1. **Current state-of –art in the field and outcomes of the project**
   a) Describe current situation of the field in worldwide context including relevant literature references
   b) Describe ability of the project proposal to expand knowledge beyond existing borders in the relevant field

2. **Project objectives**
   a) Describe project objectives for duration of the project and for its particular stages
   b) Define originality and innovativeness of objectives proposed

3. **Methodology**
   a) Describe methods for gaining project objectives
   b) Characterize methodology in the context of knowledge in given area
   c) Briefly describe your planned approach to reach the goals, in order to evaluate their feasibility

4. **Professional quality of research team, management of the team, infrastructure**
   a) Describe professional competence and complementarities of the participants, co-operating institutions in context of project proposed
   b) Describe coordination and management approach within the research team
   c) Describe existing infrastructure necessary for reaching the project goals

5. **Outcomes and impacts of the project**
   a) Describe the results and expected outcomes of the project in its particular objectives and quantify them
   b) Describe your plan for a dissemination of the project results

6. **Interaction between research and education**
   a) Describe use of the results of basic research of your project in formal or informal education.
   b) Describe your strategic plan for popularization of the project results in the society

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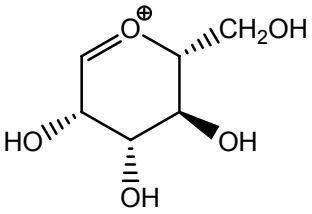
Computer modeling, synthesis and biological evaluation of selective inhibitors of Golgi mannosidase II

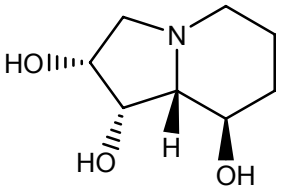
1. Current state-of –art in the field and outcomes of the project:

In breast, colon and skin cancers, the unusual quantitative distributions of complex carbohydrate structures on the cell surface are associated with disease progression, metastasis and poor clinical outcome.[1-3] This altered distribution is associated with abnormalities in the *N*-glycosylation pathway, and inhibition of a key enzyme in this pathway, human Golgi α-mannosidase II (EC 3.2.1.114, GM)[4], has shown clinical potential in cancer treatment. GM belongs to glycosyl hydrolase family 38 [5] and is central to the Golgi processing pathway, as it specifically trims two mannose residues from the branched $GlcNAcMan_5GlcNAc2$ mannose intermediate to form the core $GlcNAcMan_3GlcNAc2$ glycosyl structure, an essential precursor for the further addition of *N*-acetylglucosamine units.

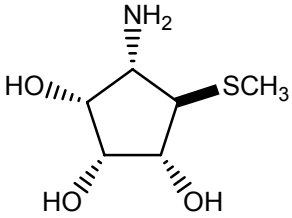
In preliminary clinical trials with late-stage cancer patients, specific inhibition of GMII by the non-toxic, orally available compound swainsonine resulted in reduced tumor growth and metastasis. [1,6-11] Swainsonine [(1*S*,2*R*,8*R*,8a*R*)-trihydroxy-indolizidine, Fig. 1] is an inzolizidine alkaloid naturally occurring in a number of Australian and North American plants. It interferes with the glycosylation pathway by noncovalent binding to the active site of GM and leads to the increased accumulation of hybrid-type glycan structures. They suppose that its biological activity is based on structural similarity with mannosyl oxocarbenium ion – GM intermediate (Fig. 1). Its inhibition constant is in the range of 20-50 nM, and thus became a lead compound for design of novel synthetic inhibitors of glycosidases.[12,13] However, swainsonine and its synthetic analogs exhibit a side effect resulting to a blockage of oligosaccharide catabolism; this arises from inhibition of a catabolic human lysosomal α-mannosidase (LM)[1,7], the relative to human Golgi α-mannosidase II.



Mannosyl-oxocarbenium ion



Swainsonine

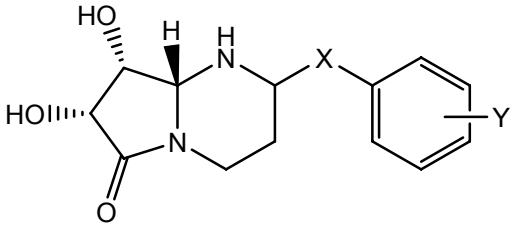


Mannostatin A

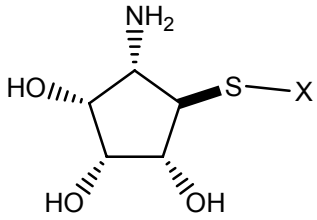
Fig. 1

Mannostatin A (Fig. 1), isolated from the soil microorganism *Streptoverticillus*, is a potent mannosidase inhibitor with a high biological activity reported (IC_{50} = 36 nM),[14] thus became another lead compound for the design of selective GM inhibitors.[15-17] Mannostatin is reversible, competitive inhibitor, which do not show the slow-binding phenomenon exhibited by swainsonine and its analogs.[15,17,18] However, mannostatins exhibit the same side effect as swainsonine, they inhibit human LM.

It is clear that for development of an effective mannosidase inhibitor will be necessary to propose a novel lead structure, which will maintain potent properties at the level of swainsonine and mannostatin and be highly selective to human Golgi α-mannosidase II with a minimal side effect toward human lysosomal α-mannosidase. For this purpose, the use of crystallography and advanced techniques of computer structure-based design can be a key factor to a successful progress. Up to date, crystal structures of two type of α-mannosidases, *Drosophila melanogaster* Golgi α-mannosidase II [4] and bovine lysosomal α-mannosidase[19], are available. For *Drosophila* GM several crystal structures with inhibitors bound at the active site of the enzyme, involving swainsonine and mannostain, are available due to a working group of Rose.[4] The *Drosophila* GM shows high sequence identity with human GM (41 % identity, 61 % similarity), and displays comparable kinetic properties and inhibitor sensitivity to mammalian GM, as well as the same substrate specificity.[4] Therefore, a 3D structure of the human GM prepared from *Drosophila* GM by means of computer homology modeling should be a good starting structure for rational structure-based design of novel inhibitors of human GM. On the other hand, low sequence identity between LM and GM (25 % identity at the active site of the enzymes)[19] give a chance for a successful design of the selective mannosidase inhibitors, what is the main effort of research teams developing novel glycosidase inhibitors and main objective of this project.



Swainsonine-based inhibitors



Mannostatin-based inhibitors

Fig. 2

2. Project objectives:

The main objective of the project is to design novel anticancer inhibitors, analogs of swainsonine and mannostatin, which will be highly selective for the human Golgi α-mannosidase with minimal side effects toward human lysosomal α-mannosidase. (Structures of both proposed inhibitors are schematically drawn in Fig. 2) For these purposes:

a) 3D structures of human GM and LM will be prepared by means of the homology modeling.

b) Then, the structure-based design based on docking techniques, molecular dynamic simulations and quantum mechanics calculations will be performed on both enzyme systems in complexes with inhibitors. The computer modeling will be performed by a working group of Dr. Tvaroška at the Institute of Chemistry, Slovak Academy of Sciences.

c) The selected inhibitors will be synthesized by a working group of Dr. Siriwardena at University of Jules Vernes at Amiens. This group co-operates with a crystallography group of Dr. Rose from the Cancer Institute and Department of Medicinal Biophysics at University of Toronto, the authors of available crystal structures of GM with inhibitors.[4]

d) Subsequently, their biological activities will be tested by a working group of Dr. Mucha at the Institute of Chemistry, Slovak Academy of Sciences. For these purposes human GM and LM will be cloned and expressed for inhibition studies.

3. Methodology:

Computer modeling. 3D structures of human GM and LM will be prepared by means of the homology modeling using the PRIME program of the SCHRODINGER package. Then, both enzyme structures will be equilibrated and analyzed by molecular dynamics (MD) simulations using the AMBER and GROMACS program packages. The optimized structures wil be subsequently used for rational structure-based design of novel swainsonine- and mannostatin-based inhibitors (Fig. 2) by means of docking modeling using the GLIDE program of the SCHRODINGER package. Then, selected structures will be optimized employing MD simulations and quantum mechanics calculations using the AMBER, GROMACS, GAUSSIAN and JAGUAR program packages. The promising candidates with the best properties predicted will be synthesized and tested their biological activity to human GM and LM. In the final refinement cycle, potent inhibitors tested will be futher analyzed and develop by chemoinformatic programs of the SCHRODINGER package.

Organic synthesis. The selected mannosidase inhibitors will be synthesized by a working group of Dr. Siriwardena at University of Jules Vernes at Amiens, which co-operates with a crystallography group of Dr. Rose from the Cancer Institute and Department of Medicinal Biophysics at University of Toronto. The proposed structures of two types of the inhibitors are schematically drawn in Fig. 2. Proposals of routes of their syntheses are worked out by the group of Dr. Siriwardena (they are not open to the public at this time because of the IP protection).

Molecular cloning , expression and purification of human Golgi α mannosidase II. Our primary target in testing of specific inhibitors will be medial human GM, the enzyme involved in protein glycosylation pathway. Previous hydropathy analysis of human enzyme indicate that human GM is a type II transmembrane protein with short cytoplasmic and transmembrane domains, which are not required for the enzyme catalytic activity. Therefore, for our purpose we will engineer gene encoding human GM preferentially for the production of its soluble form (N-terminally truncated enzyme lacking cytoplasmic and hydrophobic transmembrane domain). mRNA sequence encoding human GM is deposited in GeneBank database (accession number U31520, 3599 bp mRNA with ORF 1144 amino acid residues), thus providing information to design the specific oligonucleotides for engineering of coding region by means of PCR. The cDNA encoding human GM will be synthesized from poly (A+) RNA originated from human lymphocytes, or by amplification of cDNA library from human liver (Stratagene).[20] The non-lytic *Drosophila melanogaster* expression host system (DES)[21], or *Pichia pastoris* host (Invitrogen) will be used for production of recombinant enzyme in significant quantity and purity.

The PCR derived DNA fragment, encoding truncated form of human GM - Δ*32* will be confirmed by automated DNA sequencing and subcloned into DES vector pMT BiP His-J/V downstream of polyhistidine affinity tag and enterokinase site headed by BiP secretion signal. Transiently secreted recombinant enzyme will be purified from cultured supernatant by means of chelating sepharose charged with Ni^{2+} ions, according to the previously described protocol.[22] Protein integrity will be analyzed by SDS-PAGE and, if required, it will be blotted onto Hybon-C membranes and analyzed with antibodies directed against the protein affinity tag. Protein concentration will be determined by the Bradford method with the Bio-Rad Protein Assay kit (Bio-Rad), using bovine serum as a standard. *Pichia pastoris* expression system as an alternative host for preparation of recombinant enzyme will be employed in the case of low protein expression yield in DES.

Activity assay: measurements of the mannosidase activity will be carried out according protocol Raboille et al[23] using *p*-nitrophenyl α-D-mannopyranoside (Sigma) as a substrate. The potency and specificity of synthesized inhibitors will be expressed by calculating of IC_{50} and inhibition constants (K_i) for the purified recombinant enzyme using in principle protocol for enzyme assay in the presence of inhibitor analogs.

In order to study selectivity of inhibitory action we will also perform measurements toward human LM. For the purpose of recombinant enzyme preparation we will follow protocol from Liao et al[24] describing cloning, expression and purification of functional acid mannosidase (3.0 kb) using data deposited in Genebank under accession number U 68567.

4. Professional quality of research team, management of the team, infrastructure:

The working team is constituted of experts from different research fields: molecular modeling, organic synthesis and molecular glycobiology. All participants except for organic chemists are

affiliated at the Institute of Chemistry of Slovak Academy of Sciences and were chosen according their professional experience and results required by tasks of this multidisciplinary project. The organic chemists are affiliated at University of Jules Vernes, Faculty of Sciences, Laboratory of Saccharides, Amiens, France. Six researchers from the Institute of Chemistry will participate on the project: 1 leading senior scientists with DrSc. degree, 2 with PhD degree, 2 postgraduate students and 1 technician.

Institute of Chemistry of the Slovak Academy of Sciences, founded in 1953, is internationally recognized research establishment specialized in glycochemistry and glycobiology. Main scientific achievements are related to synthesis of biologically important saccharides, structure and functional properties of plant and microbial polysaccharides, their applications in biotechnology and medicine, structure, function, and mechanism of action of industrially important glycanases and glycosyltransferases and physiological role of carbohydrates in plant development and differentiation. The Institute has the following facilities that might be used by the project team: GC-MS Mass spectrometer, Finnigan, MALDI IV-Kratos, GC chromatograph, Hewlett-Packard, NMR spectrometer, 300 MHz, Bruker, Member of a consortium with 600 MHz NMR spectrometer (from 2004), UV spectrometer, Nicolet, Micro-analyzer, Fissons, Amino acids analyzer, HPLC chromatograph, five 8- and tree 4-processor Linux computers. Furthermore, Institute of Chemistry is equipped for insect cell culture, protein purification and molecular biology. An extensive selection of equipments for centrifugation, ultrafiltration, lyophilisation, electroforesis, blotting and low pressure chromatography is available.

Mgr. Juraj Kóňa, PhD., the principal investigator, has 8 years experience in the applications of computational methods (force field and quantum mechanics methods) on problems in organic chemistry (mechanisms of nucleophilic substitutions), biochemistry (catalytic processes of glycosyltransferases, peroxidases and aspartic proteases) and biophysics (transportation of potassium ions through transmembrane channels, activation of OxyR transcription factor), structure-based drug design (irreversible epoxide inhibitors of HIV-1 protease), homology modeling and docking techniques. He is an expert in modeling of the mechanism of chemical reactions (localization of transition states and intermediates along reaction coordinates) by means of quantum mechanics and hybrid quantum/classical mechanics methods. Experienced in molecular dynamics (MD) simulations of biophysical processes (conformational changes in proteins and active sites of enzymes) and in use of advanced computational techniques for calculations of a potential of mean force (PMF) (nonequilibrium steered MD simulations, umbrella sampling MD simulations, weighted histogram analysis method (WHAM), free energy perturbation (FEP) calculations). Author of 8 scientific papers published in international scientific journals (*J. Org. Chem, J. Chem. Soc., Int. J. Quantum. Chem., Org. Biomol. Chem. etc.*), with 16 citations and 5 contributions in international conferences.

Ing. Igor Tvaroška, DrSc., the deputy of principal investigator has over 30 years experience in molecular modeling and structural studies of biomolecules. His main interest is focused on the structure-properties relationship of biomolecules; the interpretation of the nature of the stereoelectronic effects (anomeric, exo-anomeric, and gauche effects); the development of methods for the investigation of the three-dimensional shape of carbohydrates in solution; and a description of conformational properties of oligo- and polysaccharides in solution combining NMR measurements and molecular modeling; modeling of reaction mechanisms of

glycosylhydrolases and glycosyltransferases; determination of the transition state structures; the homology modelling of enzyme structures; design of the transition state inhibitors, QSAR, ADMET, structure-based drug design. Author of 120 scientific papers including 9 chapters in books, co-author of 5 US patents, over 190 other contributions, 23 invited lectures on international conferences; over 1600 citations.

RNDr. Ján Mucha, PhD., biochemist, in the last decade mostly involved in molecular glycobiology of higher eukaryotes. He acquired theoretical and practical skills in structure – function study of glycosyltransferases from different origin using complementary molecular – biology tools in protein engineering, heterologous expression in host cells and recombinant protein purification. He made a major contribution to the field of plant protein glycosylation by cloning and characterisation of the key glycosyltransferases involved in biosynthesis of *N*-linked oligosaccharides. Results from his scientific activities in this field were published in several publications of CC listed high rank journals. He is co-inventor of two patents registered in US and EU dealing with molecular-biology methodology applicable in the heterologous production of clinically important glycoproteins in plants.

Mgr. Stanislav Kozmon, postgraduate student - computational chemist, has 4 years experience in molecular modeling of carbohydrates, lipocarbohydrates and enzymes and calculations of physical and chemical properties of organic compounds with biological activity. His main interest is focused on studies of mechanisms of organic reactions as well as enzymatic reactions using hybrid QM/MM methods, and studies of conformation analysis of compounds with biological activity in solution using molecular dynamics simulations, enzyme homology modeling and docking analysis of enzyme-substrate interactions. He is coauthor of 3 scientific papers in CC listed journals.

Mgr. Peter Both, postgraduate student, during his short scientific career he was mostly involved in biochemistry and molecular biology of higher eukaryotes. He gained a practical and theoretical knowledge in engineering, heterologous expression and characterization of recombinant glycosyltransferases of different origin.

Dr. Aloysius Siriwardena, University of Jules Vernes, Faculty of Sciences, Laboratory of Saccharides (UMR 6219, 33, rue saint Leu, 80039 Amiens, a certificate enclosed), organic chemist with profound background in syntheses of saccharides and glycosidase inhibitors (http://www.u-picardie.fr/jsp/fiche_pagelibre.jsp?STNAV=&RUBNAV=&CODE=31540425&LANGUE=0).

5. Outcomes and impacts of the project:

The following results are expected to achieve during this project:

a) The preparation of two 3D structures of the glycosyl hydrolases: the human GM and LM necessary for the structure-based design of the selective inhibitors.

b) The design of two novel inhibitors of human GM, analogs of swainsonine and mannostatin, using computer-assisted structure-based design.

c) The identification of active-site residues of human GM responsible for the biological activity and selectivity of the proposed inhibitors.

d) Syntheses of two novel selective inhibitors of human GM, analogs of swainsonine and mannostatin. Optimal synthetic routes will be developed for the syntheses of the most promising inhibitors.

e) The preparation of the recombinant human GM engineered for expression in *E. coli* system in amount sufficient for further assays.

f) Assays of synthesized inhibitors for their potency and selectivity against recombinant human GM and LM expressed by calculating of IC_{50} and inhibition constants (K_i)

g) Publications of results from the project in international scientific journals (We suppose to publish 2 articles per a year.)

h) International co-operation within the project with a working group of organic chemists of Dr. Siriwardena from University of Jules Vernes at Amiens.

i) Training in the field of design, development and testing novel glycoside inhibitors for 2 postgraduate students.

j) We will organize an advance course for postgraduate students of Institute of Chemistry in every year during the project, which will be also available in electronic form on the internet. Lectures and practices will focus on theory and applications in computer-assisted drug design.

6. Interaction between research and education:

a) Into the three-year project 2 postgraduate students, 2 computational chemist and 1 molecular biologist will be involved. Both students will have a chance to learn advanced techniques in the field of design, development and evaluation of biologically active compounds, namely, novel mannosidase inhibitors. They will learn to work in a multidisciplinary team, where an effective management and intensive communication among computational chemists, organic chemists and molecular biologists are are key factors for successful progress of the project goals.

b) The participated students will present their results at international conferences oriented on glycochemistry and computational chemistry organized in Europe within a period 2007-2009 (EUROCARB 14, EUROCARB 15, EUCO-CC7, EUCO-CC8, EUCO-CC9).

c) The principal investigator of the project will lecture an advanced course for the postgraduate students of the Institute of Chemistry. Lectures and practices will focus on theory and applications in the computer-assisted drug design. As a case study, results and experience of the project will be used for the above-mentioned lectures.

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