtual screening was used for docking calculations. A Lamarckian genetic algorithm (Amber force field) was used. The structure of mycobacterial InhA was prepared from crystal structure (pdb number: 4DRE) using Autodock Tools 1.5.4. The molecular models of ligands were prepared using Java Molecule Editor and minimized using UCSF Chimera 1.6.2. The visualization and analysis of enzyme-ligand interactions was prepared using Pymol 1.5.

The results suggest the apparent influence of π-π interactions with Phe 149 and hydrogen bonding for potential inhibitor binding within the mycobacterial InhA active site. The influence of anilide part of inhibitor molecule substitution is also discussed.

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PREPARATION OF NEW PYRAZINAMIDE DERIVATIVES FOCUSED ON MICROWAVE ASSISTED SYNTHESIS AND THEIR BIOLOGICAL EVALUATION

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Although the absolute number of new tuberculosis cases has been decreasing slowly since 2006, this infectious disease has been becoming more and more dangerous during last years.

It is caused by the fact that the new resistant strains of *Mycobacterium tuberculosis* are appearing more frequently than ever before. This reason together with rapidly growing HIV co-infection problem leads to urgent effort to invent highly effective, safer and innovative drugs.

One of the current first-line anti-tuberculosis drugs is pyrazinamide. This small template molecule, which mechanism of action was not clear for a long time, is very suitable for structure modifications due to its unique chemical properties. This research project is focused on syntheses of six series of pyrazinamide derivatives and determination of their biological properties.

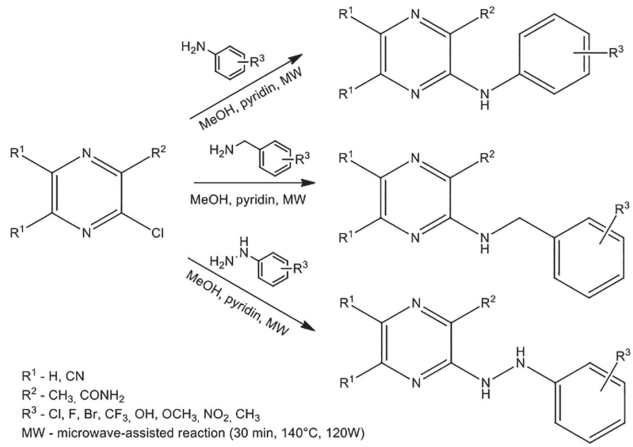
Two starting compounds (3-chloropyrazine-2-carboxamide, 5-chloro-6-methylpyrazine-2,3-dicarbonitrile) were treated with various groups of on-ring substituted anilines, benzylamines and hydrazines. These aminodehalogenation reactions were performed by using microwave reactor with focused field due to experimentally proven higher yields and shorter reaction times.

New prepared substances were characterized by melting point, ^{1}H, ^{13}C and IR spectra, elemental analysis and log *P* (resp. log *k*). *In vitro* biological screenings have followed.

Antimycobacterial evaluation was completed against few mycobacterial stems using isoniazide as standard and determining minimal inhibition concentration (MIC). The most active substance from group of currently tested compounds is 3-((4-methylbenzyl)amino) pyrazine-2-carboxamide.

Next screening was focused on herbicidal activity. The principle is measuring the inhibition of photosynthetic electron transport (PET) in spinach chloroplasts. DCMU (Diurone®) was taken as a standard determining inhibition concentration IC_{50}. Also the site of action in photosynthetic apparatus was determined by artificial electron donor 1,5-diphenylcarbazide. Two series of compounds treated with benzylamines showed good herbicidal activity. The most active substance was 5-((3,4-dichlorobenzyl)amino)-6-methylpyrazine-2,3-dicarbonitrile. The last testing included antibacterial and antifungal activity evaluation against eight bacterial and eight fungal stems using five antibiotic standards (neomycin, bacitracin, penicillin G, ciprofloxacin, phenoxymethylpenicillin) and four anti-mycosis standards (amphotericin B, voriconazole, nystatin, fluconazole). MIC was determined as 90% IC. Any substance did not show significant activity in these screenings.

It is possible to predict structure-activity relationships only for two series treated with benzylamines between herbicidal activity and lipophilicity (π) and electronic properties (σ) at this moment.



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STUDY AND APPLICATION OF RETENTION MECHANISMS OF ZIRCONIA BASED SORBENTS

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Although silica-based columns are still the most frequent in HPLC analyses, the sorbents based on metal oxides have already found their place in liquid chromatography. Compared to silica gel, the zirconia and titania phases differ in temperature and pH stability as well as in retention mechanism.

The retention properties of sorbents based on ZrO_2 was studied under different conditions (mobile phase composition, temperature). It was found, that compounds with the Lewis acid character are significantly retained in both low and high content of organic component in the mobile phase. This behavior suggests that the Lewis base and Lewis acid interactions play an important role in the retention process onto the zirconium surface. This assumption was then proved using polybutadiene coated zirconia column.

The possibility of using ZrO_2 sorbents in a selective extraction of analytes were designed on the basis of these previously obtained results. Mainly the interaction between acidic model compounds and bare zirconia or zirconia modified with ion exchangers were studied under various aqueous rich (different pH, buffer) or HILIC (98% ACN) conditions. The same procedure was done with plasma and the results were compared with the previous. The best conditions for separating analytes from plasma were determined and subsequently experimentally proved.

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SYNTHESIS AND EVALUATION OF QUATERNARY NITROGEN COMPOUNDS

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The work deals with the preparation and testing of compounds of type cationic surfactants such as disinfection and decontamination agents. Since the quaternary cationic surfactants are substances widely used in many of applications (pharmaceuticals, chemical industry, food industry etc.) are still of great interest. It was designed and prepared more than 40 surfactants based on quaternary nitrogen. Substances derived from structures commonly used (benzalkonium, cetylpyridinium or cetyltrimethylammonium). Synthetic continuously builds on my diploma thesis, where I dealt with the preparation of similar compounds. However dissertation describes the synthesis of compounds that may have to include a various nucleophilic group (hydroxyl or oxime) into a structure. It was prepared six sets of substances based on 6-hydroxyquinoline, 3-hydroxypyridine, phenylethyldimethylamine, 3-hydroxiiminomethylpyridine, 4-hydroxiiminomethylpyridine and 4-(1-aminohydroxiiminomethyl)pyridine. Each set contain seven homologues differing in two methylene units (C8-C20). Prepared structures were confirmed with analyzes of NMR, MS and EA. Furthermore HPLC method was developed to distinguish the individual homologues in the mixture.

For most compounds was measured the critical micelle concentration as a fundamental characteristic of surfactants. It was confirmed the structure relationship between the value of CMC and lipophilic chain length in the molecule.

A few selected compounds were then evaluated for antimicrobial activity expected. Some compounds significantly influenced the growth of several strains of bacteria or fungi. The minimum inhibitory and minimum microbicidal concentrations were determined afterwards.

Since structurally similar compounds are also known as the micellar catalysts (acceleration or inhibition of some reactions using micellar microenvironment), several prepared compounds were tested for this property. In collaboration was several times published the effect of cationic surfactants as the accelerators of some ester cleavage. These esters serve as the model compounds of chemical warfare agents and pesticides.

At the end based on above-mentioned properties were chosen several compounds as potential decontamination, disinfection components.

The study was supported by the European Social Fund and the state budget of the Czech Republic. Project no. CZ.1.07/2.3.00/30.0061.

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DEVELOPMENT AND VALIDATION OF RAPID UPLC METHOD FOR DETERMINATION OF RISPERIDONE AND ITS IMPURITIES IN BULK POWDER AND TABLETS

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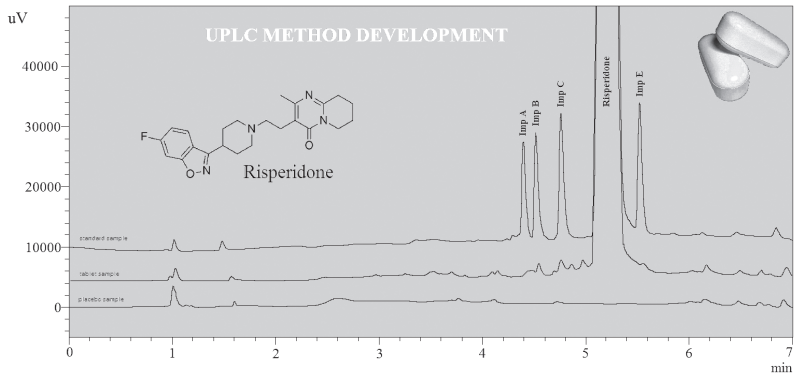
The aim of this study was to develop a new, rapid and sensitive UPLC method with UV detection for simultaneous determination of risperidone and four relative substances presented in tablets. The active substance, risperidone is the most frequently used atypical antipsychotic drug for treatment of schizophrenia, bipolar disease and behavioral disorders in youth, up to 17 years. The study is based on main impurities determined in USP35 and European Pharmacopoeia 7 (Imp A, B, C and E). Developed method is



based on binary gradient elution using silica based RP-18 chromatographic column (100 mm × 3.5 mm; 1.7 µm). Ammonium acetate buffer pH 6.8 and acetonitrile were used as mobile phases in gradient mode with flow rate of 0.5 ml/min and temperature 40 °C. Wavelength of UV detector was set to 260 nm. The developed method allows shortening analysis time up to four times comparing to conventional HPLC method used by USP35 for determination of related substances of risperidone in tablets and three times comparing to method for related substances presented in European Pharmacopoeia 7. The method was validated in accordance with ICH requirements. Measured validation parameters included selectivity, linearity, precision, accuracy, sensitivity and robustness. Developed method is suitable for use in pharmaceutical industry as well as any other analytical laboratory.

The study was supported by PRVOUK.

DEVELOPMENT OF NEW ANTIMYCOBACTERIAL DERIVATIVES OF PYRAZINAMIDE

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Worldwide, tuberculosis (TB) is the most common life-threatening infectious disease and remains a major threat to public health. In addition, increased susceptibility to TB in HIV-positive patients is another serious health issue[1]. Pyrazinamide (PZA) belongs to the most important first-line anti-TB drugs. Along with rifampicin, PZA has sterilizing activity (the ability to kill the semi-dormant mycobacteria) which is a crucial factor in shortening the duration of therapy[2].

The aim of our research project is to synthesize various compounds derived from PZA and screen them primarily for *in vitro* antimycobacterial activity (*Mycobacterium tuberculosis* H37Rv, *M. kansasii* and two different stems of *M. avium*) and additionally for antifungal and antibacterial activity. All studied compounds are also evaluated for their photosynthetic electron transport (PET) inhibition in spinach chloroplasts. Some PZA analogues have already proved good antimycobacterial activity in the previous studies.[3]

We focused on functional derivatives of 5- or 6-chloropyrazine-2-carboxylic acids, especially on substituted anilides, amides derived from benzylamines and anilides with aliphatic substitution in pyrazine part. The most active compounds exerted minimal inhibition concentration MIC = 1.56 µg/mL against *M. tuberculosis* H37Rv, which is a significant improvement compared to parental PZA (MIC = 6.25–12.5 µg/mL). During our studies we have identified structural motiffs which often carry promising antimycobacterial activity. These structure-activity relationships (SAR) will be discussed.

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DEVELOPMENT OF NOVEL THIOSEMICARBAZONE ANTICANCER AGENTS – A BIOANALYTICAL EVALUATION

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Cancer is ranked by the World Health Organization among the top 10 leading causes of death worldwide. The rising rate of global incidence as well as the resistance to current chemotherapy calls for development of novel and potent anti-tumour agents that can enhance patient response and improve cancer survival rates.

Thiosemicarbazone iron chelators are currently under intensive investigation for their ability to selectively target an essential nutrient – iron, resulting into inhibition of the growth of cancer cells.

The aim of this study was to develop modern analytical methods for the analysis of most potent thiosemicarbazones in biological materials and utilized them 1) to *in vitro* stability study in plasma; 2) to propose a formulation for their i.v. application and 3) to study the fate of these drugs in an organism.

Initially, novel HPLC-UV methods for determination of di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), 2-hydroxy-1-naphthylaldehyde thiosemicarbazone (N4mT) and their degradation products were developed and utilized in the stability study in human, porcine and rabbit plasma. The results indicated that both thiosemicarbazones are significantly more stable in plasma in comparison to their forerunners – aroylhydrazones.

Subsequently, a sensitive HPLC-MS method capable to quantify 2-benzoylpyridine-4-ethyl-3-thiosemicabazone (Bp4eT) was developed and used in a solubility study. Based on the results, the composition of the i.v. formulation for advanced preclinical experiments was proposed.

Further effort was focused on investigation of metabolism of the thiosemicarbazones. Bp4eT was incubated with human and rat liver microsomes and cytosol *in vitro* and

the structure of two phase I metabolites was proposed using LC-MS/MS. Both of the metabolites were subsequently detected *in vivo* in rats, in addition one novel metabolite was found *in vivo*. Finally, a novel HPLC-MS/MS method for the analysis of Bp4eT and its metabolites was developed and applied to pharmacokinetic study in rat plasma. The concentration-time profile of the parent compound was obtained. The amount of both metabolites assayed in plasma was only minor as compared with amount of the parent drug.

Metabolism of the newly developed and highly effective iron chelator, di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC), was studied *in vitro* by incubation with human liver microsomes and S9 fraction. The samples were analyzed using the UPLC-QTOF and the structure of ten phase I and two phase II metabolites was proposed.

Further effort will be focused on an analytical evaluation of DpC where the relevance of the *in vitro* metabolites as well as the pharmacokinetic profile of the parent compound/metabolites will be studied.

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BIOANALYTICAL ASSESSMENT OF DPC – A FIRST LOOK INTO ITS *IN VIVO* METABOLISM IN RATS AND PENETRATION INTO CANCER CELLS

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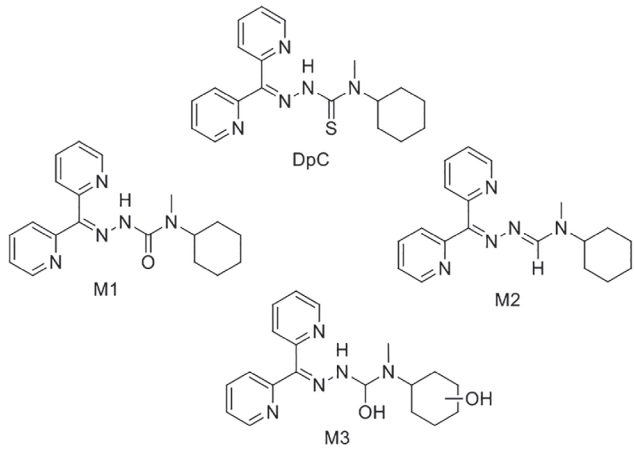
Thiosemicarbazone iron chelators rank among very potent antitumor agents, which undergo an extensive preclinical investigation in order to promote them to a clinical phase. According to results of recent studies, di-(2-pyridyl)ketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC, Fig. 1) appears to be most effective against several cancer cell lines with little to none severe adverse effects.

The main objective of this study was to obtain first insight into the *in vivo* metabolism of DpC and penetration of this compound into MCF-7 cells. Reversed – phase column (Discovery C18 75 × 4.6 mm, 3 μm) and a mobile phase composed of 2 mM ammonium acetate and acetonitrile were utilized in both experiments using IT-MS detection.

In the pilot metabolism experiment rats were administered 3 mg/kg of DpC intravenously. Plasma, urine and faeces samples were harvested in predefined times. The plasma and urine specimens were treated with solid phase extraction and faeces with liquid-liquid extraction. In our previous *in vitro* experiment we detected and identified ten phase I and two phase II metabolites of DpC. Only three of the twelve proposed metabolites (M1–M3, see Fig. 1) were detected in urine and plasma and two of them were found in faeces

(M1, M3) in this *in vivo* study. Nonetheless, their quantitative, pharmacological and toxicological significance is yet to be assessed.

In the pilot experiment cancer cells were incubated with DpC (5 μM) for six hours. The cells were harvested in defined time intervals, washed twice with PBS, precipitated with ice cold acetonitrile and analyzed. This experiment showed that this compound easily penetrates into cancer cells and prolonged exposition leads to the higher intracellular concentrations of DpC. Detailed study on the penetration properties of DpC is currently under investigation.



This work was supported by the grant of the Ministry of Health of the Czech Republic (IGA NT 12403-3/2011) and SVV 265 001.

ANION RECEPTOR BAMBUSURIL

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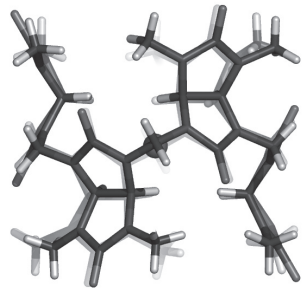
Glycoluril is a rigid bicyclic molecule that has been used as a building block of various supramolecular objects. Besides acyclic supramolecular hosts based on glycoluril, such as molecular clips, glycoluril oligomers and capsules – cucurbiturils. Recently, we prepared a new macrocyclic derivatives based on glycoluril – bambus[6]uril (BU[6]). The macrocycle is prepared by acid catalyzed condensation between 2,4-dimethylglycoluril and formaldehyde. The reaction is carried out in diluted HCl which acts not only as a catalyst but also as a template. BU[6] and chloride anion form an inclusion complex in which anion is localized in the middle of positively charged cavity of the macrocycle. The macrocycle bounds halide anions in sequence $F^- < Cl^- < Br^- < I^-$ with high affinity and selectivity in organic solvents and in aqueous media.



Support for this work was provided by the Grant Agency of the Czech Republic (P207/10/0695) and the project CETOCOEN (no. CZ.1.05/2.1.00/01.0001) from the European Regional Development Fund.

SYNTHESIS AND STUDY OF AZAPHTHALOCYANINES AND PHTHALOCYANINES IN RELATION TO THEIR PERIPHERAL HETEROATOMS SUBSTITUTION

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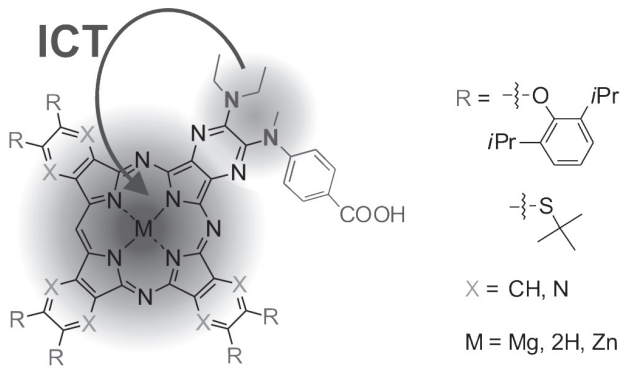
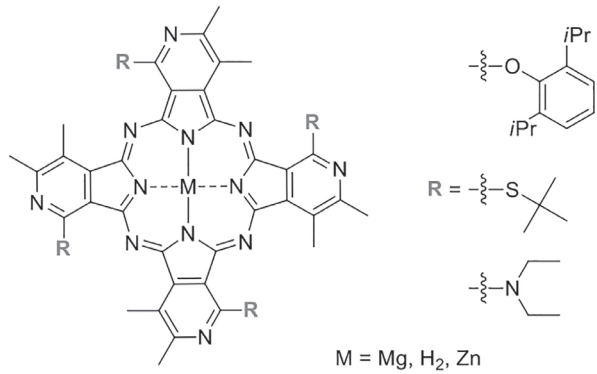
This work describes synthesis, photophysical and photochemical study of azaphthalocyanine (AzaPc) and phthalocyanine (Pc) derivatives with peripheral donor substituents for intramolecular charge transfer (ICT). In the first part of the work, we focused on influence of peripheral heteroatom substitution (oxygen or sulfur) and macrocycle composition (AzaPc, Pc) on fluorescence and singlet oxygen production. These photophysical parameters were studied in relation to ICT. ICT is responsible for deactivation of excited states that may be used in development of new sensors or dark quenchers of fluorescence[1].





In another part of the study, we synthesized new group of AzaPc - tetrapyridoporphyrazines. Till now, no substituted macrocycles of this type have been prepared. The syntheses were successful and alkyl/aryl heteroatom substituents were attached to the periphery of this AzaPc. The absorption spectrum of tetrapyridoporphyrazine macrocycles is red-shifted when compared to corresponding Pc.

References

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The study was supported by grant GA UK 68110/B-CH/2012 and SVV 265 001.

PREPARATION OF AFFINITY CARRIER WITH CONJUGATED ORACIN FOR EFFICIENT ISOLATION OF CARBONYL REDUCING ENZYMES

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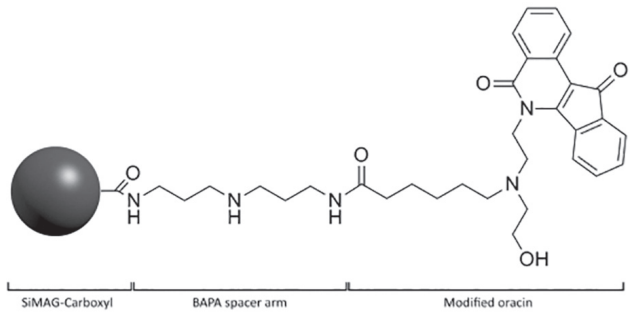
Carbonyl reducing enzymes play an important role in metabolic pathways of various eobiotics and xenobiotics. Clinically used drugs as e.g., doxorubicin, daunorubicin, haloperidol belong to xenobiotic substrates which are deactivated by reductive metabolism pathways. Enzymes reducing carbonyl compounds also contribute to development of some diseases like hormone-dependent cancers, metabolic syndrome or Cushing's syndrome. Many of these enzymes have been only poorly characterized or totally uncharacterized yet. Despite their importance in metabolism and diseases, there is still little known about them especially in the area of membrane-bound enzymes (e.g. until today only one membrane bound enzyme has been described as active towards xenobiotics, 11β-hydroxysteroid dehydrogenase type 1).

Due to their importance, it is necessary to make further studies of their substrate activity, distribution and possibilities of inhibition. Unfortunately, carbonyl reducing enzymes are usually in low concentrations in tissues so their isolation is very demanding. Purification with classical three phase purification strategy was not successful. It was not possible to obtain fraction with sufficient purity and amount of desired enzyme. The aim of the study is preparation of an original affinity carrier for selective isolation of carbonyl reducing enzymes from natural samples.

Oracin is a potential anticancer drug that has proven to be a substrate for many well-known carbonyl reducing enzymes so it seems to be universal ligand for developed affinity carrier. Firstly the molecule of oracin must have been appropriately modified so that it could be built on various carries. Three types of magnetic microparticles,



SiMAG-NH_2, SiMAG-COOH and magnetic macroporous bead cellulose have been used as matrix. After series of tests, optimal binding method of oracin on suitable matrix was chosen for further studies.

The ability of prepared affinity carrier to capture carbonyl reducing enzymes was tested on model samples. Enzymes with affinity to ligand oracin were selectively bound on the carrier and then eluted by elution buffer. It is clear that prepared original affinity carrier is well working with model enzyme samples and is ready to be used with natural samples.

This study was supported by the Grant Agency of Charles University (GAUK No. 71710/C/2010), project of the Grant Agency of the Czech Republic no. P206/12/0381 and by Charles University (SVV 265 004). The project is cofinanced by the European Social Fund and the state budget of the Czech Republic (TEAB, project no. CZ.1.07/2.3.00/20.0235.

THE EFFECT OF ANTHOCYANIDINS ON THE ACTIVITY OF HUMAN AND RAT GLUTATHIONE S-TRANSFERASES, UDP-GLUCURONOSYLTRANSFERASES AND CARBONYL REDUCTASES IN LIVER

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Anthocyanins and their aglycones anthocyanidins, a class of flavonoids, represent the largest and the most important group of water soluble pigments in plants. They are widely consumed by humans as natural compounds of vegetables, fruits, and red wine. Broad evidence of the beneficial effects of anthocyanins on human health has led to the increasing popularity of these substances in the form of food supplements and nutraceuticals. As the nutraceuticals contain concentrated bioactive agents, the occurrence of side negative effects on human health cannot be excluded. Interaction of these compounds with drug-metabolizing enzymes may affect the fate of co-administered drugs and thus exert pharmacological consequences. The aim of this study was to evaluate inhibitory potential of four anthocyanidins (delphinidin, cyanidin, malvidin, pelargonidin) on carbonyl reductases (CBR), glutathione S-transferases (GST) and UDP-glucuronosyltransferases (UGT) in human and rat liver.

The activities of CBR, GST and UGT were assayed in subcellular fractions using menadione, 1-chloro-2,4-dinitrobenzene and p-nitrophenol as specific substrates of these enzymes. The measurements were performed with or without anthocyanidins (100 μM). Half maximal inhibitory concentrations (IC_{50}) and the kinetics of inhibition were determined in human fractions for the most effective inhibitors (delphinidin for CBR and cyanidin for UGT).

The results of our study showed that anthocyanidins acted as inhibitors of all three enzymes. Activity of human UGT was decreased to the highest extent by cyanidin with

IC_{50} = 69 μM (at 200 μM substrate concentration) and competitive type of action. Delphinidin was revealed as the most potent inhibitor of CBR with IC_{50} = 16 μM (at substrate concentration 500 μM) and functioning in non-competitive manner. The inhibitory effect was the least pronounced in the case of GST. Malvidin did not affect GST activity; pelargonidin, delphinidin and cyanidin had IC_{50} about 150 μM. The inhibitory potency of anthocyanidins significantly differed in rat and human samples.

Anthocyanidins are able to inhibit CBR, UGT and GST *in vitro* in rat and human. Possible interactions of anthocyanidins (in high-dose dietary supplements) with co-administered drugs, which are UGT, CBR or GST substrates, should be taken into consideration.

The study was supported by Czech Science Foundation (Grant No. P303/12/G163).

CHARACTERIZATION OF TPR-LIKE PROTEINS IN *FRANCISELLA TULARENSIS*; IDENTIFICATION OF A NOVEL LIPOPROTEIN FTH-1662 REQUIRED FOR VIRULENCE

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Francisella tularensis is a highly infectious bacterium causing the potentially lethal disease tularemia. Proteins, which are involved in *Francisella* virulence phenotype, are still pursued. Of the potential candidates, proteins containing tetratricopeptide repeat (TPR)-like domains seems to be reasonable to study since TPR motifs form perfect module for protein-protein interaction (e.g. heat shock proteins), thus such proteins are often involved in important cell processes including virulence-associated function of bacterial pathogens. Here, we studied role of three putative TPR-like proteins FTH_0200, FTH_1662, and FTH_0778 in *Francisella tularensis* ssp. *holarctica* FSC200 virulence. Mutants defective in protein expression were prepared by targetron insertion mutagenesis. Only insertion mutagenesis of *FTH_1662* gene revealed changed phenotype, in terms of decreased virulence. FTH_1662 was shown to be crucial for intracellular replication inside primary murine bone marrow derived macrophages and represents important role in heat stress tolerance. We also found that FTH_1662 is essential for virulence in mice revealing lower bacterial growth and elimination from mice organs. Importantly, immunization with the FTH_1662 mutant strain provided protection against subsequent challenge with the wild type strain. Additionally, employing fractionation of the bacterium proteome we identified FTH_1662 protein to be a lipoprotein. Our study investigating the importance of the TPR-like proteins for *Francisella tularensis*

virulence in general, revealed a novel lipoprotein FTH_1662 to be required for virulence and heat stress tolerance.

The study was supported by grant from The Grant Agency of Charles University in Prague (project 160/50/15011), by SVV-2011-263-004, by Defense Threat Reduction Agency Medical S&T Division (Project D-CZ-10-0001) and by a Long-Term Organization Development Plan 1011.

CHARACTERIZATION OF NONMETASTATIC AND METASTATIC TYPES OF MDA-MB-231 CELLS

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Background: Breast cancer is heterogeneous disease, composed of tumor cells with different gene and protein expressions. Metastasis plays a major role in morbidity and mortality of breast cancer. The aim of present study was to investigate differences between metastatic and nonmetastatic cells in some steps of metastatic process: tumor cell invasion, proliferation, adhesion, migration and extravasation. Human breast cancer cell line MDA-MB-231 was used for this purpose. Expression of selected genes and proteins were compared in MDA-MB-231 primary tumor cells, lung metastatic cells (SCP4175) and bone metastatic cells (SCP1833) derived from MDA-MB-231 cell line.
Methods: Extracelular matrix proteins linked to FITC-dextran secreted by cancer cells during extravasation process was ascertained using transendothelial cell migration assay. The effect of inflammatory conditions, tumor necrosis factor alpha (TNF-α) stimulation of endothelial (EA.hy wt cells) and tumor cells, on the adhesive process was examined. Pro-inflammatory effect of TNFα on changes in expression of sphingosine pathway molecules and adhesion molecules was also studied using Western blotting and qPCR.
Results: Expression of individual proteins was mostly higher in metastatic cell lines in comparison to primary tumor cells. Stimulation of TNFα also increased expression of molecules in tested cancer cell lines. Adhesion effect between individual cell lines was significant. When both tumor and endothelial cells were stimulated with TNFα, increase in adhesion, which was notably higher in primary tumor cell line, was observed.

The study was supported by SVV 265 004.

S-NITROSOGLUTATHIONE CAUSES COVALENT MODIFICATION OF CBR1 AND AFFECTS ITS ACTIVITY

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In addition to oxidative reactions, carbonyl reduction contributes significantly to the phase I metabolism where reactive carbonyls are detoxified and excreted. Carbonyl reductase 1 (CBR1 or SDR21C1) is a well characterized human enzyme belonging to the short chain dehydrogenase/reductase superfamily. CBR1 is ubiquitous, 277 aminoacids long, cytosolic, monomeric, NADPH dependent enzyme, first isolated in 1973 from human brain. CBR1 participates in apoptosis, carcinogenesis and drug resistance, and has a protective role in oxidative stress, cancer and neurodegeneration. The broad substrate spectrum includes both xenobiotics and endogenous substances. The best substrates described are quinones, aldehydes and ketones, anthracyclines, prostaglandins, steroids, endogenous indol isatin or the latest substrate described, S-nitrosoglutathione (GSNO). Moreover, recently an inhibition of CBR1 was described as a consequence of covalent modification caused by GSNO. CBR1 comprises 5 cysteines (positions 26, 122, 150, 226, 227) which might be modified by GSNO. Four of those were analyzed using MALDI-TOF/TOF MS and then quantified using a using LC-SRM MS. The analysis confirmed GSNO concentration-dependent S-glutathionylation of all cysteines analyzed which was 2–700 times higher compared to wild-type CBR1 (CBR1 wt). Moreover, a disulfide bond between vicinal Cys 226 and Cys 227 was detected and we suggest a possible role of these two cysteines as a redox-sensitive cysteine pair.

To investigate changes in catalytic properties of the "modified" CBR1 and CBR1 wt, we cloned CBR1 into the pET-28b(+) vector, overexpressed in *E.coli* and purified. Next, the recombinant enzyme was incubated with 2 mM GSNO to perform the modification. After the buffer exchange, the steady state kinetics with various substrates were measured spectrophotometrically at 340 nm and by HPLC and compared to the CBR1 wt as a control. The results obtained showed, that CBR1 wt had about 2–5 times higher k_{cat} with menadione, 4-benzoylpyridine, 2,3-hexanedione, daunorubicin, 1,4-naphthoquinone, oracin and NNK compared to the "modified" form. Surprisingly, substrates containing a 1,2-diketo group in their ring structures (1,2-naphthoquinone, 9,10-phenathrenequinone and isatin) showed half to two-thirds lower k_{cat} in CBR1 wt compared to the "modified" form. We hypothesize that modification of Cys 226 and/or 227 by GSNO changes CBR1 activity by affecting the catalytic turnover rate or the nature of the rate-limiting step as well as by altering substrate binding.

The study was supported by the Grant Agency of Charles University (Grant No. 347211/C/2011), by Charles University Project SVV 265 004, by the Institutional program

of the University Hospital Hradec Králové, by Postdoctoral fellowship project No. CZ.1.07/2.3.00/30.0012. The publication is co-financed by the European Social Fund and the state budget of the Czech Republic. Project no. CZ.1.07/2.3.00/20.0235, the title of the project: TEAB.

METHODS USED IN OUR LABORATORY FOR STUDY OF CELLULAR OXIDATIVE STRESS

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Development or exacerbation of many diseases as well as natural aging process have been associated with the **oxidative stress**. This phenomenon is defined as a disturbance in the prooxidant-antioxidant balance in favour of former, due to a significant decrease in the effectiveness of antioxidant defences or an increased production of oxidizing species – especially the **reactive oxygen species** (ROS). ROS play important roles in both physiological and pathological processes, when their elevated concentrations lead to potential **damage to biomolecules** including nucleic acids, proteins and lipids. An important role in ROS production has been attributed to **transition metals**. The most abundant transition metal in human body is **iron**, which, if not appropriately bonded, catalyses unwanted free-radical reactions such as autoxidations and Haber-Weiss reaction (OH• formation) and aggravates **cellular oxidative damage**. Hence, **iron chelation** is an advantageous strategy against oxidative damage of cells. On the other hand, excessive iron depletion may result in toxicity to the cells, too. Therefore, the new strategy is oriented to the application of **iron pro-chelators activated under conditions of oxidative stress**.

Our aim has been to study the pathobiochemistry of oxidative stress, the role of free iron ions in that and effects of iron chelation.

Various methods of oxidative stress assessment have been used in our laboratory. These methods could be sorted according many aspects and allow to us studying oxidative stress from various points of view – *e.g.* oxidative stress sources, progress and/or its protection. We examine prooxidative properties of model substances (peroxides, catecholamines, anthracyclines) with/out free iron ions presence, production of ROS during oxidative action in solution or inside the cells, various cellular responses including changes in mitochondrial membrane potential, DNA changes and various pathways of cellular death. Furthermore, we study iron (pro)chelators for their affinity to bind iron ions (before and after ROS exposure), rate of their penetration inside the cells and their ability to bind free redox-active cellular iron, their potential to protect the cardiac cells against the toxicity of oxidative stress and their own toxicity.

All methods have to be appropriately selected and properly optimised but for all that they have not only advantages but also disadvantages, which must be taken into

account – such as selectivity, sensitivity, tendency to overestimate or underestimate the studied response. Therefore, further strategy is oriented towards the enrichment of repertoire of methods that can overcome imperfections of standard procedures and allow to elucidate unknown relations in oxidative stress.

The study was supported by OPVK project CZ.1.07/2.3.00/30.0061.

THE APPLICATION OF TRANSFECTION AND TRANSDUCTION TECHNIQUES FOR THE STUDY OF DRUG METABOLISM BY SDR ENZYMES IN INTACT CELLS

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Microsomal carbonyl reductases from the short-chain dehydrogenase/reductase (SDR) superfamily are NAD(P)(H)-dependent enzymes which are abundantly expressed throughout the human body. Some of these enzymes catalyze the biotransformation of endogenous compounds like steroid hormones or retinol. On the other hand, there is a huge number of superfamily members whose reducing activity toward endogenous substances as well as xenobiotics is completely unexplored. Possible reduction of carbonyl bearing drugs (e.g. warfarin) or prodrugs (e.g. oxcarbazepin) by SDR enzymes could dramatically influence the pharmacokinetic behavior of these medicaments and give rise to clinically relevant drug-drug interactions. In addition, SDR enzymes are commonly overexpressed in cancer, so they theoretically could be the reason of anthracycline resistance and, at the same time, might increase the risk of cardiotoxicity caused by the anthracycline reduced metabolite. In this study, we will investigate possible interactions of chosen drugs with microsomal SDR enzymes on the level of intact cells. At first, we will prepare plasmids containing genes for chosen SDR enzymes using classical molecular cloning techniques. In other part of our work, we will transiently transfect and/or stably transduce human cancer cells with these vectors in order to obtain SDR enzymes-overexpressing cellular models. Subsequently, incubation with drugs mentioned above will be performed employing overexpressing cells together with parent cells. Concentrations of reduced metabolites will be analysed and compared to evaluate possible involvement of SDR enzymes in the metabolism of examined drugs. Finally, in the case of positive results, the same cellular models and XTT cytotoxicity tests will be further employed to elucidate the effect of SDR enzymes on the cell resistance to anthracycline treatment.

This work is supported by OPVK project CZ.1.07/2.3.00/30.0061.

PROTECTIVE PROPERTIES OF NEW PRO-CHELATORS OF IRON AGAINST H_2O_2-INDUCED DAMAGE OF H9C2 CELLS

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Iron plays an important role in oxidative stress associated with numerous cardiovascular diseases. Therefore, shielding of free or loosely-bound iron via its chelation may be an effective therapeutic approach. Salicylaldehyde isonicotionyl hydrazone (SIH) as well as some other aroylhydrazone iron chelators have previously shown a considerable potential to protect cardiac cells against various oxidative stress-inducing agents. However, the potential favorable effects of chelators against oxidative injury may be in conditions with no systemic iron overload hindered by own chelators' toxicity due to iron depletion. Therefore we studied novel "masked" iron pro-chelators that have little or no affinity for iron until the mask is selectively removed under the conditions of oxidative stress. Aromatic hydroxyl group, which is essential for chelation of iron, is protected by boronic ester. This ester is converted to chelating active substances by the action of H_2O_2 (Charkoudian et al. J Inorg Biochem. 2008; 102: 2130–5).

Rat embryonal cardiomyoblast-derived cell line (H9c2) was used in our cardioprotection experiments. Neutral Red Uptake Assay and xCELLigence System were used for assessments of cellular viability. Calcein Assay was used to determine efficiency of pro-chelators' activation by H_2O_2 to effective chelators in buffered solution. Calcein-AM Assay was used for determination of chelation efficiency inside the cells. Epifluorescence microscopy was used for photodocumentation of cellular morphology and damage, together with fluorescent staining for mitochondrial inner membrane potential by JC-1 probe and lysosomal integrity assessment with Lysotracker Blue.

All the tested pro-chelators and reference parent chelators showed some dose-dependent decrease of cellular viability, with exception of BSIH. Fe chelators were able to significantly protect cells against injury caused by H_2O_2. Pro-chelators derived from ICL670A did not show significant protective effect. BHAPI, which is converted to HAPI protected nearly 50% of cells compared to control group at the concentrations 60 μM and 100 μM. Pro-chelators derived from SIH displayed the best protective properties. BSIH-PD and particularly the BSIH significantly protected H9c2 cells at a concentrations $\geq$ 60 μM their protective effects were comparable to parent chelators' efficiency. Our *in vitro* experiments demonstrated conversion of pro-chelators in the presence of H_2O_2 to effective Fe chelators in solution as well as in cells, except for the pro-chelators derived from ICL670A, which did not show good chelating properties.

Our results demonstrate ability of selected pro-chelators derived from well-known aroylhydrazone SIH and its novel derivative HAPI to protect cardiac cells against oxidative injury caused by H_2O_2. While retaining the useful antioxidant properties of parent chelators, they display very low potential to induce own toxicity. Therefore pro-chelators could offer solution of typical adverse effect of Fe chelators related to Fe deficiency.

The study was supported by Grant Agency of Charles University (grant No. 367911).

DEVELOPMENT OF DRUG RESISTANCE IN HELMINTHS AND ITS MECHANISM

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The resistance of helminths to anthelmintic drugs becomes a serious problem and there are many reports of resistance from several parts of the world for every year. The resistance relates almost all classes of anthelmintics and almost all families of helminths. In spite of its increasing importance, there is still a poor understanding of the molecular and genetic basis of resistance. Generally, changes in genes or in gene expression in response to drugs enable the organism of parasite to survive treatment. In small ruminants breeding, the serious problems occur with anthelmintic resistant strains of nematode *Haemonchus contortus* and trematode *Fasciola hepatica*. The aim of our project is to study resistance mechanisms, focusing on changes in drug metabolizing enzymes as increased drug metabolism i.e. drug efflux or drug detoxification may prevent the drug from reaching the target molecules. For this purpose, several strains of *H. contortus* with different sensitivity to anthelmintics are used. The relationship between drug efficacy and drug metabolism is studied. The efficacy of anthelmintics against nematodes is assayed by the Faecal Egg Count Reduction Test (FECRT) and two *in vitro* tests, the Egg Hatch Test (EHT) and the Larval Development Test (LDT). The sensitivity of these two *in vitro* tests is limited but it can be increased by using discriminating doses rather than calculating LD_{50} values. The introduction of LDT in our laboratory was our first task in this project. Consequently, this test was used for comparison of efficacy of new anthelmintic drug monepantel in different *H. contortus* strains. In next months, we would like to clarify differences in monepantel metabolism and expression of selected drug-metabolizing enzymes between sensitive and resistant strains of *H. contortus*. For enlarging of available helminths models in our laboratory, breeding of trematode *Fasciola hepatica* has started. In present, the methods for lifecycle stages collection in intermediate host *Pseudosuccinea columella* are optimized. As there are no validated *in vitro* tests of anthelmintics efficacy in *Fascioloides* and no information on the molecular basis of resistance, a large amount of research will be required. Understanding the mechanisms and genetics of anthelmintic resistance is important to efforts to overcome resistance, to slow the spread of resistance parasites, to delay the development of resistance to new anthelmintic drugs, and to better manage parasite control, including using anthelmintic combinations, with existing anthelmintics.

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STUDY OF HUMAN MEMBRANE-BOUND ENZYMES FROM SDR SUPERFAMILY, THEIR MEMBRANE TOPOLOGY AND BIOLOGICAL ACTIVITY

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It is predicted that roughly 30% of human proteins are bound to membranes, but generally, despite their important function in the body, most of them have not been sufficiently characterized yet. Short-chain dehydrogenase/reductase (SDR) superfamily comprises many of these membrane-bound enzymes. Several enzymes of the SDR superfamily have been shown to reduce endogenous and xenobiotic carbonyl compounds, others are known as oxidases or epimerases etc. Some carbonyl reducing enzymes from SDR superfamily have been attributed to the role in some serious diseases such as some types of cancer, diabetes mellitus or metabolic syndrome. But only one membrane bound carbonyl reducing enzyme has been described in biotransformation of xenobiotics so far – 11β-hydroxysteroid dehydrogenase 1.

The aim of this study is to determine membrane topology, selected posttranslational modifications, preferred cofactor and substrate specificity of four selected human microsomal enzymes of the SDR superfamily, namely dehydrogenase/reductase member 3 (DHRS3, SDR16C1), also known as retinal short-chain dehydrogenase/reductase retSDR1, dehydrogenase/reductase member 8 (DHRS8, SDR16C2), which is known under next three different names (17β-HSD11, Pan1b, retSDR2). Knowledge about these two enzymes is quite poor, it has been described just subcellular localization in endoplasmic reticulum and tissue localization based on mRNA.[1,2] Two other studied SDR enzymes are orphan, dehydrogenase/reductase member 12 (DRS12, SDR40C1) and epidermal retinol dehydrogenase 2 (RDHE2, SDR16C5). No information apart from their prediction is known.

All selected enzymes were prepared in recombinant form using Bac-to-Bac Baculovirus expression system and Sf9 insect cells. Sf9 microsomal fractions with overexpressed particular target enzymes were used for determination of their membrane topology – protease protection assay, endoglycosidase H treatment and alkaline respective detergent extraction. All results were analyzed by SDS-PAGE and western blotting. Particular microsomal fractions will be used for determination of catalytic activity toward several xenobiotics and eobiotics and also cofactor preference. Obtained results will be evaluated together with membrane topology for estimation of possible catalytic role of the above mentioned enzymes in metabolism.

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STUDY OF THE EFFECT OF *S*-NITROSOGLUTATHION (GSNO) ON CARBONYL REDUCTASE 1 (CBR1) IN CELL LINES

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Human CBR1 (carbonyl reductase 1, EC 1.1.1.184, SDR21C1) is a low molecular weight, monomeric, cytosolic, NADPH-dependent enzyme of the SDR superfamily (short-chain dehydrogenase/reductase) with broad substrate specificity for many endogenous and xenobiotic carbonyl compounds. CBR1 is abundantly expressed in almost all tissues and plays a protective role in oxidative stress, tumour metastasis, neurodegeneration and apoptosis. S-nitrosoglutathion (GSNO) represents a key nitrosothiol in biological systems and serves as a reservoir and carrier for nitric oxide. Together with reduction of GSNO by CBR1, which was described recently, the inhibition and modification of CBR1 caused by GSNO *in vitro* was proven. CBR1 contains 5 cysteine residues in its sequence (positions 26, 122, 150, 226, 227) and these cysteine residues could play an important role in this modification (S-glutathionylation, disulfide bonds between cysteine 226 and 227) and influent enzyme activity.

The goal of the proposed project is to focus on these modifications of CBR1 in selected cell cultures during physiological conditions as well as during experimentally induced conditions of oxidative stress. The results obtained will significantly help to clarify the role of CBR1 in the metabolism of GSNO at the cell culture level and will offer new point of view at the *in vivo* situation.

The experiment involves the incubation of a cell line A549 (Human lung adenocarcinoma epithelial cell line) with different concentrations of GSNO, glutathione (GSH) and oxidized glutathione (GSSG). As a model of artificially induced oxidative stress cell lines will be incubated with H_2O_2. In the next step the differential detergent fractionation of eukaryotic cells and redox two-dimensional electrophoresis will be used. By this experiment we will confirm or refute the presence of disulfide bonds between cysteines 226 and 227 and determine the conditions under which this modification occurs. To confirm or refute the modification in the form of S-glutathionylation we will use western blotting. Final analysis of the impact of the above mentioned substances will be tested using mass spectrometry. In case of positive results on the A549 cell line, the project will add an extra one or two cell line.

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THE STRUCTURAL ANALYSES OF DISULFIDE OXIDOREDUCTASE DSBA IN BACTERIUM *FRANCISELLA TULARENSIS*

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The disulfide oxidoreductase DsbA plays a key role in *Francisella tularensis* (*F. tularensis*) virulence. This enzyme introduces the disulfide bonds to newly synthetized proteins and some of the substrates are the virulence factors of *F. tularensis*. Deletion of *dsbA* gene from the bacterium leads to high attenuation of *F. tularensis*. DsbA protein consists of two functional domains: DsbA-like thioredoxin domain (DSBA) and N-terminal FKBP – type peptidyl-prolyl isomerases domain (N_FKBP). Bioinformatic studies showed that *F. tularensis* virulence may be affected by either DSBA or N_FKBP domain.

The aim of this work was to investigate the role of both domains, the amino-terminal FKBP domain and the DSBA-like thioredoxin domain in *F. tularensis* virulence. For this purpose the variant *dsbA* genes by the complementation in *trans* were created: a deletion of the N-terminal FKBP domain, and alteration of C-X-X-C active site and highly conserved *cis*-Pro residue in the DSBA domain. To see the effect of *dsbA* variants on virulence, the *in vivo* infection of Balb/c mice and *in vitro* infection of mouse monocyte-macrophage cell line J774 was performed. The data shows both *in vivo* and *in vitro* attenuation of these mutants. The analysed disulfide oxidoreductase activity of recombinant proteins showed high impact of all mutations on DsbA protein activity. Moreover, the chaperone-like activity of *F. tularensis* DsbA was proved. Taking all together, both domains show high importance in DsbA protein and thus in *Francisella tularensis* virulence.

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BIOCHEMICAL PROPERTIES OF HUMAN DEHYDROGENASE/REDUCTASE (SDR FAMILY) MEMBER 7

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Human genome project has provided enormous volume of data about existence of proteins that have not been known heretofore. Despite of a great effort of scientists throughout the world, there still remains a significant fraction of genes with known

nucleotide sequences but uncharacterized products of their translation. Members of short-chain dehydrogenase/reductase superfamily (SDR) belong to such orphan proteins as well.

The SDR is a large group of enzymes with more than 160,000 members in all forms of life. So far, 77 SDR proteins in 47 families have been identified in humans (according Swiss-Prot database). These proteins (with different subcellular localization) are involved in the metabolism of diverse range of compounds, including steroid hormones, retinoids, prostaglandines, lipids and xenobiotics. One of the catalytic activities of SDR members is carbonyl reduction. This reaction takes an important part in the Phase I of the biotransformation. There are several SDR enzymes exhibiting carbonyl reducing activity towards different xenobiotics. These ones are mainly cytosolic. On the other hand there is not too much known about microsomal enzymes. It is assumed that they create more than 1/3 of SDR according data from human genome. Presently, only one microsomal carbonyl reducing enzyme, 11β-hydroxysteroid dehydrogenase 1, has been described to be involved in the biotransformation of xenobiotics with carbonyl group.

Human dehydrogenase/reductase (SDR family) member 7 (DHRS7) belongs to poorly characterised members of SDR superfamily. It was predicted to be a membrane-bound enzyme but any other information about its properties or its function in human body has been missing. Our research group prepared recombinant form of human DHRS7 with taking advantage of baculovirus expression system followed by expression of target protein in insect cells Sf9. We confirmed its microsomal localization and specified its transmembrane orientation in endoplasmic reticulum and some of its posttranslational modifications maintained in insect cells. In addition we established its basic biochemical characteristics such as preference for cofactors and catalytic efficiency towards selected eobiotic and xenobiotic substrates.

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UHPLC/MS/MS IDENTIFICATION OF MONEPANTEL METABOLITES FORMED *IN VITRO* IN OVINE HEPATOCYTES

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Monepantel (MOP), belonging to a new class of anthelmintics drugs known as amino-acetonitrile derivatives. It was approved for use in veterinary practice in Czech Republic

in year 2011. So far, biotransformation and transport of MOP in target animals have been studied insufficiently, although the study of metabolic pathways of anthelmintics is very important for efficacy and safety of therapy and evaluation of risk of drug–drug interactions. The aim of this study was to identify the MOP metabolites and to suggest the metabolic pathways of MOP in sheep. For this purpose, primary culture of ovine hepatocytes was used as a model *in vitro* system. After incubation, medium samples and homogenates of hepatocytes were extracted separately using solid-phase extraction (SPE). Analysis was performed using hybrid quadrupole-time-of-flight (QqTOF) analyzer with respect to high mass accuracy measurements in full scan and tandem mass spectra for the confirmation of an elemental composition. The obtained results revealed S-oxidation to sulfoxide and sulfone and arene hydroxylation as MOP phase I biotransformations. From phase II metabolites, MOP glucuronides, sulfates and acetylcysteine conjugates were found. Based on the obtained results, scheme of metabolic pathway of MOP in sheep has been proposed.

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INITIAL ASSESSMENT OF NITRITES AND NITRATES AS POTENTIAL PROTECTIVE AGENTS AGAINST ANTHRACYCLINE CARDIOTOXICITY

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Even though half a century old, anthracyclines (doxorubicin, daunorubicin or epirubicin) remain among the backbone of modern combination cancer therapy. Unfortunately, the clinical usefulness of anthracycline antineoplastic drugs is limited by their cardiotoxicity. Its pathogenesis is still poorly understood, although the induction of myocardial oxidative stress has been among the most popular theories. The treatment of this reactive oxygen species-induced damage is very difficult, thus preventive cardioprotective approaches are preferable. Dexrazoxane is the only cardioprotective agent approved for clinical use. The use of inorganic nitrites has also been shown as cardioprotective in the myocardial ischemia-reperfusion injury probably due to NO release and/or S-nitrosylation of key myocardial proteins. Similarly the oral administration of inorganic nitrate, which is converted to nitrite in GIT, can protect the heart from acute anthracycline cardiotoxicity. Since there is a lack of data on efficacy of these nitrites/nitrates, as well as their impact on athracycline anticancer activity remains unknown, we decided to evaluate it more.

In vitro cardiotoxity of studied compounds (sodium nitrite and organic nitrate 3-morpholinosydnonimine), was examined on isolated rat ventricular neonatal cardiomyocytes using 48-hour after exposure. Sodium nitrite showed no significant own toxicity up to the 30 mM concentration. 3-morpholinosydnonimine had no cardiotoxic effect in a concentration range of 0.05–2 mM.

Potential cardioprotective effect of these compounds was studied on developed model of anthracycline toxicity (induced through incubation with 1.2 μM daunorubicin or doxorubicin). Two modes of protective experiments were executed: cardiomyocytes were each time preincubated for 3 hours with potential protective agent and then co-incubated with anthracycline for 48 hours or only for 3 hours with 48-hour wash-out period. Preliminary results prompted no cardioprotective efficacy of sodium nitrite and of 3-morpholinosydnonimine against anthracycline cardiotoxicity however further investigation is inevitable. Another cardioprotective experiments, at present performed on isolated cardiomyocytes, concern with the model oxidative stress induced by incubation of cells with H_2O_2. Analogical study of potential cardioprotective efficiency of tested compounds will be performed on H9c2 cell line (derived from rat cardiac myoblasts) with both antracycline and hydrogen peroxide insult as well as evaluation of sodium nitrite and 3-morpholinosydnonimine impact on the anticancer activity of anthracyclines (combination index determined by the Chou and Talalay method performed on human promyelocytic leukemia cells HL-60).

This project is done in cooperation with Faculty of Medicine of the Charles University. Potential cardioprotective effects are also studied *in vivo* on well established model of chronic anthracycline cardiotoxicity in rabbits.

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ASSESSMENT OF NOVEL DEXRAZOXANE AND ADR-925 ANALOGS FOR PROTECTION OF CARDIOMYOCYTES AGAINST ANTHRACYCLINE AND HYDROGEN PEROXIDE TOXICITY

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Anthracycline (ANT) antibiotics belong to the most frequently used antineoplastic agents. However, their cardiotoxic effect is still within the dose-limiting factors in clinical practice. Although this adverse effect is known for decades, its mechanism has not been fully elucidated. Traditionally, reactive oxygen species (ROS) formation is believed to be the main cause of this adverse effect. However, the role of ROS has been recently critically reviewed and is widely discussed. Anthracyclines possess topoisomerase II poisoning activity, which leads to DNA damage, which is now the main hypothesis of the outstanding ANTs antineoplastic activity. To date, dexrazoxane (DEX) is the only clinically approved cardioprotectant against ANTs. Its metal chelating metabolite ADR-925 may inhibit the

ROS formation. Nevertheless DEX is also known to be catalytic topoisomerase II inhibitor, which could be able to protect from the DNA damage.

The aim of this study was to investigate the structure-activity relationships of the DEX cardioprotection in the model of isolated neonatal rat cardiomyocytes (NVCMs). Cells were isolated from the 3-day old Wistar rats and incubated with drugs in different time schedules using various concentration combinations in clinically relevant range. Significant protective action against the DAU cardiotoxicity was observed with DEX and two other topoisomerase II catalytic inhibitors sobuzoxane (SOB) and merbarone (MER). This protection was time-dependent, increasing with the time of preincubation. On the other hand they were unable to provide protection against hydrogen peroxide. The novel synthetized analogs of ADR-925, KH-TA4 and JR-159, showed no protection. Moreover, KH-TA4 showed its own toxicity towards cardiac cells and when preincubated for 3 and 6 hours it significantly aggravated the toxicity of DAU. Conversely, KH-TA4 was protective against this oxidative insult after 3 to 6 hours of preincubation. The novel structures MK-15 and ES-5 developed as the analogues of DEX parent structure were also unable to provide protection both against DAU and hydrogen peroxide toxicity. The reason for this finding is yet unknown and experiments on the influence of these substances on the topoisomerase activity will be undertaken.

In conclusion, our data indicate that, rather than by metabolism to the chelating ADR-925, DEX may protect the heart against ANT cardiotoxicity as the parent compound. Further studies are nevertheless necessary to further elucidate these findings.

MONITORING OF 25-HYDROXY VITAMIN D BY UHPLC-ESI-MS/MS METHOD IN HUMAN SERUM

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Vitamin D is a hormone produced from sterols in the body by photolytic reaction of ultraviolet light on the skin. However, there are some people who obtain this vitamin in the nutrient from their diets, such those live in the northern latitudes, or spend most of their days indoors[1]. Vitamin D deficiency has manifested in the general population and can lead to increased risk of cancer, diabetes mellitus, chronic pain, osteoporosis and hypertension[2].

Importance of laboratory monitoring of vitamin D metabolites levels in serum has increased and its measurement can be utilized diagnostically. Mainly 25-hydroxy vitamin D (25-OH-D) is the primary circulating form and is considered as a clinical marker of general status of vitamin D in human serum[3]. The endogenously derived 25-OH vitamin D_3 (cholecalciferol) accounts for approximately 95% of the total circulating 25-OH vitamin D pool. In contrast, 25-OH vitamin D_2 (ergocalciferol) is derived from plant sources and normally represents a minor component unless supplements containing significant amounts of ergocalciferol are used by the patient[4].

In the presented study metabolites 25-OH D_3 and 25-OH D_2 were monitored in human serum. Biological samples were collected from three groups of patients from Faculty Hospital Hradec Králové. In the first group were elderly patients (n = 22), in the second group patients with familiar hypercholesterolemia treated with LDL apheresis (n = 26) and in the third group patients with age-related macular degeneration treated with hemorheopheresis (n = 18).

All the measurements were carried out on the chromatographic system UHPLC Nexera, coupled with mass spectrometry detector LC-MS 8030 (Shimadzu, Kyoto, Japan). Core shell analytical column Kinetex (1.7 µm, 3 mm × 100 mm, Phenomenex, Torrance, USA) was used for separation.

Monitoring of 25-OH D_3 and 25-OH D_2 in human serum of elderly patients and also patients with familiar hypercholesterolemia and age-related macular degeneration provided new pathophysiologic knowledge.

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AUTOMATION OF SAMPLE PRETREATMENT AND SEPARATION PROCEDURE FOR DETERMINATION OF BIOACTIVE COMPOUNDS IN BIOLOGICAL SAMPLES

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Automated sample preparation methods are nowadays imperative to meet compressed analytical timeline and to reach low detection and quantitation limits using quick and effective methodologies. The presentation will be devoted to the development of automated methods for dynamic chemical fractionation and monitoring of bioaccessibility of bioactive compounds in biological fluids and dietary supplement products.

An automated method for the simultaneous determination of the beta-blockers, namely, atetonol, pindolol, acebutolol, metroprolol, labelol and propanolol in biological samples will be discussed. Automated sample pre-treatment including sample clean-up, preconcentration using solid phase extraction (SPE) with micro bead injection Lab-On-Valve (BI-LOV) technique will be explained. Determination was optimised using high performance liquid chromatography that is planned to be coupled on-line to sample pre-treatment step. Separation conditions are following: gradient elution from 15% A: 85% B to 35% A: 65% B (A, acetronitrile: B, phosphate buffer with pH 3.8) in 10 min using high resolution monolithic column C-18. Signal for detection was scanned at 220 nm.

The future work focused on bioaccessibility of vitamin-B-complex in dietary supplements will be mentioned in this presentation as well.

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OPTIMIZATION OF A CHROMATOGRAPHIC METHOD FOR DETERMINATION OF PESTICIDES AND INSECTICIDES IN AQUEOUS SAMPLES

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Nowadays there is a growing concern over the contamination of different environmental compartments by toxic organic compounds. For this reason it is necessary to develop sensitive and selective analytical methods for the determination of these compounds at trace levels. Coupled column chromatography is a powerful technique because it increases the peak capacity and improves the resolution. Moreover the possibility to inject a large volume of sample can lead to increase the sensitivity in trace analysis. The development of the new method using large volume injection into coupled column reversed phase liquid chromatography with ultraviolet detection for the simultaneous determination of two different groups of organic pollutants in aqueous samples – pesticides and insecticides – is presented. The pesticides analyzed are carbamate types: aldicarb, carbaryl, carbofuran, oxamyl, fenoxycarb, pirimicarb. These organic compounds are derived from carbamic acid. The insecticides analyzed are pyrethroid type: baythroid, kadethrin, flumethrin, cypermethrin, deltamethrin, fenpropathrin, permethrin, these compounds are a large group of synthetic insecticides based on the structure and properties of the pyrethrins naturally occurred in *Chrysanthemum cinerariaefolium*.

In this work we discuss the importance of select the adequate analytical column considering the nature of the phase stationary, the length and the diameter of the column and how affect other chromatographic parameters in the separation of these compounds.

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TRENDS IN THE SAMPLE PRE-TREATMENT USING THE SEQUENTIAL INJECTION ANALYSIS SYSTEM

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Obtaining the sample free of interfering compounds and compatible with subsequent analysis is crucial for the success of whole analysis. Therefore the sample pre-treatment operation followed by a separation, derivatization, or selective detection became one of the key steps for analysis of a complex sample with a several determined substances[1]. The sequential injection analysis (SIA) method widely explored to perform all of the necessary steps in one system.

The SIA is a not-segmented flow method for analysis of solution, sample pre-treatment and analyte separation with subsequent detection. Method is based on stream propelled by a piston pump connected by tubing to central port of selection valve. To the side ports of selection valve, sample and reagent containers, the detector and possibly other necessary devices are connected and the system is highly modifiable[2]. Reproducible sample injection, controlled dispersion and precise timing of single steps enable automation of sample analysis[3].

The trends in sample pre-treatment are now focused on the solid phase extraction (SPE) and its miniaturization and automation. To the SIA system a SPE is applied usually in form of sorbent filled columns or is performed in the format of Bead injection on Lab On Valve. The spectrum of sorbents used for SPE is extended of many new kinds of sorbents such as restricted access materials, molecularly imprinted polymers and immunosorbents which are topic of this work. Micro-extraction by packed sorbent (MEPS) is discussed as the newest trend in SPE automated in SIA. The MEPS column contents small amount of sorbent (1–4 mg) which is suitable for manual performance or automated extraction in SIA systems1[4].

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METHOD FOR ANALYSIS OF SEVEN BENZOIC AND CINNAMIC ACID DERIVATIVES IN DIETARY SUPPLEMENTS BY TRANSIENT ISOTACHOPHORESIS – CAPILLARY ZONE ELECTROPHORESIS.

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A novel transient isotachophoresis-capillary zone electrophoresis for preconcentration and determination of seven phenolic acids (caffeic, cinnamic, p-coumaric, ferulic, protocatechuic, syringic and vanilic acid) in the ethanolic extract of medicinal herbs was developed and validated. Effects of several factors such as control of EOF, pH, buffer composition and conditions for sample pre-cleaning and pre-concentration were investigated. Presented method shows excellent detection limits in range from 11 to 31 μg/L[1]. Validated method has been utilized for analysis of four commercially available nutraceuticals containing *Epolobium parviflorum*. *Epilobii herba* was chosen as a model sample because of its popularity in adjuvant therapy of Benignous prostatic hyperplasia, which is one of the most common diseases traditionally cured with nutraceuticals containing polyphenols of natural origin.

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DETERMINATION OF VITAMIN D IN HUMAN BREAST MILK

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Vitamin D is a fat-soluble vitamin that is essential for the healthy development of whole human body. In the last decade it can be observed a growing interest of scientists about vitamin D which plays an important role not only in calcium and bone metabolism but in regulating and influencing a whole organism.

Vitamin D and its metabolites are found in human breast milk in very low concentrations and therefore are difficult to determine. Vitamin D passes easy into breast milk, contrary, 25-OH-D passes very badly, and 1,25-$(OH)_2$-D does not appear to go through at all[1]. Mother's milk contained vitamin D at 30% of woman's circulating concentration of

vitamin D and 25-OH-D at 1% of circulating concentration of 25-OH-D[2]. Low levels of vitamin D in children can lead to soft, thin, and brittle bones; this disease is known as rickets. However, concentration of liposoluble vitamins in breast milk are very low and depends on many factors involving woman such as diet, age, different stages of lactation and others[3].

Due to the fact, that human breast milk is complex biological matrix, containing many interfering substances, there is a very important sample pre-treatment. The extraction process of milk samples is commonly based on liquid-liquid extraction with hot saponification and extraction with hexane, followed evaporation at reduced pressure in a vacuum rotatory evaporator and with subsequent dissolution of the residue in methanol or acetonitrile. For increasing sensitivity derivatization of target compounds was employed in several studies. For the analytical part of determination of vitamin D and its metabolites in milk are widely used HPLC with UV detection and competitive protein binding assay (CPBA). However, as commonly in analytical chemistry, LC-MS become most used.

The goal of this study was to develop simple and fast sample preparation method for analyses of two derivatives of vitamin D, 25-OH-D_2 and 25-OH-D_3 in human breast milk suitable for routine clinical laboratory and optimized it. The sample preparation method was based on precipitation, centrifugation and filtration steps. Determination of target analytes was carry out using the UHPLC set coupled with core shell analytical column Kinetex (Phenomenex, Torrance, USA) and tandem mass spectrometry.

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HPLC METHOD FOR DETERMINATION OF REDUCED GLUTATHIONE, CARNOSINE AND TAURINE WITH PRE-COLUMN DERIVATIZATION

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A new gradient reversed-phase HPLC method with precolumn derivatization was developed and validated for simultaneous determination of biological active compounds – reduced glutathione, carnosine and taurine. As the separation and detection of these components

is complicated either by the large differences in compound's polarity and by low absorption in UV region, therefore pre-column derivatization with 1-fluoro-2,4-dinitrobenzene (DNFB) was used. Chromatographic separation was achieved with a fused core analytical column Supelco Ascentis Express C_{18} (100 mm × 4.6 mm, 2.7 μm). A 30 mM triethylamine in acetonitrile with 30 mM triethylamine aqueous solution (pH 2.5, adjusted by glacial acetic acid) was used in a gradient elution mode at a flow rate of 0.8 mL min^{-1}. The injection volume of the sample after derivatization was 5 μL and the column temperature was maintained at 30 °C using detection wavelength at 375 nm. The validation characteristics included system suitability, accuracy/recovery, precision, linearity, range and solution stability. The standard calibration curves were found to have a linear relationship ($r^2 > 0.99$) for all compounds. The mean percentage recoveries obtained for reduced glutathione, carnosine and taurine were 99.61, 99.38 and 99.43%, respectively. Precision was < 2% for retention times and peak areas. The usefulness of this method was demonstrated by successful application for the analysis of combined eye drop formulation containing reduced glutathione, carnosine and taurine (Fig. 1).

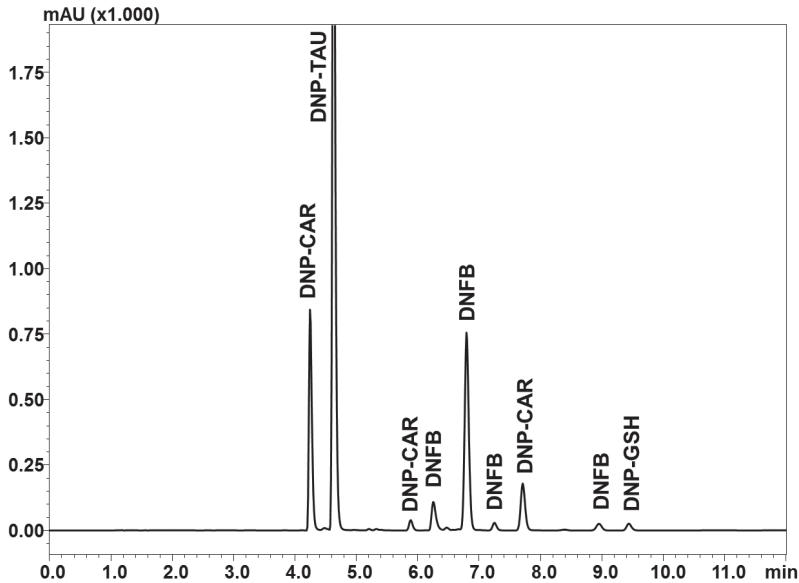

Fig. 1. Chromatogram of derivatives of GSH (DNP-GSH), CAR (DNP-CAR) and TAU (DNP-TAU) in eye drop formulation. DNFB peaks represent unreacted derivatization reagent.

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MONITORING OF ALPHA-TOCOPHEROL AND RETINOL IN PATIENTS TREATED BY EXTRACORPOREAL ELIMINATION OF LIPOPROTEINS

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The aim of this study was to observe the markers of lipoperoxidation and serum antioxidative capacity by monitoring of retinol and alpha-tocopherol in serum, lipoprotein fractions and erythrocyte membrane. A group of patients with familial hypercholesterolemia and patients with age-related macular degeneration treated by extracorporeal elimination of lipoproteins were included in this study.

Familial hypercholesterolemia is a serious genetic disorder, characterized by very high plasma concentrations of low density lipoprotein (LDL) cholesterol, tendon xanthomas and increased risk of premature coronary heart disease. For patients who do not respond to pharmacologic hypolipidemic treatment extracorporeal elimination of lipoproteins is needed. LDL apheresis is a procedure for selective removal of LDL cholesterol. Age-related macular degeneration affects older population and results in a loss of vision. Hemorheopheresis is method based on influencing of microcirculation by improving of rheological conditions. The treatment principle is to remove a defined spectrum of molecules (LDL-cholesterol, fibrinogen, IgM and others), resulting in decreased blood viscosity.

Retinol and alpha-tocopherol are important antioxidants that help to remove free radicals away from the human body. Retinol participates in many functions through the body, such as gene expression, vision, embryonic development and reproduction, bone metabolism, haematopoiesis or immune functions. Alpha-tocopherol is the major liposoluble antioxidant in human body. It protects lipid parts of cell membrane, blood fats, cholesterol and other fatty substances against oxidation. It is considered to be protective factor against cardiovascular diseases, cancer, age-related macular degeneration, central neurodegenerative diseases and diabetes mellitus.

Retinol and alpha-tocopherol levels were monitored before and after treatment of LDL apheresis and rheopheresis, respectively in serum, lipoprotein fractions and erythrocyte membrane. Lipoprotein fractions were obtained by gradient ultracentrifugation. Vitamins were extracted by liquid-liquid extraction procedure by n-hexane and then analyzed by High Performance Liquid Chromatography.

Our results showed that the levels of important antioxidant agent alpha-tocopherol decreased after the LDL apheresis and rheopheresis procedures, but the antioxidant capacity of serum, lipoprotein fractions and erythrocyte membrane were not changed.

The study was supported by the European Social Fund and the state budget of the Czech Republic, project TEAB no. CZ.1.07/2.3.00/20.0235, by the Charles University in Prague – Project SVV 2012-265-002 and by the Project of Ministry of Health, Czech Republic for conceptual development of research organization 00179906.

ENHANCEMENT OF SENSITIVITY AND SEPARATION EFFICIENCY OF CAPILLARY ELECTROPHORETIC TECHNIQUE BY USING COMBINATION OF COMPLEX FORMATION AND LARGE VOLUME SAMPLE STACKING WITH POLARITY SWITCHING

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Method of capillary electrophoresis with UV detection for the separation of phenolic compounds and aromatic carboxylic acids was developed. Addition of complex-forming reagent (tungstate playing the role of central ion) into the background electrolyte (BGE) was used to improve the separation of such mixtures. Twelve analytes (apigenin-7-glucoside, umbelliferone, apigenin, naringenin, rutin, hyperoside, quercetin, luteolin, p-coumaric acid, chlorogenic acid, cinnamic acid and gallic acid) were separated in the BGE of pH* 7.7 (adjusted by TRIS) containing 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 2.4 mM sodium tungstate and 25% of methanol (*V/V*). The separation was conducted in a fused silica capillary (inner diameter 75 μm, total length 60.2 cm, effective length 50 cm). Operational temperature was 25 °C, voltage applied for separation was +25 kV, and the detection wavelength was 275 nm. The sample injection time for conventional CE method was 6 s at the pressure of 48.3 mbar. Additionally the online pre-concentration technique of large volume sample stacking (LVSS) with polarity switching was employed to compensate for relatively low sensitivity of UV detection in CE. The LVSS step involved long sample zone injection (99 s at 151.7 mbar) and application of reversed voltage of −25 kV for approximately 2.5 min for excessive sample matrix zone removal and pre-concentration of the analytes. Thereafter the polarity switching (to +25 kV) was applied to realize conventional CE separation of the pre-concentrated analytes. The method seems to be suitable for the determination of low amounts of phenolic analytes in complex natural product samples.

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ENHANCING THE SENSITIVITY OF THE DETERMINATION OF 8-HYDROXY-2'-DEOXYGUANOSINE IN URINE BY CAPILLARY ELECTROPHORESIS WITH UV DETECTION

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8-Hydroxy-2′-deoxyguanosine (8OHdG) is a product of oxidative DNA damage. Elevated levels of urinary 8OHdG correlate with oxidative stress and cancerogenesis[1]. A low limit of detection of the analyte (down to tens of nmol/L) is required when assessing 8OHdG within the healthy population. Development of a sensitive capillary electrophoretic (CE) method with UV detection suitable for determination of such concentrations of 8OHdG in urine is the objective of this study.

High sensitivity detection cells were tested to extend the optical path length. At the same time a large volume sample stacking technique (LVSS) was applied to achieve on-line pre-concentration of the sample. A separation method using either bubble cell or Z-cell in the combination with LVSS was developed, validated and compared.

All separations were carried out in conventional fused-silica capillaries. Background electrolyte of pH* 9.2 (adjusted with 1 M NaOH) contained 0.15 M boric acid and 10% (v/v) of acetonitrile. 2′-Deoxyguanosine was used as internal standard (IS) and the detection wavelength was 282 nm. Optimization of the CE method was pursued with model aqueous solutions containing 8OHdG and the IS. The limit of detection (LOD) for bubble cell + LVSS was 83.3 nM and the LOD for Z-cell + LVSS was 35 nM.

The preparation of urine samples before the CE separation combined with LVSS such as extractions involving ion-exchange principals will be discussed.

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AUTOMATION OF MICROEXTRACTION PROCEDURES USING FLOW SYSTEM; APPLICATION IN DETERMINATION OF METSULFURON METHYL

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Recently, miniaturization and automation of extraction techniques (solid phase extraction as well as liquid-liquid extraction) gain a lot of interest.

Performing liquid-liquid extraction in flow system brings many advantages. First of all, the solvent consumption is radically reduced. Decreased amount of organic solvent (in order 10–100 microliters) increases the pre-concentration factor and enables for determination of the analyte at very small concentration level (at optimized conditions).

Dispersive liquid – liquid microextraction is a technique which, apart from extraction solvent, employs a dispersive solvent soluble both in organic and aqueous phase to create dispersion and thus increase the surface area between extraction solvent and aqueous phase. In this way, the extraction becomes more efficient and the determination more sensitive as well.

An automated method for determination of metsulfuron methyl (pesticide) in water with UV detection is under study. In this method, extraction of the analyte in micro-format is performed in flow system. The studies involve testing of different extraction solvents (with density lower than that of water, such as alcohols) and dispersive solvents. The optimal geometry of the extraction cell and a mixing device are also investigated and will be discussed.

The study was supported by The Ministry of Education of the Czech Republic (project Mobility 7AMB12AR008) and Argentine Ministry of Science, Technology and Innovative Production and the European Social Fund and the state budget of the Czech Republic, project no. CZ.1.07/2.3.00/20.0235, the title of the project: TEAB.

SAMPLE PREPARATION AND LC-FD ANALYSIS OF PTERIDINES WITH EMPHASIS ON THEIR STABILITY

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Pteridines are a group of heterocyclic compounds composed of fused pyrimidine and pyrazine rings containing a wide variety of side chain substituents which greatly influence their biological activity and solubility. Pteridines are excreted by humans in urine[1] and their levels have been shown to elevate significantly when the cellular immune system is activated by certain diseases. For instance, neopterin (NEO) and its reduced form, 7,8-Dihydroneopterin (NH2), are produced in large amounts by activated monocytes/macrophages after stimulation with interferon-γ secreted by activated T-lymphocytes[2]. Concentrations of NEO in human fluids can therefore be used as a marker of cell-mediated immune response[3]. The determination in urine becomes increasingly popular because of its non-invasiveness.

The aim of our study was to develop sample preparation method (Solid Phase Extraction – SPE), for the analysis of NEO, biopterin and their derivatives by means of Hydrophilic Liquid Chromatography with Fluorescence Detection (HILIC-FD). The main emphasis was put on the stability of dihydroforms during the sample processing and storage, because of three oxidation states of pteridines with variable stability. Therefore, it was necessary to preserve the original oxidation states of the individual pterins during the sample preparation and analysis. Dithiothreitol (DTT), as a stabilizing agent, its various concentrations, and various pH values (3.8–9.8) of working solutions were tested. Dihydrobiopterin (BH2) and NH2 were considered to be more susceptible to the influence of pH value than biopterin (BIO) and NEO. The best stability during 24 hours was observed at pH 6.8 for both BH2 and NH2 and quite corresponding results were obtained for their oxidized analogues. Also various concentrations of DTT (0.5–8%) were found out to have different effect on the stability of NH2, BH2 and NEO in contrast to BIO. Very similar results were noticed for BIO with different DTT concentrations. The best results for BH2 and NH2 were observed with 4% and 8% DTT. The development of a new sample preparation method was focused on the selection of SPE sorbent and farther optimization of SPE conditions (conditioning, elution, flow rate etc.), and on the stability as well. This is more difficult and important in HILIC where the composition of dilution solvents is significant for reasonable peak shapes.

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UHPLC-MS/MS MULTISTATIN METHOD FOR THE DETERMINATION OF STATINES, THEIR METABOLITES AND INTERCONVERSION PRODUCTS IN SERUM

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Statins are inhibitors of microsomal 3-hydroxy-3methylglutaryl-coenzym A (HMG-CoA) reductase and reduce the levels of total cholesterol, low-density lipoprotein cholesterol and plasma triglycerides. In recent studies anticancer effects of some statins were found. For these reason the development of methods for the determination of more statins was requested for the monitoring of their levels in serum.

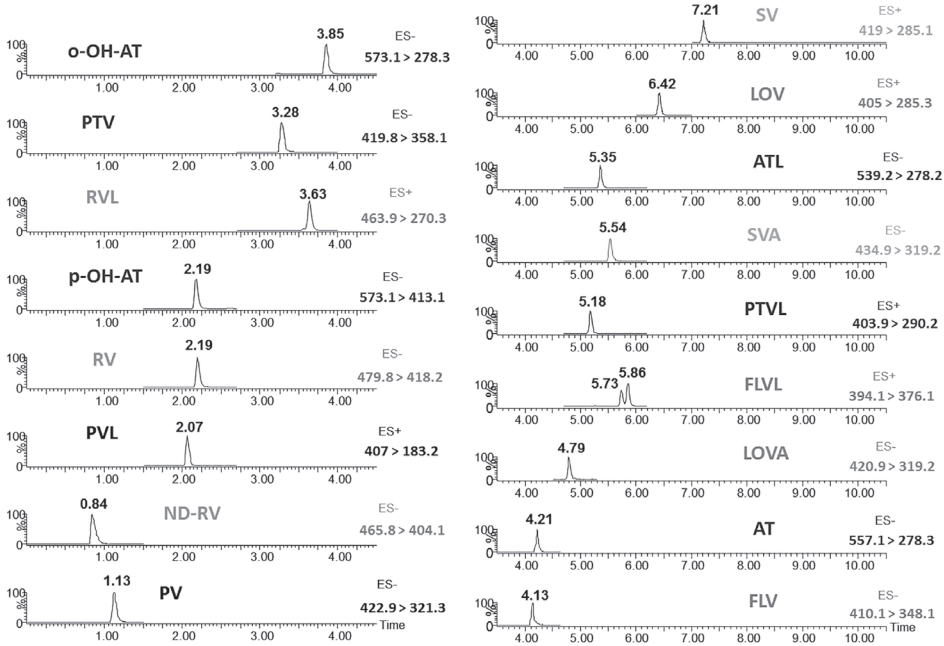
The aim of this work was the development and validation of one method for the determination of 7 statins, their metabolites and interconversion products in human serum (17 analytes in total).

The optimalization of chromatographic separation was initiated by systematic method development using UHPLC with PDA detection. The choice of the optimal conditions of UHPLC method was made with regard to MS detection subsequently used for the determination of statins. Microextraction by packed sorbent (MEPS) with combination of protein precipitation was chosen as the suitable sample preparation technique.

The optimal conditions of MEPS-UHPLC-MS/MS method for the determination of statins, their metabolites and interconversion products were found. The ammonium acetate pH 4.0 at flow rate 0.3 ml/min and gradient elution were used. Electrospray ionisation (ESI) in polarity switching mode was applied. Individual parameters of ESI and triple quadrupole were optimized. Quantification of all analytes was performed using one SRM (selected reaction monitoring) transition and using 13 internal standards. PP was performed using 100 µl of acetonitrile added to 50 µl of sample. The supernatant was removed and diluted by 1900 µl of 0.01 mM ammonium acetate pH 4.5 after the precipitation and centrifugation. The whole volume of this solution was applied into the MEPS C8 sorbent. Analytes were eluted by mixture of acetonitrile: 0.01 mM ammonium acetate pH 4.5 (95: 5, v: v).

Novel analytical method enabled simultaneous determination of 17 analytes within 10.5 minutes. The validation data indicated good linearity ($r > 0.998$ for all analytes), sensitivity (LOQ ≤ 5 ng/ml for most analytes), repeatability of retention time (< 1.0% RSD for all analytes), repeatability of peak area (< 7.0% for all analytes), recovery (100 ± 25% for all analytes except fluvastatin lactone), precision (RSD < 12% for all analytes) and matrix effects (< 25% for all analytes except for lovastatin, pravastatin lactone and rosuvastatin lactone at the LLOQ concentration level).

Fast and simple MEPS-UHPLC-MS/MS method for the determination 7 statins, their metabolites and interconversion products in serum was developed and validated. The method is universal for the analysis of all clinical used statins and will be applied to the real samples (1) of patients treated by statins and (2) real samples for the study of pharmacokinetic profiles.



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DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR DETERMINATION OF PROPRANOLOL HYDROCHLORIDE AND SODIUM BENZOATE IN PAEDIATRIC ORAL LIQUID PREPARATIONS

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Propranolol is a non-selective beta blocker. Its main indication has changed from therapy of cardiovascular diseases (such as hypertension) to therapy of haemangiomas in paediatric patients during last few years. Unfortunately there is no appropriate formulation of propranolol for paediatric patients available in the Czech Republic. The cooperation of the Faculty of Pharmacy in Hradec Králové and Hospital Pharmacy in Prague – Motol

led to development of eleven versions of oral liquid preparations with propranolol as an active substance and sodium benzoate as a preservative. The need of conducting stability studies of these eleven preparations has resulted into the development and validation of HPLC method for the determination of their ingredients. A simple, selective and sensitive HPLC-UV method for quantification of propranolol hydrochloride and sodium benzoate was developed and fully validated. Separation was performed by Supelco Discovery® C18 (25 cm × 4.6 mm, particles 5 μm) column. UV/VIS absorbance detector was set at wavelength 230 nm. Isocratic elution with mobile phase flow rate 1.8 mL min^{-1} was used. Column oven was conditioned to 25 °C. Mobile phase was prepared by dissolving 1.6 g of sodium dodecyl sulphate and 0.31 g of tetrabutylammonium dihydrogen phosphate in 450 mL of ultrapure water; 1 mL of sulphuric acid (95–97%) and 550 mL of acetonitrile were added. Sodium hydroxide solution (2.1 M) was used for adjusting pH to value 3.3 (± 0.05). Retention times of sodium benzoate, propranolol hydrochloride and butylparaben (internal standard) were 2.2, 3.3, and 4.1 min respectively. Newly developed method is suitable for simultaneous determination of propranolol hydrochloride and sodium benzoate and has been successfully applied for stability testing of extemporaneous paediatric oral formulations.

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SOCIAL AND CLINICAL PHARMACY

HISTORY OF JEWISH PHARMACY (JUDENAPOTHEKE) IN PRAGUE

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In this paper I decided to outline the problems of the unique existence of the very specific Jewish pharmacy – so called Judenapotheke in Prague, whose existence lasted in various forms since the Middle Ages to the end of the 19th century. Its exceptionality consisted in the fact, that it – by contrast to other pharmacy owned by the Czechs – was not controlled by the Medical Faculty of Prague University. Its activities were limited, because it could serve just only for Jewish doctors and patients. The last stage (the 18th and 19th centuries) in the existence of this pharmacy was very important. Already in the first half of the 18th century (1725) the distinguished Prague Jewish family Jeitteles became the new owner of pharmacy. They bought it from other Prague Jewish family, family Kisch. In this era, i.e. during the rule of the Emperor Joseph II, the shop received the Emperor's Privilege, according to it could sell pharmaceuticals not only to the Jews. On the other side, since the beginning of the 18th century it fell within the Medical Faculty of Prague University cognizance. Thanks to the privilege mentioned above the pharmacy could exist in the face of the Emperor's Decree, which proscribed to all Jews to operate the pharmacy in the Austrian Empire in 1829. However, for the whole era the shop had to have Christian leader of pharmacy. In this connection it is necessary to say that the owners of the shop had many other activities. They got scientific degrees and positions and were very active in the various clubs, leagues and associations. One of the owners of the shop, Benjamin Jeitteles, became the first tyron of the Jewish origin in the Czech Lands. The history of this pharmacy very well illustrates the emancipation of the Jewish minority in the Czech Lands in the 19th century. In 1873, when the Czech apothecary, PhMr. Alois Pecka bought the shop, the history of its Jewish ownership ended.

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BEGINNINGS OF RATIONALIZATION IN THE CZECHOSLOVAK PHARMACY PRACTICE – COMMISSION FOR RATIONALIZATION AND STANDARDIZATION IN MEDICINE, VETERINARY MEDICINE AND PHARMACY

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In agreement with the development in the U. S. and Western Europe, the growing interest in the issue of work rationalization (scientific management) occured in the early 20th century Czechoslovakia. It also led to the establishment of Masaryk Academy of Work (MAP) in 1920. In 1926, a Commission for Rationalization and Standardization in Medicine, Veterinary Medicine and Pharmacy (RANOK) was established within Science-medical Department of MAP, to deal with issues of rationalization and standardization in health care. The aim of this research is to study the existence and work of the Comission and its role in the pharmacy practice rationalization efforts in Czechoslovakia. The presented work, focused mainly on Section of Pharmacy of RANOK, deals with important dates, staff members, areas of interests and inteded tasks, and attained results of the section's activity. The work is based on the study of archive records (Masarykova akademie práce fonds; Masaryk Institute and Archives of the ASCR), period journals (*Praktický lékař*, *Časopis československého lékárnictva*, *Praktický lékárník* etc.) and other sources. RANOK was established on the initiative of Adolf Měska, MD, in 1926. It started to work only in 1928 when professor Otakar Rybák, MD, was appointed as a chairman. RANOK had two sections. Section of Medicine was the older and more active one, having higher number of coworkers. Section of Pharmacy was constituted at the turn of 1929. Six eminent pharmacists were members of the section, led by the pharmacist Vincenc Bosák. The section should have dealt with standardization of pharmacy devices, equipment and work processes, designs of sample pharmacies and other proposals leading to more effective work at pharmacies. In 1931, two types of file cabinets were developed and described in the pharmaceutical journals. Apparently, this was the only one result of the section's work. After Bosák resigned in 1931, activity of the section ceased. Section of Veterinary Medicine, the third intended part of the Comission, was never established. In 1937, RANOK was dissolved. Although Section of Pharmacy didn't significantly influenced the pharmacy practice in Czechoslovakia, it represents the first institution which formulated and discussed questions of possible standardization and rationalization in this field.

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ANTIBIOTIC USE AND KNOWLEDGE IN THE COMMUNITY OF YEMEN, SAUDI ARABIA AND UZBEKISTAN

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Inappropriate overuse and misuse of antibiotics has resulted in a dramatic increase of antimicrobial resistance worldwide. Available data suggest that resistance has reached unacceptable levels in the pathogens most common in developing countries and that trends show further increases. Antibiotics are frequently purchased without proper indication, in insufficient quantities, or when contraindicated. It is associated with poor access to medical care, insanitary, overcrowded conditions, which promote the development, maintenance and spread of antibiotic resistance.

The objective of this study was to estimate the frequency and reasons for self- medication with antibiotics by a population of Yemen, Saudi Arabia and Uzbekistan.

A pre-tested questionnaire was used to collect data from a sample of 1200 adult persons selected from three cities that is Ibb (Yemen), Riyadh (Kingdom of Saudi Arabia) and Tashkent (Uzbekistan). Data were analyzed using descriptive statistics.

Among the 1200 respondents, 1089 (91%) reported treatment with antibiotics. 342 (31%) of them had taken an antibiotic according to the prescription and 747 (69%) without a medical prescription. The most common reasons for self-administration of antibiotics were cough (40%). The major indicators for auto-medication per country were cough (26%) in Yemen, gynecological inflammations (33%) in Saudi Arabia and influenza (18%) in Uzbekistan. It was found that 49% stop taking antibiotics when feel better and 47% of respondents were storing antibiotics at home. Gender, age and educational level of secondary school or higher increased self-administration of antibiotics.

The prevalence of self-medication with antibiotics in adult people in the studied countries is alarmingly high. A majority of the patients had respiratory and gastrointestinal symptoms. Most of the antibiotics were obtained from pharmacies without the requirement of a prescription. Therefore, intervention from health authorities is needed to urgently stop this practice.

The study was supported by Charles University in Prague (Project SVV 265 005).

THERAPEUTIC DRUG MONITORING OF CARVEDILOL AND INFLUENCE OF HUMAN CYTOCHROME P450 2D6 POLYMORPHISMS ON CARVEDILOL THERAPY IN PATIENTS WITH CIRRHOSIS

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Carvedilol is highly lipophilic β_1, β_2, and α_1 – adrenoreceptor blocker without intrinsic sympathomimetic activity. It has also antioxidant properties. Carvedilol is a racemic mixture of R-(+) and S-(–) enantiomers. Carvedilol is primarily metabolized by CYP2D6. As the concentration of the drug increases, metabolism spills over to CYP3A4, CYP1A2, CYP2C9, CYP2C19, which are high-capacity, low-affinity enzymes.

CYP2D6 polymorphisms have been shown to cause variation in carvedilol pharmacokinetics. There is up to ten-fold difference in plasma concentration of carvedilol among people with different genotypes. Poor metabolizers have increased risk of drug-induced side effects and have longer half-life and higher minimum plasma concentration of carvedilol. Plasma level of carvedilol is also sensitive to co-medication with inhibitors and inductors of CYP2D6.

The aim of the project is to determine the activity of CYP2D6, specifically polymorphism affecting the metabolism of a lipophilic beta-blocker (particularly carvedilol) by cirrhotic patients with acute variceal bleeding and propose optimization of the therapy with respect to genotype of a patient. A gene is considered to be polymorphic when the variant allel is present in at least 1% of the population, however, the real prevalence is often much higher. Polymorphism can affect the function of many enzymes which results in varying level of their metabolic activity.

By specifying a particular type of metabolisers, we will be able to optimize therapy of carvedilol in cirrhotic patients.

We will use high-performance liquid chromatography with mass spectrometry to quantify the steady state concentration of carvedilol in human plasma. The pharmacokinetic program MwPharm will help us to demonstrate pharmacokinetics of carvedilol, taking into account measured concentration. We will compare the measured level of carvedilol in plasma with level in healthy population. CYP2D6 polymorphism will be detected by polymerase chain reaction (PCR).

The dosage of carvedilol will be adjusted with regard to both polymorphism and plasma concentration. We believe that individualization of the beta-blocker therapy will contribute to rational therapy.

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UPTAKE OF THE NEW ANTI-RANKL MONOCLONAL ANTIBODY DENOSUMAB IN THE TREATMENT OF OSTEOPOROSIS

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Since August 2011 a new biological anti-osteoporosis drug denosumab has been available in the Czech Republic (CR). This monoclonal antibody represents an innovative mechanism of action, inhibiting osteoclast formation, function and survival through preventing the RANKL/RANK interaction, thereby decreasing bone resorption. The drug is applied subcutaneously twice a year.

Similarly to other recently introduced original drugs with dual (strontium ranelate) and osteoanabolic (teriparatide, intact parathormone) mechanism of action, and due to its higher price, denosumab is subject of special prescribing limitations (only secondary prevention of osteoporotic fractures and 2nd line treatment).

The objective was to analyze current trends in consumption of the new anti-RANKL monoclonal antibody denosumab in the treatment of osteoporosis since its introduction onto the market in CR.

The prescription-based database of the General Health Insurance Company (VZP) of the CR that covers approximately 62% of the Czech population was used as the data source. An insured person with a recorded prescription for denosumab in the period of interest was defined as a patient. The obtained epidemiological and costing data were also confronted with the prediction in the budget impact analysis in the public reimbursement dossier accessible in the State Institute for Drug Control files.

We have identified 3119 (158) patients (men) treated with denosumab between August 2011 and July 2012. The median age of patients was 73 years. Of the patients prescribed denosumab in the first half-year, 84% received the second dose within the observed period of one year, of which 85% within the authorized period of 6 months (±1 month). This new drug was most often prescribed by physicians trained in internal medicine (47%) and rheumatology (30%). The cumulative costs for VZP of the drug were CZK 27.3 mil in the first year.

The uptake of denosumab has been rapid and agrees with the predictions presented by the manufacturer before the launch of the drug. This preliminary data also suggest that both the prescribing doctors and the patients filling the prescriptions follow the dosing schedule.

The project was supported by project SVV 265 005.

CORRELATES OF SELF-CARE ADHERENCE IN ADULTS WITH TYPE 1 DIABETES MELLITUS

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Treatment adherence is essential for optimal compensation of patients with diabetes mellitus. Pharmacist plays an important role in diabetes management. The main aim of the study was to map the adherence to self-care recommendations and to identify the influencing factors in adults with type 1 diabetes mellitus.

The observational cross-sectional study was conducted in the Diabetes Centre of the University Hospital in Hradec Králové, the Czech Republic. Self-administered questionnaires and medical records were used as the data sources. The study protocol was approved by the local Ethical Committee. Mann-Whitney test and Spearman correlations were used for statistical evaluation.

Diabetes self-care adherence was measured by the Self Care Inventory-Revised (La Greca; 2005) and treatment satisfaction by the Diabetes Treatment Satisfaction Questionnaire-status version (Bradley; 1994).

A total of 111 patients were enrolled in the study. The mean age of the study subjects was 42.4 years, 59.5% of patients were females and 53.2% of patients used insulin pump. The mean IFCC HbA1C was 68 ± 15 mmol/mol and the mean insulin dosage was 0.6 ± 0.3 IU insulin/kg/day. Self-care adherence was associated with a higher level of treatment satisfaction, a higher level of education, female gender, and lower insulin dosage, but was not associated with the incidence of hypoglycemic events or any other insulin therapy-related problems, number of concomitant Rx drugs used or family history of type 1 or 2 diabetes mellitus.

Self-care adherence in adults with type 1 diabetes mellitus is difficult to predict based on socio-demographic and clinical characteristics. Treatment satisfaction is one of the key factors that need to be targeted to maximize benefits to patients.

The study was supported by SVV 265 005.

RELATIONSHIP BETWEEN DRUG COMPLIANCE AND QUALITY OF LIFE IN RHEUMATIC PATIENTS

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Rheumatic disorders are chronic inflammatory diseases with a specific course and treatment which is still only symptomatic in many cases and its target is usually an achievement and maintenance of remission, reduction of symptoms and improvement in patients' quality of life (QoL). In chronic patients poor compliance to medication is common even though it represents a key requirement for successful treatment. The relationship between compliance and QoL has never been tested, however, we hypothesize two possibilities. The first is based on the role of compliance in the treatment and we suppose that compliant patients reach higher QoL than non-compliant ones. On the other hand the second hypothesis considers higher tendency to be compliant just at the moment when patients' QoL is poor. Therefore, the aim of our study was to elucidate the relationship between drug compliance and QoL in patients with different rheumatic disorders.

A cross-sectional study was conducted. We recruited patients ≥ 18 years of age with rheumatoid arthritis (RA), spondylarthritis (SA), juvenile idiopathic arthritis (JIA) and systemic scleroderma (SSc). Data were collected by questionnaires Short Form 36 version 2 (SF-36v2)[1], Compliance Questionnaire Rheumatology (CQR)[2], Health Assessment Questionnaire (HAQ)[3] and by general questionnaire focused on demographic patient's characteristics.

289 (83.8%) patients completed all key questions and were included in the analysis. The median (min to max) of age was 56 years (18–82), most of patients were women (76%). 178 patients (61.6%) were treated for RA, 47 patients (16.3%) for SA, 41 (14.2%) for SSc and 23 (8%) for JIA. The lowest median (min to max) of CQR score was reached in patients with JIA (66.7; 42.1–100) and the highest in the RA group (82.5; 26.3–100), the difference was significant ($p < 0.01$). 51.6% of all patients reached CQR score < 80. QoL score was lower than the average for the general population. SSc patients reached the significantly lowest physical score of QoL compared to RA, JIA ($p < 0.001$) and SA ($p < 0.05$).

No significant relationship between compliance and QoL was found, however, a trend to higher compliance in patients with lower QoL and greater disability was seen.

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MEDICATIONS AS A CAUSE OF HOSPITALIZATIONS IN THE ELDERLY

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Elderly population is a group of people who need special care. Pharmacokinetics and pharmacodynamics of drugs are changing in the elderly. Big problem in the elderly are poly-morbidity and polypharmacotherapy. Adverse drug reactions (ADRs) are more frequent and associated with increased consumption of drugs.

The aim of this project is determinate the prevalence of the hospitalizations geriatric patients due to medications.

The project is a retrospective cross-sectional study. All patients were hospitalized in two departments of the Clinic of Gerontology and Metabolism, University Hospital in Hradec Králové during the year 2010. Hospital admission had to be unplanned, all medical records had to be searchable in the hospital computer system and the age of patients had to be over 65 years. We recorded age, gender, social background and duration of hospitalization for all hospitalized patients and medications, diagnoses and Charlson Comorbidity Index only for patients who were hospitalized because of adverse reactions.

653 hospitalizations were included in the study. Mean age of the patients was 85.3 ± 4.2 years. Women were 386 (59.1%) and 267 (40.9%) were men. Women were older (85.8 ± 3.9) than men (84.6 ± 4.4). ADRs caused 79 (12.1%) from 653 hospital admissions. The mean duration of hospitalization of all patients was 9.4 days. Patients, who were hospitalized because of ADR, used 7.8 drugs per patient. Diuretics (38% from 79 cases) were the most common drugs, which led to hospital admission. On second place were ACE inhibitors and digoxin (12.7%) and on third were non-steroidal-anti-inflammatory drugs and warfarin (10.1%).

The current results suggest a similar trend like in the rest of the world. The range of prevalence of ADR related hospitalizations in the world is between 3% and almost 35%.

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USE OF THEORY OF PLANNED BEHAVIOR IN MINIMIZING RISK OF SELF-MEDICATION

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The theory of planned behavior explains relation between belief, attitude and intention to behave certain way and the real behavior. Attitude, subjective norms and perceived behavioral control shape together intention to behave and the real behavior (in this case it is over-the-counter drugs recommending). The theory of planned behavior belongs to theories with the highest predictability and it has been successfully used in many healthcare studies. European multicentric study OTC SocioMed Group with center at University of Crete in Greece is concerned with factors influencing pharmacist`s behavior regarding to recommending over-the-counter drugs.

Aim of this study is to identify and interpret risk factors of self-medication.

Standard questionnaire of Theory of planned behavior with 95 questions was used.

Pharmacists in the region Hradec Králové and Kroměříž were asked to fill in the questionnaire in the spring 2011. The obtained data has been analyzed with statistic software SPSS version 19.

Results show strong correlation between positive attitude to OTC drugs and their recommending. Pharmacists who consider drugs as useful and as a good practice recommend these 5.5 times more than pharmacists with negative attitude.

Strong correlation has been also found between perceived behavioral control and recommending OTC drugs. Pharmacists who are confident of right decision and ability to rational recommend OTC drugs to their patients, recommend these medicaments 3.3 times more than pharmacists who are sure less or not at all.

Between subjective norms and recommending OTC drugs has been found only weak correlation.

Study based on the Theory of planned behavior identified factors influencing pharmacists decision about OTC drugs recommending. Strong factors are trust and knowledge about the drugs and confidence of right decision. As a result of this study OTC SocioMed Group we can assess the pharmacists as responsible.

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SOCIAL PHARMACY AS A FIELD OF STUDY IN PHARMACY EDUCATION

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Social Pharmacy is an interdisciplinary subject with the aim to integrate drugs into the broader perspective and to include legal, ethical, economic, political, social, communicative, and psychological aspects into their evaluation in order to contribute to the safe and rational use of drugs.

The aim of the study was to compare differents in education of Social Pharmacy on Faculties of Pharmacy in the world (mainly European and American faculties). It compared the number and proportion of subdisciplines such as Pharmacoepidemiology, Pharmacoeconomics, Pharmacy Practice, Pharmacy Management, Education and Operations of Research, Health and Drug Policy, Ethics, Pharmacy Legislation and Regulations, Pharmaceutical Care, Psychology, Communication and others.

Basic information for questionnaire survey were obtained from the detailed analysis of the curricula (study plans) of 15 faculties. The subjects were divided according to the basic division into 4 groups: basic, medical, pharmaceutical and social.

The questionnaire survey was carried out from October to November 2012. The questionnaire was sent electronically to 371 faculties (deans or heads of departments).

The study involved 51 faculties (return rate was 13.7%) in total and shows significant regional differences. E.g. Pharmacy Management has as a subject almost 88% of faculties in North America, but only 37% of faculties in Europe. The opposite difference is in subject Industrial Pharmacy (47% in North America and 25% in Europa). All conclusions are presented by arranged tables and graphs.

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HEALTHY ADHERER EFFECT – AN UNMEASURED CONFOUNDER IN MEASURING OF ADHERENCE.

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Evaluation of adherence to the therapy is the subject of many studies. Also because of different methodologies it could be a reason for discrepancies between those studies and hence unavailability to compare acquired outcomes. We can observe wide variability of measurement instruments and definitions of adherence and limits associated with therapeutic intervention and patient character. One of the unmeasured factors related to the patient is the so called healthy adherer effect.

The aim is to characterize the healthy adherer effect, its occurrence in different designed studies and their solution to prevent this phenomenon.

We searched for literature from PubMed database. Eligible articles were selected based on predefined key words.

Healthy adherer effect is usually defined as a confounder related to patient behavior to the medication regime. Patients with high adherence to the therapy have potentially high adherence to healthy behavior (e.g. regular follow-up with health care professionals, vaccination, diet or exercise). Previous studies noted this effect when reported decreased risk of mortality with high adherence to the drug therapy even to placebo. Studies with placebo and objective health outcomes show good approach to reveal healthy adherer effect. In observational studies is recommended to adjust adherence to drug therapy using differential drug effects of evidence-based pharmacotherapies.

Healthy adherer effect is the unmeasured confounder related to health seeking behavior which was observed between high adherence to placebo and lower mortality. Researchers should be more careful about this when set up a trial and subsequently interpret final results.

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PROSPECTIVE ANALYSIS OF INTRAVENOUS DRUG INCOMPATIBILITIES IN AN INTENSIVE CARE UNIT IN UNIVERSITY HOSPITAL

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Drug incompatibilities are relatively common in inpatients and may result in morbidity or mortality of patients and add to the cost of the therapy. The objective of our long-term research project is to identify the real condition of drug incompatibilities *in vitro*

in critically ill patients in an intensive care unit (ICU). Based on acquired data, standard operating procedures (SOP) will be made with the aim to minimalize the occurrence of the incompatibilities.

Prospective analysis of 50 patients with multi-organ failure, intoxications and serious infections. All patients were admitted to the ICU at department of gerontology and metabolism in university hospital between December 2010 and June 2011 with at least 2 different intravenous drugs. This analysis led to identify the frequency of incompatibilities between intravenous drugs.

From 412 drug pairs given to the 50 patients on ICU, 3.64% of drug pairs were incompatible. Into the most frequent incompatible drugs pertains insulin, ranitidine, furosemide and ciprofloxacin. The newly developed SOPs class these and other incompatible drugs (mainly catecholamines, antibiotics and parenteral nutrition) to the group of drugs that should be separated from all other administered drugs. The use of an idle i.v. line or the administration of incompatible drugs in different times was recommended for this group as well.

This project intended to identify the real state of drug incompatibilities before the implementation of the new SOP. In comparison with other studies (Bertsche T., 2008; Taxis K., 2004; Gikic M., 2000) made in this field, a lesser number of potentially incompatibilities were observed in this study. The next study with the observation of incidence of incompatibilities after SOPs implementation and training of physicians will be following soon.

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DRUG-RELATED PROBLEMS IN PHARMACEUTICAL CARE

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Drug-related problem (DRP) is an event or circumstance involving pharmacotherapy that actually or potentially interferes with desired health outcomes. DRPs are common and decrease quality of health care and increase health costs. DRPs may be related to medication errors or adverse drug reactions. Medication review is an evaluation of medicines of patient with the aim of managing the risk and optimizing the outcome of drug therapy by detecting, solving and preventing DRPs. The aim of pharmaceutical care is to promote optimal therapeutic value of drugs, i.e., to reduce the effect of DRPs in the sense of maximize effect and minimize risk (including drug non-compliance), while maintaining optimal cost.

Objective: The aim is to show benefit and irreplaceability of pharmacist in the healthcare system of Czech Republic on the outcomes of medication review carried out by pharmacists.

Methods: Retrospective analysis of data from selected Czech pharmacies and clinical pharmacists' activities completed by comparison with literature.

Results: 44 DRPs and the same amount of drugs with negative effect on weight were identified by pharmacist during individual consultations in pharmacy, which were provided to 41 patients with risk of overweight and obesity from June 2006 to December 2009. Contraindications or drug interactions were found in 28%, resp. 24% patients requiring orlistat from May to December 2009. 64 DRPs were identified in 128 visitors of pharmacy during three months who want to consult their blood pressure. 331 DRPs were identified by pharmacist in dispensing 3761 drugs during 60 days. 142 DRPs were found at medication review in 70 randomly patients of rehabilitation centre.

Conclusion: The outcomes showed that pharmacist is able to detect and solve DRPs during consultation and dispensation in pharmacy or working on ward of health facilities. The question remains how to use these skills in building up safe medication practice in the Czech Republic.

The study was supported by the Charles University in Prague (SVV 265 005).

THE EVALUATION OF ANTIBIOTIC TREATMENT IN URINARY TRACT INFECTIONS, POTENTIAL INFLUENCE OF THE AGE AND GENDER ON THE TERAPEUTICAL DECISION. DATABASE STUDY

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We assessed the individual prescriptions of antibiotics used in treatment of uncomplicated infections of lower urinary tract (UTI). We evaluated the repeated use of these antibiotics, because the decreased effectiveness of antibiotic therapy after previous antibiotic treatment of urinary or respiratory infection in individual patients may last 3–6 months even up to 1 year. We used the General Insurance Company (VZP) database, which lacks the diagnose information. Therefore only nitrofurantoin and norfloxacine prescriptions indicate concrete diagnose of lower urinary tract infection. Nevertheless this research is advisable for the size of studied population.

We selected "new" patients with UTI from the database, who didn't use any of antibiotics, recommended by Czech Medical Association of J. E. Purkyně for UTI treatment in previous 12 months and newly got nitrofurantoin or norfloxacine within the time period 1. 1. 2008 – 30. 6. 2009. We assessed their first prescription and searched for the following antibiotic prescriptions and evaluated the type of the first repeated one and the number of repeated prescriptions in the following 180 days.

We identified 174,368 new patients in the database. Norfloxacine was found on the first prescription at more than 10% cases of new women patients older than 55 years, nitrofuranotin at 7% cases. Older men got norfloxacine and nitrofurantoin at 4.8% and 4.3% resp.

Slightly more (63%) patients, who received nitrofurantoin do not need next antibiotic treatment for UTI in following 180 days (norfloxacin 61.8%), OR = 1.0532, CI = 1.0313 to 1.0756. 32% of patients older 55 years, received the same type of antibiotic on the first repeated prescription (within 180 days) as was their first antibiotic (nitrofurantoin, norfloxacine či SMX-TMP). This proportion was lower (about 23%) in younger patients and for other antibiotic groups.

Conslusion and Discussion: Number of postmenopausal women receiving norfloxacine to their first UTI onset is quite high, considering that norfloxacine should never be the first choice antibiotic. Additionaly *E. coli* isolates from older women are less sensitive to fluoroquinolons. Nitrofurantoin seems to have slightly protective effect regarding reccurent UTI. The prescription of the same antibiotic repeatedly is not correct, however in case of nitrofurantoin do not indicate marked prescribing error as in case of beta-lactams and SMX-TMP (both disputable if respiratory or urinary infection) or norfloxacine.

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SELF-REPORTED USE OF OSTEOPOROSIS GUIDELINES AMONG GENERAL PRACTITIONERS

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Management of osteoporosis (OP) at primary level is of great importance. Based on attitudes and activities of general practitioners (GPs) towards OP it should be specified the role of pharmacists in this field. Researchers trained in rational pharmacotherapy should assist with formulation and revision of evidence-based guidelines.

The study objective was to assess use of OP clinical guidelines among general practitioners in the Czech Republic.

A cross-sectional survey among GPs was performed to analyse their opinions about OP, activities in OP management and its barriers. In 2007, an anonymous postal 24-item questionnaire was distributed to 1393 GPs randomly selected from a database of the Institute of Health Information and Statistics of the Czech Republic. Main outcome measures were use of OP guidelines and its correlates.

The mean age of respondents (n = 525, return rate 38%) was 52 years (range 30–83) and 59% of them were women. As much as 50% of respondents use the guidelines repeatedly. The guidelines were never used by 8% of respondents. Adherence to the guidelines correlated significantly with knowledge about OP ($p = 0.002$). Controlled for age, use of guidelines correlated significantly with quality of care indicators (suspicion of OP, reasons for referral to a specialist, initial check-up; $p < 0.05$ for each). Use of other sources of information (professional literature, workshops and conferences, manufacturer's information) did not correlate with the indicators or correlated only with one of them.

In order to increase knowledge about OP in GPs and to provide primary care of higher quality, it is necessary to motivate GPs to use clinical guidelines.

The project was supported by project SVV 265 005.

PHARMACEUTICAL BOTANY AND PHARMACOGNOSY

ALKALOIDS OF *NERINE BOWDENII* WATSON (AMARYLLIDACEAE) AND THEIR BIOLOGICAL ACTIVITY

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Nowadays, AD is the most predominant cause of dementia in the elderly. The etiology of AD is still unknown, but *postmortem* studies have shown two characteristic pathologic hallmarks, senile plaques (SPs) and neurofibrillary tangles (NFTs). In AD patients, deficit of the neurotransmitter acetylcholine (ACh) in the cortex results in a degradation of cholinergic functions' level, and thus the memory impairments. Not only acetylcholinesterase (AChE) participates in the cholinergic regulation of central nervous system (CNS) in humans, but also butyrylcholinesterase (BuChE), which is able to hydrolyze ACh, as well as other esters. BuChE is associated with the NFTs and SPs and its activity increases in the AD brain, where it co-localizes with Aβ fibrils. In severe AD, levels of AChE and choline acetyltransferase are decreased by approximately 90%, while the concentration of BuChE increases.

Very important family is Amaryllidaceae, which produce structurally unique alkaloids with a wide range of interesting physiological effects. These alkaloids are restricted to this family and are best known due to galanthamine, alkaloid used for treatment of Alzheimer's disease (AD). It is a selective and reversible inhibitor of acetylcholinesterase that increases the level of acetylcholine in the brain.

The genus *Nerine* is the second largest within Amaryllidaceae with ca 30 species. *Nerine bowdenii* is an autumn-flowering perennial bulbous plant group, whose species inhabit areas with summer rainfall and cool, dry winters. Previous investigations led to the isolation of more than 30 Amaryllidaceae alkaloids. Some of these have been tested for their AChE inhibitory activity, but not against BuChE and PEP.

The extract of alkaloids was prepared by percolation of fresh bulbs (10 kg) by 95% EtOH and it was fractionated by column chromatography. Nowadays, 4 alkaloids were isolated, which were tested for their inhibition activity against AChE, BuChE and PEP.

Cholinesterase inhibitory activity was tested *in vitro* using human erytrocyte AChE and plasmatic BuChE. The activity was measured spectrophotometrically by Ellman's method. The values of IC_{50} were determined and compared with values of IC_{50} of reference substances or substances used in therapy (galanthamin, rivastigmin, huperzin A).

PEP catalyzes the degradation of proline-containing neuropeptides such as vasopressin, substance P, and thyrotropin-releasing hormones that are involved in the processes of learning and memory.

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BERGENIA MOENCH. GENUS – CONTENT MATTERS AND BIOLOGICAL ACTIVITY

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Bergenia, a genus included in the family *Saxifragaceae*, is a valuable source of healing matters. About 10 *Bergenia* species are known all over the world. Scientific research is focused on five species mainly distributed in the mountains of Central and East Asia: *Bergenia ciliata* (Haw.) Sternb., *Bergenia stracheyi* Engl., *Bergenia crassifolia* (L.) Fritsch, *Bergenia ligulata* (Wall.) Engl. and *Bergenia himalaica* Boriss. These taxons belong to the widely used medicinal herbs in the traditional Chinese, Nepalese and Indian medicine, for therapy of cough and pulmonary diseases, to stop bleeding, to increase imunity and to dissolve kidney or bladder stones. In the Czech republic this species is commonly grown but it's not use for medical therapy. Individual parts of plant demonstrate interesting biological activity, anti-bacterial, adaptogenic, anti-viral and cytoprotective effect. *Bergenia* is a valuable resource of interesting chemical compounds. This plant contains several active compounds such as polyphenol bergenin (C-glucoside of 4-O-methyl gallic acid), its derivative norbergenin, catechin and other polyphenols. The main attention is focused on bergenin for its antiviral, antibacterial and antioxidant effect. Another active compound of this species is arbutin, phenolic monoglucoside of hydroquinone. The main aim of our study is the evaluation of the content of the secondary metabolites in different taxons of *Bergenia* genus (*Bergenia crassifolia* (L.) Fritsch., *Bergenia ciliata* (Haw.) Sternb., *Bergenia* x *ornata* Stein.). Garden of Medicinal Plants, Mendel university in Brno, Faculty of Horticulture in Lednice has provided plant material. For the evidence and determination of total tannin, arbutin and bergenin spectrophotometric and HPLC methods were used. Analysis of 79 samples of *Bergenia* plant revealed that the highest content of arbutin (35.08 mg/g) was in the leaves of *Bergenia* x *ornata* Stein. collected in May 2012 and the lowest (7.44 mg/g) was in the leaves of *Bergenia ciliata* (Haw.) Sternb. collected in October 2011. The highest content of total tannin was revealed in the leaves of *Bergenia crassifolia* (L.) Fritsch. collected in October 2011 (38.8 mg/g), the lowest content was in the leaves of *Bergenia ciliata* (Haw.) Sternb. (19.3 mg/g). The extracts prepared from single parts of plant will be also tested for potential immunological and antioxidant activity.

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ALKALOIDS FROM *FUMARIA OFFICINALIS* L. (FUMARIACEAE) AND THEIR CHOLINESTERASE INHIBITORY ACTIVITY

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Alzheimer's disease (AD) is the most common form of dementia among the elderly. This progressive, degenerative disorder of the brain is characterized by a loss of memory and cognition, together with a declined ability to perform basic activities of daily living. The current approved therapy includes the anticholinesterase inhibitors and the NMDA blockers. Cholinesterase inhibition is the most used therapeutic treatment for the symptoms of AD, although spectrum of used anticholinesterase compounds is relatively limited. In the screening of natural inhibitors of acetylcholinesterase, alkaloid extracts from species of Fumariaceae have demonstrated AChE inhibitory activity[1].The main compounds of genus Fumaria are isoquinoline alkaloids belonging to different structural types[2].

Herbs of *Fumaria officinalis* L. were selected on the basis of bio-guided spectrophotometric Ellman's method[3] as a source of isoquinoline alkaloids for study of their selected biological activities. The herbs were extracted with ethanol and the mixture of summary tertiary alkaloids was fractionated in alumina chromatography column using step gradient elution with petrol, chloroform and ethanol. Repeated column chromatography, preparative TLC and crystallization led to the isolation of nine isoquinoline alkaloids (protopine, fumaricine, parfumine, sinactine, fumariline, *O*-methylfumarofine, cryptopine, bicuculline and stylopine) so far. The chemical structures of isolated compounds were determined on the basis of spectroscopic techniques and by comparison with literature data. Isolated alkaloids were tested on ability to inhibit human erythrocyte acetylcholinesterase and serum butyrylcholinesterase. Established IC_{50} values of these compounds were compared with standards galanthamine, huperzine A and eserine.

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IN VITRO PLATELET ANTIAGGREGATORY ACTIVITY OF FLAVONOIDS

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Flavonoids constitute one of the most common group of polyphenolic compounds widely present in plants and foods. They are ubiquitous in nature and according to the chemical structure they can be divided into several classes (flavanols, flavanons, flavones, isoflavones and anthocyanins). They have been largely studied because of their potential beneficial effects on human health including antioxidant, anti-inflammatory, antitumor and antiplatelet activity.

Since the data on antiplatelet activity of flavonoids are not in the full agreement, the aim of the study was to test a series of flavonoids in order to confirm their antiplatelet activity as well as find out the mechanism of action.

The effect of flavonoids on three different levels of arachidonic based aggregation, namely on thromboxane receptors, thromboxane synthase and cyclooxygenase-1, was tested by use of commercial sets and procedures previously reported by us (Macáková K. et al., Biochimie 2012).

Although experiments with all selected flavonoids have not been finished yet, it is apparent that inhibition of thromboxane synthase is not the main target of flavonoids. The flavonoids did not markedly influence the enzyme activity in contrast to the standard 1-benzylimidazole.

Analogously, the majority of flavonoids did not inhibit the cyclooxygenase-1, but isoflavonoids genistein and daidzein seem to be substantially active. Preliminary experiments showed that they can be similarly efficient as acetylsalicylic acid.

The main mechanism of antiplatelet activity of flavonoids appears to lie in the antagonism at the thromboxane receptor. In particular, the 7-hydroxygroup seems to be the most important structural characteristic for the activity. However, it should be mentioned that not all flavonoids are antagonists at the mentioned receptor.

Additional experiments are necessary for the establishment of the structure-activity relationship.

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CURRENT KNOWLEDGE IN PLANT DEFENSE – WHERE DO WE STAND?

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Plant secondary metabolites are of interest because of their benefits to human and animal health for many centuries. Recent research has also recognized the role of secondary metabolites in plant defense and ecological interactions. This extends beyond simple considerations of plant protection because secondary metabolites are also involved in the regulation of events of signaling cascades that lead to defense. The goal of our research is to elucidate specific part of the molecular mechanism underlying defense signaling in *Arabidopsis thaliana*, focusing on the spatiotemporal dynamics of the cytoskeleton and its interactions with phospholipase during defense responses. Cytoskeletal components together with a wide range of associated proteins mediate transport inside cells, which is essential in the early stages of defense reaction. Phosphatidic acid (PA) generated by the hydrolytic activity of phospholipase Dd represents an important second messenger in stress signaling, and both the cytoskeleton and phospholipase Dd are involved in plant signaling cascades in various ways. Using live-cell imaging and image analysis we discovered that depolymerization of actin cytoskeleton after treatment with salicylic acid takes part in a signaling cascade leading to the expression of pathogen related (PR) proteins. We found that treatment with PA prevented actin depolymerization and resulted in inhibition of PR proteins expression, indicating a connection between the actin cytoskeleton and phospholipase De. Examination of tobacco BY-2 cells, *Arabidopsis thaliana* cells and plants transiently or stably transformed with GFP-PLDd reporter revealed at least four unique patterns located beneath the plasma membrane, with one of them showing filament – like structures colocalized *in vivo* with microtubules. These findings will help to elucidate where and when particular elements of defense cascades operate.

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INVESTIGATION OF PERMEATION OF NANOPARTICLES THROUGH SUBLINGUAL MEMBRANES *IN VITRO*

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A great number of new, important, physiologically active substances are of macromolecular character (e.g. peptides, nucleotides, enzymes, proteins, hormones). The therapeutic usage of such substances is limited due to a series of problems (such as enzymatic degradation, aggressive environment in the gastrointestinal tract, slow and poor absorption, and high first-pass effect) which hinder their effective oral administration. There are also considerable complications linked with their administration via parenteral route (i.v., s.c., i.m.), particularly invasiveness, painfulness and poor patient compliance.

Sublingual route of administration has a handful of advantageous properties: thin barrier, well accessible and well perfused, relatively low enzymatic activity, avoidance of first-pass effect, quick onset of action, pH near the neutral range (5.8–7.6), non-invasiveness, good patient compliance. There are, of course, some drawbacks as well: relatively small area, washing off by the saliva, some enzymatic activity. Mechanism of permeation of the macromolecules and nanoparticles through sublingual membrane is little known.

The permeation of Chromeon 470 marked nanoparticles through variously treated porcine sublingual membrane (1 cm^2) into buffered acceptor phase (pH 7.4) *in vitro* was evaluated. Either freshly cut up membranes or membranes frozen for 1 hour or long-time frozen membranes were used, in both cases preserved by sodium azide.

A higher rate of nanoparticle permeation was observed in sodium azide preserved frozen membranes than in membranes that were not frozen. Surprisingly, the nanoparticles permeated better from diluted than from non-diluted donor samples.

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EFFECT OF FILLERS AND LUBRICANTS ON PARAMETERS OF COMPACTION EQUATION

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This study deals with the evaluation of the compaction process of fillers and their mixtures with lubricants using the parameters of the compaction equation. Compaction equations are mathematical functions, which describe compaction process, and are able to divide it into phases. Three-exponential equation, that was used, was developed previously at our department of pharmaceutical technology. This equation describes dependence of volume reduction on compacting pressure, and is able to divide the compaction process into three phases, that run simultaneously. The first phase describes particle rearrangement, the second phase describes elastic deformations, and the third phase describes plastical deformations, and creation of interparticle bonds. This equation describes the compaction process by parameters ai, that describe the volume reduction, and parameters ti, which characterize speed of volume reduction of corresponding phase of compaction process. Furthermore it is possible to calculate parameters Ei and pHi. Parameters Ei indicates the energy consumed by the relevant phase. Parameters pHi indicate at what pressure takes place one half of volume reduction.

With the help of the parameters of this of equation we described and compared the behavior of three fillers during pressing. The fillers that we used have different binding mechanisms. As fillers we used microcrystalline cellulose, dicalcium phosphate dihydrate and lactose. Then we evaluated mixtures of fillers with magnesium stearate, sodium stearyl fumarate and modificated colloidal silicon dioxide. We used the parameters of compaction equation to evaluate the influence of the gliding agents at the compaction process. Finally we described the effect of concentration of the lubricants on the values of parameters of compaction equation.

ELECTROPSUN MEMBRANES FOR IMPROVED SOLUBILITY AND BIOAVAILABILITY OF POORLY SOLUBLE DRUGS

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Many important drugs are poorly soluble in aqueous medium, leading to a lack of absorption from the gastro-intestinal tract. This problem is solved by increasing the drug dose or by using of non-chemical procedures which increase the solubility, such as crystal modification and amorphization, pH modification, complexation, micronization, hot-melt extrusion, self-emulsification, spray-drying, freeze drying and others.

Electrospinning technology producing fibers of submicron scale diameter was also successfully tested for enhancement of drug solubility, but it is not very well explored yet.

The presented work is focused on the utilisation of free liquid electrospinning technology (Nanospider^{TM}) for formulation of drug loaded nanofibre membranes containing substance D, which is almost insoluble in water. Potential increase in solubility of the drug after this processing was evaluated.

Dissolution of substance D was observed under the conditions commonly used for the evaluation of the solubility in BCS procedures, using an artificial intestinal juice and continuous sampling for HPLC analysis.

Results of *in vitro* tests demonstrate a very high increase in dissolution rate as well as a great increase in the total amount of dissolved substance D. Based on the obtained data, it is also very tempting to assume, that the observed increase in solubility could lead to a subsequent increase in bioavailability of the drug after oral administration. The method of drug incorporation into nanofibre carrier under study allows, in principle, to improve p.o. administration of substances of very low water solubility. The method can be adopted for drugs intended for administration onto variety of mucosal membranes.

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INFLUENCE OF DIFFERENT TYPES OF FILLERS ON THE PARAMETERS OF PLASTICITY AND ELASTICITY

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Fillers are pharmaceutical excipients in the production of tablets, which are used to enhance the volume of the active substance to the required weight. Their properties significantly affect the properties of the whole tablet. They are used crystalline and polymeric substances. They differ in their viscoelastic properties. Crystalline materials are brittle and even at low pressure its particles fragment. For polymers, fragmentation does not occur. At low pressures, there is a slight elastic phase, under higher pressure come to plastic deformation. These characteristics then influence the process of compressing.

The parameters of plasticity and elasticity were described by stress relaxation test. This test records the decrease in pressing power in a tablet at its constant height and was used to evaluate the time-dependent deformation for microcrystalline cellulose (Avicel PH 200), lactose (Lactochem Fine Crystals) and dicalcium phosphate dihydrate (Emcompress). During 180 s period was monitored the decrease in compressing power and was described by the three exponential equation. From this equation were obtained the parameters of elasticity. Parameters of plasticity were calculated. These parameters divide the process into three simultaneous processes. Each process is characterized by the interaction of particles in various stages of deformation.

The lowest values of elasticity and plasticity in all processes were found in Emcompress. This is due to the nature of bonds that creates (weak bonding interactions – Van der Waals force). Higher values of these parameters have lactose. In addition to Van der Waals forces, there is also a part of hydrogen bonds. The highest values are at Avicel, which creates mainly hydrogen bonds. It was also found the linear nature of the relationship between elasticity and plasticity parameters.