„Expression, isolation and inhibition of the bacterial ureases activity”

Urease (urea amidohydrolase, E.C. 3.5.1.5) is an enzyme that catalyzes hydrolysis of urea to ammonia and carbamate which decomposes to generate a second molecule of ammonia and carbon dioxide. In aqueous solutions, the released carbonic acid and the two molecules of ammonia are in equilibrium with their deprotonated and protonated forms, respectively. This reaction causes significant increase of pH due to the release of large quantities of ammonia. This effect has considerable consequences in two types of severe bacterial infections of human body: (i) enzymatic decomposition of urea allows survival of *Helicobacter pylori* in stomach and duodenum, (ii) alkalinization of urine during infections by *Proteus mirabilis* and related species of urinary tract causes fast formation of stones. The crucial catalytic role of enzymes in biological processes makes them good molecular targets for designing of novel inhibitory molecules that may serve as the starting points for drug discovery or development of compounds of practical application in agriculture, medicine and food industry.

In the present work the potency of novel classes of highly specific inhibitors of bacterial urease were examined. The design of specific inhibitors of urease is very difficult because: (i) small space available in the enzyme active site and (ii) movable flap constituting a significant part of the active site. In this work was apply several design strategies for the discovery inhibitors of bacterial ureases. The first of these were explore the influence of heavy metal ions on catalytic activity of urease. The second strategy was based on a potentially promising modification of the previous scaffolds, bis(aminomethyl)phosphinic acid. The last approach were explore compounds capable of binding to the cysteine residue present at the entrance of the active site (ebselen derivatives and analogues, Michael acceptors, phosphonic/phosphinic acids with Michael acceptor group, Morita-Baylis-Hillman acetates). Although cysteine is not directly involved in the catalysis of enzymatic reaction, its placement allows effective inhibition. An additional part of the study was to obtain crystal structure of urease in the complex with selected inhibitors.

The starting point of study was to obtain a catalytically active enzyme from the cells of *Bacillus pasteurii* and *Proteus mirabilis* showing a natural ability to produce urease recombinant Helicobacter pylori urease. Expression conditions were tested the for improved high production yield of recombinant urease. Escherichia coli Rosetta were induced using six different concentrations of IPTG (0.25, 0.5, 0.75, 1.0, 1.25, 1.5 mM), four different concentrations of nickel ions (II) (0.3. 0.5. 0.75. 1.0 mM) and six different induction temperatures (from 20 to 30^{o}C overnight). The cell-free

supernatant was analyzed by SDS and qualitative test for urease activity to determine the effects of the temperature, inducer and Ni^{2+} ions concentrations on the productivity, activity and the solubility of the protein.

Enzyme purification was performed using the sequence of four (or five) different chromatographic columns including following steps: ion-exchange chromatography (Q Sepharose, Resource Q, Mono Q), hydrophobic interaction chromatography (Phenyl Sepharose), gel filtration (Sephacryl S-300, Superdex S-200) and affinity medium — on the Cellufine Sulfate gel. The use of multi-step chromatographic approach led to almost homogenic urease preparation. Under the specified experimental conditions a highly purified preparation of bacterial ureases from *Sporosarcinia pasteurii*, *Proteus mirabilis*, *Helicobacter pylori* were obtained. The purified fractions of enzyme were subjected to standard biochemical analysis in order to establish pH-stability of urease and its affinity to urea. Optimum pH for analyzed urease is in the range of 7 and 8, and K_M values is 23.4 ± 1.9 mM for SPU; 25.87 ± 3.08 mM for PMU and 0.38 ± 0.02 mM for HPU. Specific activity of HPU was determined as 2451U/mg for SPU, 2051 U/mg for PMU and 1654.46 U/mg for HPU. The purified enzyme was further used to establish inhibitory properties of selected inhibitors.

The first group of urease inhibitors were heavy metal ions. The kinetics of heavy metal ions inhibition of bacterial urease (*Sporosarcinia pasteurii* and *Helicobacter pylori*) was studied by progress curve analysis in a reaction. The inhibition of *Sporosarcina pasteurii* urease was found to be biphasic and reversible for heavy metal ions such as: Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+}, Fe^{3+} while Cu^{2+}, Zn^{2+}, Fe^{3+} have demonstrated of irreversible inhibition. In the case of *Helicobacter pylori* urease all of the above heavy metal ions (Ni^{2+}, Co^{2+}, Mn^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Fe^{3+}) have demonstrated of reversible inhibition. The observed reversible inhibition was time-dependent and could be best described by a slow-binding mechanism. The sensitivity of urease to heavy metal ions is due to the presence of multiple cysteine residues, of which one, conserved principally in all known ureases, is located in the mobile flap of the active site of the enzyme.

In the case of low molecular weight urease inhibitors more complex studies have been performed. Starting from the bis(aminomethyl)phosphinic acid as the lead compound, several structural modification of the molecule, based on computer-aided modeling, were proposed and their correctness was further verified by enzymatic analysis with the use of native bacterial (*Sporosarcina pasteurii* and *Proteus mirabilis*) and recombinant bacterial (*Helicobacter pylori*) ureases. The aminomethyl(N-n-hexylaminomethyl)phosphinic acid from the group of 16 analyzed compounds was found to be the most potent inhibitor, with a K_i = 108 nM against the *S. pasteurii* enzyme, K_i = 202 nM against *P. mirabilis* and K_i = 290 nM against *H. pylori*. Microbiological tests

performed on the living cells of uropathogenic strain *Proteus mirabilis* confirmed inhibitory properties of this class of compounds towards intracellular enzyme, suggesting it is ability to pass across bacterial membrane. Taking into account high hydrolytic stability of aminophosphonates even at low pH, we assume their possible use as the ancillary antimicrobial agents that could improve effectiveness of the standard antibiotic treatment of infections caused by ureasepositive pathogens of gastric and urinary tract.

Examination a series of Michael acceptors containing ketone, carboxylic ester, amide and acid functions led to the discovery a very potent urease inhibitors. Interestingly, several compounds with high inhibitory activity was found among carboxylic acids. In general, carboxylic acids are much less reactive then corresponding esters, amides and ketones. The reactivity of selected examples against thiols was tested using glutathione as a model substrate and clearly the confirmed above-mentioned rule. It is worth to note, that acetylene dicarboxylic acid seems to be particularly interesting due to a nanomolar inhibitory activity (K_i = 42.5 nM) combined with very low thiol reactivity (log*k*GSH = -2.14). The exploration of its derivatives could lead to further optimization of these parameters.

The next group inhibitors were Morita-Baylis-Hillman (MBH) acetates. Although their structure is similar to Michael acceptors, the mechanism of reaction with thiols (including cysteine residue) is different. Studies of simple examples of MBH acetates led to the discovery of very potent urease inhibitors. Addition of –SH group to the double bond of MBH acetates results in relocation of double bond and elimination of acetate. Due to this mechanism, the reaction is irreversible. The value of k_{inact}/K_i is in the range from 75.3 to 94.8 $M^{-1}s^{-1}$. Interestingly, the activity of acids and related esters is similar, although their reactivity towards thiols is significantly different (esters are much more reactive than corresponding acids).

Phosphonic/phosphinic acid based inhibitors of bacterial ureases have been studied since several years and highly active compounds have been found. Molecular modelling studies indicated that metal binding abilities combined with the structural similarity to transition state of the enzymatic reaction are crucial parameters responsible for high inhibitory activity. This class of compounds was classified as extended transition state analogues. In this part of study it has been proposed to extend structurally phosphonic/phosphinic acids previously studied by addition of a Michael acceptor group. Such structures should simultaneously interact with nickel ions (by phosphonic/phosphinic acid functions) and cysteine residue. Such mode of action has never been shown for any known urease inhibitor, but my research result in highly active and selective inhibitors.

The inhibitory activity in low micromolar range was found for some derivatives (the inhibition constants are in the range from 5.17 to 36 μM).

The last group of inhibitors is ebselen and their derivatives. Ebselen is an organoselenium compound, which is capable reacting with thiols. Biological activities, related to this reactivity, including antimicrobial, antiviral, antifungal and anti-inflammatory, has been already shown for this molecule and its derivatives. Interestingly, metabolism and toxicity of ebselen was studied in detail and it was shown that this compound could be safely used in humans. The studies have shown that ebselen, analogous diselenide and few derivatives exhibit extremely low nanomolar reversible inhibitory activity. Thus, these compounds rank among most active inhibitors of bacterial ureases with the inhibition constants are in the range from 2.11 nM to 222 μM).

Despite of the unsuccessful outcome of initial crystallographic studies seeking to obtain crystal structure of *Sporosarcina pasteurii* and *Helicobacter pylori* urease in complex with one of the inhibitors, the obtained Sporosarcina pasteurii urease crystals with bound citrate molecule in the active site.