Published in final edited form as:

ChemMedChem. 2018 February 20; 13(4): 373–383. doi:10.1002/cmdc.201700607.

N-Benzyl substitution of polyhydroxypyrrolidines - the way to selective inhibitors of Golgi α -mannosidase II

Dr. Sergej Šesták^a, Dr. Maroš Bella^a, Tomáš Klunda^a, Dr. Soňa Gurská^b, Dr. Petr Džubák^b, Florian Wöls^c, Dr. Iain B. H. Wilson^c, Dr. Vladimír Sladek^a, Assoc. Prof. Marián Hajdúch^b, Dr. Monika Poláková^{*a}, and Dr. Juraj Kóna^{*a}

^aInstitute of Chemistry, Center for Glycomics, Slovak Academy of Sciences, Dúbravská cesta 9, 845 38 Bratislava, Slovakia

^bLaboratory of Experimental Medicine, Institute of Molecular and Translational Medicine, Faculty of Medicine and Dentistry Palacky University and University Hospital in Olomouc, Puškinova 6, 775 20 Olomouc, Czech Republic

^cDepartment of Chemistry, University of Natural Resources and Life Sciences, A-1190 Vienna, Austria

Abstract

Inhibition of biosynthesis of complex *N*-glycans in Golgi apparatus influence progress of tumor growth and metastasis. Golgi α -mannosidase II (GMII) has become a therapeutic target for a drug with anticancer activities. One critical task for successful application GMII drugs in medicine treatment is to reduce their unwanted co-inhibition with lysosomal α -mannosidase (LMan) by which suffer all known potent GMII inhibitors. A series of novel *N*-substituted polyhydroxypyrrolidines was synthesized and tested with modeled GH38 α -mannosidases from *Drosophila melanogaster* (GMIIb and LManII). The most potent structures inhibited GMIIb ($K_i = 50\text{--}76\text{ }\mu\text{M}$ as determined by enzyme assays) with a significant selectivity index of $\text{IC}_{50}(\text{LManII})/\text{IC}_{50}(\text{GMIIb}) > 100$. They also showed the inhibitory activity in vitro assays with MTS test towards cancer cell lines (leukemia, $\text{IC}_{50} = 92\text{--}200\text{ }\mu\text{M}$) and low cytotoxic activity towards normal fibroblast cell lines ($\text{IC}_{50} > 200\text{ }\mu\text{M}$). In addition, they did not show any significant inhibitory activity towards GH47 *Aspergillus saitoi* $\alpha 1,2$ -mannosidase. An appropriate stereo configuration of $-\text{CH}_2\text{OH}$ and benzyl functional groups on the pyrrolidine ring of the inhibitor may lead to an inhibitor with the required selectivity to the active site of a target α -mannosidase.

Keywords

pyrrolidines synthesis; swainsonine; Golgi mannosidase II; cytotoxicity; molecular modeling

monika.polakova@savba.sk (M. Poláková), juraj.kona@savba.sk (J. Kóna).

This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). This work is currently citable by using the Digital Object Identifier (DOI) given below. The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

Introduction

Swainsonine **1** [(1*S*,2*R*,8*R*,8*aR*)-trihydroxy-indolizidine] is an alkaloid naturally occurring in a number of Australian and North American plants.[1] It was found that it interferes with the glycosylation pathway where it specifically inhibits glycoside hydrolases from the GH family 38.[2] Swainsonine has attracted attention as a potential anticancer agent as it inhibits tumor growth and metastasis, augments natural killer and macrophage-mediated tumor cell killing, and stimulates bone marrow cell proliferation.[2g, 3] The effect of swainsonine on oligosaccharide processing was elucidated [2b, 2d, 2f, 4] as it binds reversibly to the active site of Golgi α -mannosidase II (GMII) (EC 3.2.1.114)[5] and modulates *N*-glycoprotein synthesis resulting in the accumulation of hybrid type of *N*-glycans.[2e, 6] Clinical trials of short duration have demonstrated that swainsonine has a low toxicity in humans and is well tolerated.[2g, 7] On the other hand, swainsonine and all other known α -mannosidase inhibitors have an additional serious side effect that potentially precludes their use as therapeutics. These compounds also inhibit the broad-specificity lysosomal α -mannosidase (LMan) (EC 3.2.1.24, GH family 38)[8] resulting in the accumulation of mannose-containing oligosaccharides in tissues, serum and urine.[2g, 4]

To apply swainsonine-mechanism-based compounds as therapeutics, selective inhibitors of GMII with minimized side effect to LMan are required. However, the potent inhibitors of GMII exhibited negligible or no selectivity.[5a, 5f, 9] It was observed that 3- and 5-substituted swainsonine derivatives exhibited a weak selectivity towards GMII [$IC_{50}(\text{LMan})/IC_{50}(\text{GMII}) < 20$] at the nanomolar and micromolar levels, while 6- and 7-substituted derivatives exhibited even poorer activity and selectivity at the micromolar and millimolar levels.[5f, 10] Some polyhydroxypyrrolidine derivatives also showed weak selectivity.[9a, 9b]

The active site of *Drosophila melanogaster* Golgi α -mannosidase II (dGMII) is made up of three sugar-binding sites: the catalytic, holding, and anchor subsites.[5a, 5b, 11] dGMII needs Zn^{2+} ion co-factor for catalytic activity which resides at the bottom of the catalytic subsite.[5b] Zn^{2+} ion is in octahedral coordination with four amino acid residues (side chains of His90, Asp92, Asp204, His471) and with an inhibitor (bidentate ligand). All potent dGMII inhibitors have to bind properly to the bottom of the active site by interaction with Zn^{2+} and neighboring amino acid residues otherwise the potency of the inhibitor is dropped dramatically.[9a, 9b, 9e, 9f, 9i-k, 10c, 12] The critical role of Zn^{2+} ion and aspartic acid residues of the catalytic subsite for the interactions with the dGMII inhibitors was recently confirmed by quantum mechanics calculations.[13] From crystal structures of available fruit fly dGMII[5b, 11a] and bovine bLMan[8a] it is evident that the active site of both enzymes are structurally and chemically almost identical in radius of 10 Å around Zn^{2+} ion co-factor. This is one of reasons why structurally small potent GMII inhibitors like swainsonine inhibit both enzymes effectively with no significant selectivity observed. Thus, our strategy in the design of a selective GMII inhibitor was based on a previous proposal,[5a, 12c] and consists of two basic points: (i) design of the core unit of the inhibitor (key interactions with catalytic subsite which is identical in both dGMII and bLMan); and (ii) design of structural linker (specific interactions with holding or anchor subsites of dGMII which are missing in bLMan).[5a, 8a, 11a] For these purposes, polyhydroxypyrrolidines with *N*-substitution were

prepared. It is known that certain polyhydroxypyrrolidines derived from natural 1,4-dideoxy-1,4-imino-D-mannitol (the structure **24**), an azafuranose analogue of mannose, are inhibitors of both GMII and LMan at the micromolar or millimolar level with a weak selectivity index to GMII observed for some *N*-substituted derivatives [9a, 9b] (for more details about the design of the structure of the inhibitors see the section Molecular modeling). Our research group has recently published a series of articles with mannose derivatives to find a structural feature of the inhibitor with impact on selectivity.[9p-r] These studies revealed that a selective GMII inhibitor should have a linker of the benzyl type and modified 6-OH functional group. Structural design was further developed and the present paper reports a study with six novel *N*-arylalkyl substituted pyrrolidines from which, to the best of our knowledge, some of them are the first GH38 inhibitors with a significant selectivity index of $IC_{50}(LManII)/IC_{50}(GMIIb) > 100$ (for more details on the modeled GH38 enzymes, LManII and GMIIb, see the section Enzyme assays – experimental).

Results and Discussion

In the following subsections, the results from synthesis, biological assays and molecular modeling are described and discussed in connection with the selected Golgi and lysosomal enzymes from the GH family 38 which are abbreviated as follows: GMII (human Golgi α -mannosidase II),[9a, 9b] dGMII (fruit fly Golgi α -mannosidase II, the homologue of mammalian Golgi α -mannosidase II),[5b, 14] GMIIb (fruit fly Golgi α -mannosidase II with some distinct differences in comparison to other invertebrates and vertebrates),[15] LMan (human lysosomal α -mannosidase),[8b, 9a, 9b] dLMan (fruit fly lysosomal α -mannosidase, CG6206 gene product),[5f] LManII (fruit fly lysosomal α -mannosidase, one of the acidic class II mannosidases),[15] JBMan (Jack bean α -mannosidase, the plant homologue of the acidic class II mannosidases)[16] and bLMan (bovine lysosomal α -mannosidase).[8a] The fruit fly GMIIb, LManII and JBMan were used for enzymatic assays in this study and our previous studies:[9p-r] GMII, dGMII, LMan, dGMII and JBMan in other previous studies, [5f, 9a-o] while dGMII and bLMan are the enzymes with available crystal structures.[5a, 5b, 8a, 12c, 17]

Synthesis

Computer-assisted design[18] was used to predict a core structure and a selectivity structural linker of the dGMII inhibitor (for more details see also the section Molecular modeling). The synthesis of target compounds started from 2,3-*O*-isopropylidene derivatives **2** and **3** which were prepared from D-ribose according to the previously published procedure (Scheme 1).[19]

Highly efficient reductive ring opening of the lactols **2** and **3** with NaBH₄ was performed in EtOH providing corresponding diols **4**[20] and **5**. Standard mesylation of diols **4** and **5** led smoothly to dimesylated derivatives **6**[20] and **7**. The cyclization of the dimesylate **6** with benzyl amine to access fully protected 1,4-imino-L-lyxitol **8** was conducted previously in refluxing toluene within 24 h.[20] Another substrate very similar to **7** was cyclized to 1,4-imino-L-lyxitol in neat benzyl amine under reflux for 18 h.[21] However, optimization of reaction conditions showed that optimal reaction time and temperature for the cyclization of

dimesylates **6** and **7** in neat benzyl amine were 7 h at 120 °C. In addition, the work-up procedure was significantly simplified by extraction of excess BnNH₂ with cold 0.5M citric acid[22] instead of its tedious removal by evaporation. Acid-sensitive protective groups were stable under the condition used and the ring closures were performed on a gram scale (~ 3.5 g, yield 87%). Simultaneous removal of isopropylidene and trityl/silyl protective groups from **9** under acidic conditions (6M HCl/MeOH) afforded *N*-benzyl pyrrolidine **10** in moderate yield. In addition, debenzylation of **10** under catalytic hydrogenation conditions followed by treatment with diluted HCl provided the core pyrrolidine **11**[21] as its hydrochloride (Scheme 1).

Following the optimized conditions for the cyclization reaction, pyrrolidines **12-14** were prepared from dimesylate **7** and the corresponding benzyl amine. Simultaneous hydrolysis of trityl and acetonide protecting group furnished target pyrrolidines **15-17** in moderate yields (Scheme 2).

Another approach based on the removal of *N*-benzyl group from **8** under standard conditions (H₂, Pd/C in methanol) followed by *N*-benzylolation of the resulting pyrrolidine **18** with *p*-iodo- or *p*-bromobenzyl bromide gave pyrrolidine **19** and **20** in 89% and 74% yield, respectively. Their treatment with 6M HCl in methanol afforded target pyrrolidines **21** and **22** (Scheme 3).

Biological assays

A series of seven polyhydroxylated pyrrolidines **10**, **11**, **15-17**, **21** and **22** was evaluated towards the class II α -mannosidases (GH family 38) GMIIb, LManII and JbMan and the class I α -mannosidases (GH family 47) AspMan. All *N*-substituted pyrrolidines showed inhibitory activity towards the target enzyme, GMIIb, with IC₅₀ values in the range from 52 μ M to 770 μ M (Table 1).

However, unsubstituted pyrrolidine **11** exhibited only moderate inhibitory activity (IC₅₀ = 270 μ M). Among the *N*-alkylated pyrrolidines, phenethyl derivative **15** and pyridin-4-ylmethyl derivative **17** were found to be the least potent inhibitors in comparison with *N*-(4-substituted benzyl) analogues **10**, **16**, **21** and **22**. The latter two *N*-(4-halobenzyl)pyrrolidines **21** and **22** were the most potent GMIIb inhibitors with IC₅₀ values 52 and 55 μ M, respectively. These data suggest that *N*-alkylation plays an important role in the inhibitory activity of the *N*-substituted polyhydroxylated pyrrolidines. For the compounds **10**, **21** and **22** with the lowest IC₅₀ values, their inhibition constants were also determined (Table 1). It was observed that *K_i* values were essentially the same as IC₅₀ values, not differing more than by 10%.

A selective GMII inhibitor is required to exhibit none or significantly reduced inhibitory activity towards LMan. All tested polyhydroxylated pyrrolidines were found to be weak LManII inhibitors with IC₅₀ values at the millimolar level (IC₅₀ in range of 1.2 mM to >8mM). *N*-Substituted derivatives were slightly weaker inhibitors of LManII in comparison with the parent pyrrolidine **11**. It is noteworthy that *N*-phenethyl derivative **15** showed approximately 3-fold higher inhibition capacity than **11**, suggesting that two-carbon spacer

between pyrrolidine and benzene ring is a preferred arrangement for LManII inhibition. Thus, it is not the suitable structural fragment of an effective GMIb inhibitor.

Based on the IC_{50} values for LManII and GMIb, a selectivity index was calculated for each derivative as a ratio of $IC_{50}(\text{LManII})/IC_{50}(\text{GMIb})$. The most potent inhibitors of GMIb **21** and **22** had the highest selectivity index 117 and 136, respectively. *N*-Benzyl pyrrolidine **10** exhibited a slightly lower index of 80. To the best of our knowledge, these structures are the first known GH38 inhibitors with the significantly high selectivity index since all known GH38 inhibitors had the selectivity index <20 (for some example see **23**, **25** and **26** in Table 1) insufficient for clinical treatments. The selectivity index of **16** was also satisfactory and higher than that of the weakest GMIb inhibitors **17** and **11**. On the contrary, the significantly reduced selectivity was observed for *N*-phenethyl derivative **15**. It seems that a length of hydrocarbon chain between pyrrolidine unit and phenyl function is a crucial factor for retaining the selectivity of the GMIb inhibitors. The similar inhibitory properties were also observed in our previous study with sulfur-containing α -D-mannopyranosides having the phenylalkyl functional group.[9p]

As published previously [5f] swainsonine **1**, the nanomolar inhibitor of both GMII and LMan, gave an extremely poor selectivity index of 2. A substitution at the position 5 in its analogue **23** led to an increase in the selectivity index to a value of 11. However, a swainsonine derivative with the benzyl functional group itself at the position 5, which could mimic the inhibitor **10** with the high selectivity index, is yet to be described. For such a structure another increase of the selectivity index could be expected to maintain its potency at the nanomolar level. The core structure of the pyrrolidine inhibitors presented here is closely related to the structure of natural 1,4-dideoxy-1,4-imino-D-mannitol **24**, namely its *N*-benzyl derivative **25** (Table 1). When the potency and selectivity of **24** is compared to the inhibitor **11**, the deletion of $-\text{CH}(\text{OH})-\text{CH}_2\text{OH}$ moiety in **24** and the additional attachment of $-\text{CH}_2\text{OH}$ at the opposite site of the ring in **11** led to a dramatic decrease in potency towards LManII (from 13 μM to 7500 μM) and only to a slight decrease in potency towards GMIb (from 100 μM to 270 μM) resulting to the required selectivity index. Similarly, when the potency and selectivity of the *N*-benzyl derivatives **25** and the inhibitor **10** are compared, the benzyl function further improved the potency and selectivity (in case of **10**) and only the selectivity (in case of **25**). These results suggest that a combination of an appropriate stereo configuration of $-\text{CH}_2\text{OH}$ and benzyl functional groups on a mannose mimicking core (polyhydroxylated pyrrolidine ring) may lead to an inhibitor with the required selectivity for the active site of GMIb.

To verify the high selectivity index and weak inhibitory properties of our pyrrolidine series towards LManII, the inhibition assay was also measured with the α -mannosidase from *Canavalia ensiformis* (Jack bean) (JBMan) (EC 3.2.1.24, GH family 38), widely used as a model for acidic α -mannosides.[2b, 5f, 9e, 9q-r, 10, 12a-b] This assay provided similar results as compared to LManII (Table 1). All tested structures did not inhibit JBMan at the 2mM concentration of the inhibitor except for **24**. These results further support the validity of the selectivity index of the *N*-benzyl polyhydroxylated pyrrolidines. Since it is known[2b] that **24** and its pyrrolidine analogues are also strong inhibitors of class I α -mannosidases, further verification of the selectivity index of our compounds (**10**, **11**, **16**, **21**, **22**) towards

GMIIB was performed in an enzymatic assay with *Aspergillus saitoi* α -1,2-mannosidase (AspMan) (EC 3.2.1.113, GH family 47). All tested structures, except for unsubstituted derivative **11** ($IC_{50} = 1$ mM), did not inhibit AspMan at the 1-2 mM concentration of the inhibitor. These results indicate that the most selective inhibitors (**10** and **21**) may maintain their specificity towards Golgi α -mannosidases with no inhibitory effects towards acidic lysosomal and endoplasmic reticulum α -mannosidases. Since the employed model GMIIB from fruit fly showed some distinct biochemical differences in comparison to mannosidase III or mannosidase II^x from other invertebrates and vertebrates,[15] additional enzymatic assays with Co²⁺ independent forms of Golgi mannosidase II, GMII, are required for any future refinements of inhibitor design.

Compounds **1**, **10**, **15**, **16**, **21**, **22** were tested *in vitro* for their cytotoxic activity on an eight cancer cell lines: A549 (human lung adenocarcinoma), CCRF-CEM (T-lymphoblastic leukemia), CEM-DNR (T-lymphoblastic leukemia, daunorubicin resistant overexpressing the LRP protein), K562 (acute myeloid leukemia), K562-TAX (acute myeloid leukemia, overexpressing the P-glycoprotein), HCT116 (human colorectal cancer with wild-type p53), HCT116p53^{-/-} (human colorectal cancer with deleted p53), U2OS (Human Bone Osteosarcoma Epithelial Cells) and on the two non-malignant cell lines BJ (human fibroblast) and MRC-5 (human lung fibroblasts). Cytotoxic activities are presented in Table 2.

In general, cytotoxic activities were observed in high concentrations, and none of the tested compounds were cytotoxic to the non-malignant cell lines BJ and MRC, and solid tumor cell lines HCT116, HCT116p53^{-/-} and A549. In opposite, all compounds were cytotoxic to chemosensitive CCRM-CEM acute lymphoblastic cell line as well as resistant line CEM-DNR with IC_{50} in range of 87-132 μ M. The pyrrolidines **10**, **16** and **21** showed slightly higher cytotoxicity for CEM-DNR compared to the non-resistant CCRF-CEM. A similar trend was also observed for the cell line K562 and its resistant counterpart K562-TAX. In addition, pyrrolidine **22** and swainsonine **1** did not inhibit non-resistant K562 at all. The tested *N*-substituted pyrrolidines showed slightly higher cytotoxicity as swainsonine **1** (90 – 130 μ M versus 110 – 140 μ M) indicating that the pyrrolidine structures and the standard **1** may have similar pharmacokinetics properties.

Molecular modeling

The main goals of molecular modeling were to design of a selective GMII inhibitor, and to explain observed selective binding to GMII of the synthesized polyhydroxypyrrolidines. In proposals of new inhibitors computational design was combined with available experimental data of known GH38 inhibitors. We knew from our previous studies [9p, 9r] that appropriate attachment of the benzyl functional group and deletion of 6-hydroxy group on the mannose core of the inhibitor increased selectivity of the inhibitors towards GMIIB. Because the mannoside inhibitors showed only a weak inhibition at the millimolar level, a new core structure of the selective inhibitor had to be designed. The polyhydroxypyrrolidine ring was selected based on structural analogy with α -mannosidase inhibitors swainsonine and 1,4-dideoxy-1,4-imino-D-mannitol. Positions and stereo configurations of functional groups (hydroxy, hydroxymethyl and benzyl) were optimized by molecular docking. The proposed

structures (compounds **10**, **21** and **22**) were further refined by QSAR models with quantum mechanics interaction energy descriptors developed in our laboratory.[18] Other compounds (**11** and **15-17**) synthesized in this paper were not proposed by molecular modeling. They were prepared to compare their potency and selectivity with the proposed structures **10**, **21** and **22** as well as to understand an interaction pattern for selective binding to GMIIb of the proposed inhibitors.

To understand the observed selectivity towards GMIIb of the polyhydroxypyrrolidines we focused on two factors which would influence differential binding of inhibitors to GMIIb and LManII (with GMII and LMan as surrogates with known 3D structures); i) possible different ionization states of amino group of the inhibitors and active-site amino acids; and ii) quantum mechanics effects between the inhibitor and active-site amino acids of the enzyme. For this purposes pK_a calculations in water and in both enzymes, docking, hybrid QM/MM geometry optimizations and interaction energy FMO-PIEDA calculations were performed.

pK_a calculations—Polyhydroxypyrrolidines contain an amino functional group incorporated in a ring moiety of the inhibitor. The pK_a values of such amino group may range from 5 to 9 depending on the position of the nitrogen atom in the ring as well as on other structural factors (number and position of hydroxyl groups in the ring, the conformation of the ring, chemical external environment of the amino group, etc.).[24] pH of GMII in Golgi apparatus is about 6 while pH of LMan is more acidic (4.5-5.0) (for more details about pH of enzymatic assays see the section 4.2. Enzyme assays). Thus, inhibitors with a pK_a value of 5-6 units could bind to GMII in neutral form (as an amine) and to LMan in ionized form (as an ammonium cation). This could induce different interaction strength and binding mode as was proposed recently.[13] The calculated pK_a values of the synthesized inhibitors in aqueous solution and in both enzymes are compiled in the Table 3.

The values in aqueous solution are close to a value of 7 being in the range from 6.3-7.9. This indicates that some inhibitors may prefer protonated form and some neutral ones in aqueous solution. For comparison, there are also pK_a value calculated[13] for swainsonine **1** and pyrrolidine **24**, for which also experimental pK_a value are available.[24a] In the case of dGMII, the inhibitors prefer protonated forms except for **11**, **1** and **24**. In contrast, for the 'acidic' bLMan, all inhibitors prefer protonated forms with pK_a values being in the range from 5.9 to 8.2. In case of the neutral form of the inhibitors the catalytic acid Asp341 in dGMII (and Asp319 in bLMan) is in direct contact with the inhibitor, the neutral (–COOH) form would also be favoured, while for the protonated form of the inhibitors, the ionized form (–COO[–]) of this Asp residue is preferred. Interestingly, the adjacent Asp340 residue in dGMII (Ser318 in bLMan) changed its pK_a value depending on the protonation states of Asp341 and the inhibitor. Although these results do not explain a connection between measured IC₅₀ of the inhibitors and their protonation states, pK_a calculations clearly indicate that in the active sites, namely in dGMII, there is a balance between protonation states of the inhibitor and some active-site ionizable amino acids. This balance may vary between inhibitors and be dependent on the binding mode, thereby influencing the potency and observed selectivity of the pyrrolidine inhibitors synthesized in this study.

Molecular docking—By molecular docking it was found that polyhydroxypyrrolidines bind to the active site of both dGMII and bLMan in a similar manner as swainsonine (Figure 1). However, the pyrrolidine ring of our compounds is shifted away from Asp204 and the Zn^{2+} ion as compared to the indolizidine ring of the swainsonine. In Figure 1a there is in superposition of the bound swainsonine (from the crystal structure with ID PDB: 3BLB[5f, 17]) with the computationally docked compound **21** in the active site of dGMII. Probably due to a steric effect of hydroxymethyl moiety of **21**, its pyrrolidine ring may not bind so tightly to the bottom of the active site. For example, the distances between the oxygen of Asp204 and the ring nitrogen of the inhibitors are 2.75 Å (for swainsonine) and 3.51 Å (for **21**). The different position of the amino groups of both analyzed inhibitors may induce different interactions with the active-site residues, namely with Asp204, Asp341 and Zn^{2+} , and influences binding affinities and pK_a values of the pyrrolidine inhibitors.

A superposition of the docked compound **21** in the active site of GMII and bLMan clearly indicates the similar binding mode of both pyrrolidine and benzyl moieties (Figure 1b).

FMO – PIEDA binding analysis—The molecular system under study via the FMO method consisted of 24 fragments including the ligand and the structural water which is always present in the dGMII cavity[5f] for these ligands (for more details see the Experimental section - Molecular modeling). Each fragment corresponded to one of the following amino acids residues: His90, Asp92[−], Trp95, Asp204[−], Phe206, Arg228⁺, Tyr267, Ser268, Tyr269, Asp270[−], Asp340[−], Asp341, Tyr407, Asp409[−], Trp415, His470, His471, Asp472[−], Tyr727, Arg876⁺, Gly877, H₂O, Zn^{2+} , and the ligand. Since, as discussed earlier, the main source of GH38 specificity is expected to result from the different binding of the substituent groups attached on the pyrrolidine core. It therefore appears useful to point out residues of the binding site that interact with different parts of the pharmacophores. Firstly, the core structure which is essentially identical to the unsubstituted ligand **11** interacts predominantly with the charge negative residues Asp92, Asp204, Asp472, the charge neutral Tyr727 and Phe206 and the charge positive Zn^{2+} ion. Secondly, more specifically, the interaction of the side-chain $-\text{CH}_2-\text{OH}$, which is present also in **11**, is mediated through Asp341, Tyr269 and Asp340. Lastly, the residues in direct interaction with the benzyl linker are Arg228, Arg876, H₂O, Trp415 and Tyr267.

The strongest attractive forces (energies) stem from the Coulomb electrostatic interactions with charged residues. The E_{es} is around -90 to -150 kcal mol^{−1} between the ligand and Asp92 and Asp204. E_{es} is below -150 to -220 kcal mol^{−1} for the interaction with Asp472. This observation is in good qualitative and quantitative agreement with the results for swainsonine by Sladek et al.[13] It is noteworthy that the interaction with Asp204 is considerably weakened if substituents are introduced into the unsubstituted structure **11** (Table S1 of Supplementary data). E_{es} drops from about -156 kcal mol^{−1} in **11** to some -90 to -110 kcal mol^{−1} for the substituted derivatives. Furthermore, the charge transfer + polarisation term $E_{\text{ct+mix}}$ of all studied ligands is the largest for **11**. Both may be easily rationalised if one considers the difference in the Asp204...NH-ligand separation being ~ 1.6 Å for **11** and ~ 2.5 Å for the remaining ligands. It is due to the steric hindrance of the bulky substituent groups that prevent the ligand from approaching Asp204 much closer.

The main repulsive contribution is from the charge positive Arg228 and Arg876 with E_{es} around 50 and 30 kcal mol⁻¹, respectively. Zn²⁺ is a special case - its total interaction with the ligand is also repulsive. However, it is due to the exchange energy term E_{ex} rather than E_{es} . This can also be interpreted in the same way as for swainsonine,[13] since the interaction with the diol group on the ligand appears to be essential for successful binding in the enzyme. Even though Arg228 and Arg876 are in Coulomb repulsion with the ligand, the benzyl ring exhibits non-negligible dispersion interactions with both residues, which we believe to be important in the stabilization within the enzyme cavity. The E_{disp} with Tyr269 is in all cases around -5 kcal mol⁻¹, which is somewhat more than for swainsonine[13]. Similarly, E_{disp} interaction of **11** with Phe206 is stronger than for swainsonine with some -15 kcal mol⁻¹. The remaining ligands show E_{disp} interaction with Phe206 around -6 to -9 kcal mol⁻¹. The largest E_{disp} originates in the interaction with Trp95 with some -30 to -40 kcal mol⁻¹.

Conclusions

Using available experimental data and computational modeling the structures of the selective inhibitor of fruit fly Golgi α -mannosidase IIb with minimal side-effects towards fruit fly lysosomal α -mannosidase II were designed, synthesized and tested in enzyme and *in vitro* cell line assays. It was found that two functional groups, namely benzyl and hydroxymethyl functions in appropriate stereo configurations, at the core ring of the inhibitor can dictate potency and different binding to the target and side-effect enzymes. It can be concluded that *N*-benzylation of the core polyhydroxylated pyrrolidine unit leads to increased IC₅₀ (and K_i) as well as a selectivity index towards GMIb about 3-fold in comparison with the non-substituted **11**. Further increasing of the potency and the selectivity index can be reached by appropriate substitution at the position 4 on the benzene ring where **21** and **22** had the significant selectivity index >100. Compared with known GMII, dGMII and GMIb inhibitors, as natural swainsonine (**1**) or 1,4-dideoxy-1,4-imino-D-mannitol (**24**), the structures **10**, **21** and **22** are the first known selective GMIb inhibitors with minimal side effect towards the lysosomal α -mannosidase II. Since their potency is several orders of magnitude lower than that of swainsonine, further structural refinements will be required to obtain the selective and sufficiently potent inhibitor. Also, additional enzymatic assays with human GMII will be necessary to validate the high selectivity index found for **10**, **21** and **22** in assays with GMIb. The observed cytotoxic activity of the tested compounds in 72-hour MTS assay is rather low, demonstrating low toxicity on non-tumor cell lines, on the other side is hard to predict its real antitumor potential by the simple cytotoxic test. Such activity should be more likely monitored by synergistic assay with conventional antitumor drugs as doxorubicin, 5-FU or in combination with X-ray irradiation and irinotecan as was published previously for swainsonine and tunicamycin.[25] Experiments with similar design might be a valuable tool in the identification of the real therapeutic potential of GMIb inhibitors in future.

Based on the results from molecular modeling the potency and the selectivity of the synthesized pyrrolidines might be influenced by the binding position of the pyrrolidine ring (the position of the ring nitrogen to Zn²⁺ and other active-site amino acid residues) and its protonation state giving different interactions with the key active-site residues (Zn²⁺,

Asp341, Asp204, Asp92 and Asp472 in case of dGMII) as was demonstrated in our previous work.[13]

Experimental Section

General chemistry

TLC was performed on aluminium sheets precoated with silica gel 60 F₂₅₄ (Merck). Flash column chromatography was carried out on silica gel 60 (0.040-0.060 mm, Merck) with distilled solvents (hexanes, ethyl acetate, chloroform, methanol). Dichloromethane was dried (CaH₂) and distilled before use. All reactions containing sensitive reagents were carried out under an argon atmosphere. ¹H NMR and ¹³C NMR spectra were recorded at 25 °C with Varian VNMRs 400 MHz, Bruker AVANCE III HD 400 and Bruker AVANCE III HDX 600 spectrometers. Chemical shifts are referenced to either TMS (δ 0.00, CDCl₃ for ¹H) or HOD (δ 4.87, CD₃OD for ¹H), and to internal CDCl₃ (δ 77.00) or CD₃OD (δ 49.00) for ¹³C. Optical rotations were measured on a Jasco P2000 polarimeter at 20 °C. High resolution mass determination was performed by ESI-MS on a Thermo Scientific Orbitrap Exactive instrument operating in positive mode.

The lyxitol **10**, **11**, **15**, **16**, **17**, **21** and **22** used in biological tests were lyophilized before the use. *p*-Nitrophenyl α-D-mannopyranoside (*p*NP-Man*p*) and jack bean α-mannosidase were purchased from Sigma; swainsonine from Calbiochem; *Aspergillus oryzae* α1,2-mannosidase from Prozyme.

Enzyme assays

Assays with Class II α-Mannosidases (GH family 38)—The isolation and purification of recombinant *Drosophila melanogaster* Golgi (GMIIb) and lysosomal (LManII) α-mannosidases was carried out as described by Nemová et al.[15] The α-mannosidase from *Canavalia ensiformis* (Jack bean) (JBMan) was purchased from Sigma. Mannosidase activity of these enzyme preparations were measured using *p*-nitrophenyl-α-D-mannopyranoside (*p*NP-Man; Sigma; 100 mM stock in dimethylsulphoxide) as a substrate at 2 mM final concentration in 50 mM acetate buffer at the relevant previously-defined optimal pH) (Jack bean α-mannosidase, JBMan, at pH 5.0, GMIIb at pH 6.0, and LManII at pH 5.2) and 0.5 μL of the enzyme (0.05 μg of protein for JBMan), in a total volume of 50 μL for 1-2 h at 37 °C. GMIIb was assayed in the presence of 0.5 mM CoCl₂.

The inhibitor was preincubated with the enzyme in the buffer for 5 min at room temperature and the reaction was started by addition of the substrate. The reactions were terminated with two volumes (0.1 mL) of 0.5M sodium carbonate and the production of *p*-nitrophenol was measured at 405 nm using a multimode reader Mithras LB943 (Berthold Technologies).

The average or representative result of three independent experiments made in duplicate is presented. The IC₅₀ value was determined with 2 mM *p*NP-Man. The *K_i* values were determined from Dixon plots of assays performed with *p*NP-Man at the indicated concentration (0.5-4 mM).

Enzyme assays with Class I α -Mannosidase (GH family 47)—As *p*NP-Man*p* is not a substrate for GH family 47 enzymes, a MS-based assay was developed using a 2-aminopyridine-labelled Man₅GlcNAc₂ *N*-glycan (Man α 1,2Man α 1,2Man α 1,3(Man α 1,6)Man β 1,4GlcNAc β 1, 4GlcNAc-PA; purified by RP-HPLC of the *N*-glycome of *Trichomonas vaginalis*.[26] Approximately 5 pmol of the *N*-glycan was incubated with 0.3 μ L (4 μ U) *Aspergillus saitoi* α 1,2-mannosidase in the absence or presence of 0.25–2.0 mM inhibitor, 50 mM ammonium acetate buffer (pH 5) in a total of 5 μ L; all assays for 1 or 2 hours at 37 °C. The reactions were heat inactivated for 5 minutes prior to MALDI-TOF MS (Bruker Autoflex Speed); the peak heights of the *m/z* 989, 1151 and 1313 [M+H]⁺ ions (i.e., corresponding to the substrate and products with one or two α 1,2-mannose residues removed) were integrated to estimate the conversion of substrate.

In vitro MTS assays

Cell lines—All cells (if not indicated otherwise) were purchased from the American Tissue Culture Collection (ATCC). The CCRF-CEM line is derived from T lymphoblastic leukemia, evincing high chemosensitivity, K562 represent cells from an acute myeloid leukemia patient sample with bcr-abl translocation, U2OS line is derived from osteosarcoma, HCT116 is colorectal tumor cell line and its p53 gene knock-down counterpart (HCT116p53^{-/-}, Horizon Discovery Ltd, UK) is a model of human cancers with p53 mutation frequently associated with poor prognosis, A549 line is lung adenocarcinoma. The daunorubicin resistant subline of CCRF-CEM cells (CEM-DNR bulk) and paclitaxel-resistant subline K562-TAX were selected in our laboratory by the cultivation of maternal cell lines in increasing concentrations of daunorubicin or paclitaxel, respectively. The CEM-DNR bulk cells overexpress MRP-1 and P-glycoprotein protein, while K562-TAX cells overexpress P-glycoprotein only. Both proteins belong to the family of ABC transporters and are involved in the primary and/or acquired multidrug resistance phenomenon.[27] MRC-5 and BJ cell lines were used as a non-tumor control and represent human fibroblasts. The cells were maintained in nunc/corning 80 cm² plastic tissue culture flasks and cultured in cell culture medium according to ATCC or Horizon recommendations (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% fetal calf serum, and NaHCO₃).

Cytotoxic MTS assay—MTS assay was performed at Institute of Molecular and Translational Medicine by robotic platform (HighResBiosolutions). Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (25000 – 35000 cells/mL based on cell growth characteristics). Cells were added by automatic pipettor (30 μ L) into 384 well microtiter plates. All tested compounds were dissolved in 100% DMSO and four-fold dilutions of the intended test concentration were added in 0.15 μ L aliquots at time zero to the microtiter plate wells by the echoacoustic non-contact liquid handler Echo550 (Labcyte). The experiments were performed in technical duplicates and three biological replicates at least. The cells were incubated with the tested compounds for 72 h at 37 °C, in a 5% CO₂ atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed by using the MTS test. Aliquots (5 μ L) of the MTS stock solution were pipetted into each well and incubated for additional 1–4 h. After this

incubation period, the optical density (OD) was measured at 490 nm with an Envision reader (Perkin Elmer). Tumor cell survival (TCS) was calculated by using the following equation: $TCS = (OD_{\text{drug-exposed well}} / \text{mean } OD_{\text{control wells}}) \times 100\%$. The IC_{50} value, the drug concentration that is lethal to 50% of the tumor cells, was calculated from the appropriate dose-response curves in Dotmatics software.[27]

Molecular modeling

Docking with Glide—The crystal structures of dGMII (PDB ID: 3BLB),[5f, 17] and bLMan (PDB ID: 1O7D) [8a] were used as 3-D enzyme models of human GMII and LMan for docking of synthesized pyrrolidines with the GLIDE program [28] of the Schrödinger package. Protonation states of amino acid residues of enzymes were calculated for the pH = 6.0 ± 1 (dGMII) and 4.5 ± 1 (bLMan) by the Protein Preparation Wizard of the Schrödinger package.[29] For docking with dGMII all molecules of water at the active site of dGMII were deleted except one (WAT1820, numbering according to PDB ID: 3BLB)[17]. This water has been shown to be conserved in crystal structures of dGMII either with intact substrates or inhibitors.[5f, 9d, 11a] In all docking calculations the catalytic acid (Asp341 in dGMII and Asp319 in the case of bLMan) was modelled in the protonated form in accordance with its catalytic role as a general acid. The receptor box for the docking conformational search was centred at the Zn^{2+} ion co-factor at the bottom of the active site with a size of $39 \times 39 \times 39$ Å using partial atomic charges for the receptor from the OPLS2005 force field except for the Zn^{2+} and side chains of His90, Asp92, Asp204, Arg228, Tyr269, Asp341 and His471 (analogous residues were selected for bLMan). For these structural fragments the charges were calculated at the quantum mechanics level with the DFT (Density Functional Theory) method (M06-2X) [30] using hybrid quantum mechanics/molecular mechanics (QM/MM) model (M06-2X/LACVP**:*OPLS2005*) with the QSite [31] program of the Schrödinger package. The grid maps were created with no Van der Waals radius and charge scaling for the atoms of the receptor. Flexible docking in standard (SP) precision was used. The partial charges of the ligands were calculated at the DFT level (M06-2X/LACVP**) using the Jaguar program [32] of the Schrödinger package. The potential for nonpolar parts of the ligands was softened by scaling the Van der Waals radii by a factor of 0.8 for atoms of the ligands with partial atomic charges less than specified cut-off of 0.15. The 5000 poses were kept per ligand for the initial docking stage with scoring window of 100 kcal mol⁻¹ for keeping initial poses; the best 400 poses were kept per ligand for energy minimization. The ligand poses with RMS deviations less than 0.5 Å and maximum atomic displacement less than 1.3 Å were discarded as duplicates. The post-docking minimization for 10 ligand poses with the best docking score was performed and optimized structures were saved for subsequent analyses using the MAESTRO [33] viewer of the Schrödinger package.

p*K*_a calculations—The p*K*_a values of ligands in water were calculated at the quantum mechanics level, including empirical corrections from the p*K*_a prediction module[34] of the Jaguar program[32] of the Schrödinger package. p*K*_a values of ligands bound in the active site of dGMII and bLMan were calculated at the empirical level considering an enzyme pH optimum of 6.0 (dGMII) and 4.5 (bLMan) using the PROPKA v.2 program.[35]

Quantum mechanics calculations—The geometries of complexes (GMII-inhibitor) were optimized at the hybrid QM/MM level using the meta hybrid Minnesota functional with doubled amount of nonlocal exchange (M06-2X) [30] with the Los Alamos national laboratory effective core potential (LACVP**) [36] basis set employing the Qsite program [31b] of the Schrödinger package. The QM part contained ligands, Zn^{2+} and the following active-site amino acids (side chains of His90, Asp92, Trp95, Asp204, Phe206, Arg228, Ser268, Tyr267, Tyr269, Asp270, Asp340, Asp341, Asp409, Trp415, His471, Asp472, Tyr727, Arg876, backbone of Gly877 and WAT1820). The MM part of the system (the rest of the enzyme) was treated with the OPLS2005 force fields.[37] The starting geometries were taken from molecular docking (see the section Docking with Glide).

The virtue of the Fragment Molecular Orbital – Pair Interaction Energy Decomposition Analysis (FMO-PIEDA) technique is to enable evaluation and analysis of pair interactions embedded within the electrostatic potential of the surroundings.[38] This method was successfully used to analyse the change of the binding motif of dGMII ligands when they bind in different protonation states.[13] In this study, we intended to explore its possible use in such way, that individual energy terms could be used in simple QSAR models. That is, to correlate any of the energy terms with experimentally assessed G values. The FMO – PIEDA allows the following energy decomposition

$$E_{\text{tot}} = E_{\text{els}} + E_{\text{exch}} + E_{\text{ct-mix}} + E_{\text{disp}}$$

Here E_{int} or E_{tot} is the total pair interaction energy, E_{els} is the electrostatic energy, E_{exch} stands for the exchange energy, $E_{\text{ct+mix}}$ is the charge-transfer + mixing energy (which is the not fully recovered polarization energy) and E_{disp} is the dispersion energy. E_{exch} is always repulsive as it represents the Pauli exchange term. The electrostatic/Coulomb term can be either positive or negative while the mixing and dispersion terms are negative, thus attractive and stabilizing the interactions. In practice E_{disp} is often used to explain ligand binding in enzymes.[39] We shall focus on E_{int} and E_{disp} . It should be noted that these calculations at the current state of implementation are not corrected for basis set superposition effect (BSSE).[40] Some authors argue this not being a problem at close-to-equilibrium separations of the interacting moieties.[41] General remarks on the FMO accuracy may be found elsewhere.[38b, 42]

FMO-PIEDA analysis was carried out in the GAMESS package [43] using the Def2-SVP basis sets.[44] For each residue (amino acid), its own fragment was assigned and all were in the same layer (i.e. same method applied). The single point calculations were done at the RI-MP2 level.[45] PIEDA background is documented elsewhere.[38a, 46] The HOP – Hybrid Operator Projection technique was adopted in the generation of fragments for the covalently bounded amino acids.[46b] Metal atoms in enzymes represent a challenge for QM description.[47] The approach regarding the zinc atom in dGMII was described earlier.[13]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by the Slovak Research and Development Agency (the project APVV-0484-12), Scientific Grant Agency of the Ministry of Education of Slovak Republic and Slovak Academy of Sciences (the project VEGA-2/0064/15), the Slovak State Programme Project No. 2003SP200280203 and the Research & Development Operational Programme funded by the ERDF (Amplification of the Centre of Excellence on Green Chemistry Methods and Processes, CEGreenII, Contract No. 26240120025) and the Austrian Science Fund (P29466). The MTS study was supported by grants from the Czech Ministry of Education, Youth and Sports (Grant numbers: LO1304, LM2015063) and IGA-LF-2017-030 (Palacky University). A part of the calculations were performed in the Computing Centre of the Slovak Academy of Sciences using the supercomputing infrastructure acquired in project ITMS 26230120002 and 26210120002 (Slovak infrastructure for high-performance computing) supported by the Research & Development Operational Programme funded by the ERDF.

References

- [1]. Colegate SM, Dorling PR, Huxtable CR. *Aust J Chem.* 1979; 32:2257–2264.
- [2]. a) Dorling PR, Huxtable CR, Colegate SM. *Biochem J.* 1980; 191:649–651. [PubMed: 6786280] b) Moremen KW. *Biochim Biophys Acta Gen Subj.* 2002; 1573:225–235. c) Tulsiani DR, Harris TM, Touster O. *J Biol Chem.* 1982; 257:7936–7939. [PubMed: 6806288] d) Cenci di Dello I, Dorling P, Winchester B. *Biochem J.* 1983; 215:693–696. [PubMed: 6419731] e) Tulsiani DR, Touster O. *Arch Biochem Biophys.* 1983; 224:594–600. [PubMed: 6408990] f) Tulsiani DRP, Broquist HP, Touster O. *Arch Biochem Biophys.* 1985; 236:427–434. [PubMed: 3917650] g) Goss PE, Baker MA, Carver JP, Dennis JW. *Clin Cancer Res.* 1995; 1:935–944. [PubMed: 9816064]
- [3]. a) Olden K, Breton P, Grzegorzewski K, Yasuda Y, Gause BL, Oredipe OA, Newton SA, White SL. *Pharmacol Ther.* 1991; 50:285–290. [PubMed: 1754603] b) Dennis JW, Koch K, Yousefi S, Vanderelst I. *Cancer Res.* 1990; 50:1867–1872. [PubMed: 2106389]
- [4]. Novikoff PM, Touster O, Novikoff AB, Tulsiani DP. *J Cell Biol.* 1985; 101:339–349. [PubMed: 3926777]
- [5]. a) Rose DR. *Curr Opin Struct Biol.* 2012; 22:558–562. [PubMed: 22819743] b) van den Elsen JMH, Kuntz DA, Rose DR. *EMBO J.* 2001; 20:3008–3017. [PubMed: 11406577] c) Tulsiani DRP, Opheim DJ, Touster O. *J Biol Chem.* 1977; 252:3227–3233. [PubMed: 863880] d) Dewald B, Touster O. *J Biol Chem.* 1973; 248:7223–7233. [PubMed: 4200586] e) Moremen KW, Touster O, Robbins PW. *J Biol Chem.* 1991; 266:16876–16885. [PubMed: 1885615] f) Kuntz DA, Nakayama S, Shea K, Hori H, Uto Y, Nagasawa H, Rose DR. *ChemBioChem.* 2010; 11:673–680. [PubMed: 20209559]
- [6]. Tulsiani DRP, Touster O. *J Biol Chem.* 1983; 258:7578–7585. [PubMed: 6408079]
- [7]. a) Goss PE, Reid CL, Bailey D, Dennis JW. *Clin Cancer Res.* 1997; 3:1077–1086. [PubMed: 9815786] b) Goss JW, Baptiste J, Fernandes B, Baker M, Dennis JW. *Cancer Res.* 1994; 54:1450–1457. [PubMed: 8137247]
- [8]. a) Heikinheimo P, Helland R, Leiros HKS, Leiros I, Karlsen S, Evjen G, Ravelli R, Schoehn G, Ruigrok R, Tollersrud OK, McSweeney S, et al. *J Mol Biol.* 2003; 327:631–644. [PubMed: 12634058] b) Liao YF, Lal A, Moremen KW. *J Biol Chem.* 1996; 271:28348–28358. [PubMed: 8910458]
- [9]. a) Winchester B, Aldaher S, Carpenter NC, Dibello IC, Choi SS, Fairbanks AJ, Fleet GWJ. *Biochem J.* 1993; 290:743–749. [PubMed: 8457203] b) Dibello IC, Fleet G, Namgoong SK, Tadano K, Winchester B. *Biochem J.* 1989; 259:855–861. [PubMed: 2499316] c) Bols M, Lopez O, Ortega-Caballero F. *Comprehensive Glycoscience.* 1st ed. Kamerling JP, editor Vol. 1. Elsevier; Oxford: 2007. 815–867. d) Kuntz DA, Zhong W, Guo J, Rose DR, Boons GJ. *ChemBioChem.* 2009; 10:268–277. [PubMed: 19101978] e) Fiaux H, Kuntz DA, Hoffman D, Janzer RC, Gerber-Lemaire S, Rose DR, Juillerat-Jeanneret L. *Bioorg Med Chem.* 2008; 16:7337–7346. [PubMed: 18599296] f) Siriwardena A, Strachan H, El-Daher S, Way G, Winchester B, Glushka J, Moremen K, Boons GJ. *ChemBioChem.* 2005; 6:845–848. [PubMed: 15800866] g) Li B, Kawatkar SP, George S, Strachan H, Woods RJ, Siriwardena A, Moremen KW, Boons GJ. *ChemBioChem.* 2004; 5:1220–1227. [PubMed: 15368573] h) George S, Li B, Strachan H, Ayres R, Collins R, Siriwardena A, Moremen KW, Boons GJ. *Glycobiology.* 2002; 12:668–668. i) Englebienne P, Fiaux H, Kuntz DA, Corbeil CR, Gerber-Lemaire S, Rose DR,

- Moitessier N. *Proteins*. 2007; 69:160–176. [PubMed: 17557336] j) Fiaux H, Popowycz F, Favre S, Schutz C, Vogel P, Gerber-Lemaire S, Juillerat-Jeanneret L. *J Med Chem*. 2005; 48:4237–4246. [PubMed: 15974577] k) Kavlekar LM, Kuntz DA, Wen X, Johnston BD, Svensson B, Rose DR, Pinto BM. *Tetrahedron-Asymmetry*. 2005; 16:1035–1046. l) Kuntz DA, Ghavami A, Johnston BD, Pinto BM, Rose DR. *Tetrahedron-Asymmetry*. 2005; 16:25–32. m) Wen X, Yuan Y, Kuntz DA, Rose DR, Pinto BM. *Biochemistry*. 2005; 44:6729–6737. [PubMed: 15865418] n) Chen W, Kuntz DA, Hamlet T, Sim L, Rose DR, Pinto BM. *Bioorg Med Chem*. 2006; 14:8332–8340. [PubMed: 17010621] o) Kawatkar SP, Kuntz DA, Woods RJ, Rose DR, Boons GJ. *J Am Chem Soc*. 2006; 128:8310–8319. [PubMed: 16787095] p) Poláková M, Šesták S, Lattová E, Petruš L, Mucha J, Tvaroška I, Kó a J. *Eur J Med Chem*. 2011; 46:944–952. [PubMed: 21295890] q) Poláková M, Stanton R, Wilson IBH, Holková I, Šesták S, Machová E, Jandová Z, Kó a J. *Carbohydr Res*. 2015; 406:34–40. [PubMed: 25658064] r) Poláková M, Horák R, Šesták S, Holková I. *Carbohydr Res*. 2016; 428:62–71. [PubMed: 27152630]
- [10]. a) Pearson WH, Guo LY. *Tetrahedron Lett*. 2001; 42:8267–8271. b) Hembre EJ, Pearson WH. *Tetrahedron*. 1997; 53:11021–11032. c) Shah R, Carver J, Marino-Albernas J, Tvaroška I, Tropper FD, Dennis J. United States Patent Application Publication. US 2003/0236229 A1. 2003. d) Fujita T, Nagasawa H, Uto Y, Hashimoto T, Asakawa Y, Hori H. *Org Lett*. 2004; 6:827–830. [PubMed: 14986985]
- [11]. a) Shah N, Kuntz DA, Rose DR. *Proc Natl Acad Sci U S A*. 2008; 105:9570–9575. [PubMed: 18599462] b) Zhong W, Kuntz DA, Ernber B, Singh H, Moremen KW, Rose DR, Boons GJ. *J Am Chem Soc*. 2008; 130:8975–8983. [PubMed: 18558690]
- [12]. a) Bleriot Y, Dintinger T, Genregandpierre A, Padrines M, Tellier C. *Bioorg Med Chem Lett*. 1995; 5:2655–2660. b) Heck MP, Vincent SP, Murray BW, Bellamy F, Wong CH, Mioskowski C. *J Am Chem Soc*. 2004; 126:1971–1979. [PubMed: 14971930] c) Kuntz DA, Tarling CA, Withers SG, Rose DR. *Biochemistry*. 2008; 47:10058–10068. [PubMed: 18759458] d) Kumar NS, Kuntz DA, Wen X, Pinto BM, Rose DR. *Proteins*. 2008; 71:1484–1496. [PubMed: 18076078]
- [13]. Sladek V, Kó a J, Tokiwa H. *Phys Chem Chem Phys*. 2017; 19:12527–12537. [PubMed: 28470253]
- [14]. Rabouille C, Kuntz DA, Lockyer A, Watson R, Signorelli T, Rose DR, van den Heuvel M, Roberts DB. *J Cell Sci*. 1999; 112:3319–3330. [PubMed: 10504337]
- [15]. Nem ovi ová I, Šesták S, Rendi D, Plšková M, Mucha J, Wilson IBH. *Glycoconjugate J*. 2013; 30:899–909.
- [16]. a) Kumar BSG, Pohlentz G, Schulte M, Mormann M, Kumar NS. *Glycobiology*. 2014; 24:252–261. [PubMed: 24295789] b) Howard S, Braun C, McCarter J, Moremen KW, Liao YF, Withers SG. *Biochem Biophys Res Commun*. 1997; 238:896–898. [PubMed: 9325188]
- [17]. Kuntz DA, Rose DR. Golgi Mannosidase II in complex with swainsonine at 1.3 Å. The Research Collaboratory for Structural Bioinformatics (RCSB): RCSB-Rutgers, RCSB-San Diego Supercomputer Center, and University of Wisconsin-Madison; PDB ID: 3BLB <http://www.rcsb.org>. [Accessed June 04, 2012]
- [18]. Bobovská A, Tvaroška I, Kó a J. *J Mol Graph Model*. 2016; 66:47–57. [PubMed: 27035259]
- [19]. a) Vamshikrishna K, Srihari P. *Tetrahedron*. 2012; 68:1540–1546. b) Cho JH, Bernard DL, Sidwell RW, Kern ER, Chu CK. *J Med Chem*. 2006; 49:1140–1148. [PubMed: 16451078] c) Furst R, Lentsch C, Rinner U. *Eur J Org Chem*. 2013:2293–2297.
- [20]. Kim DK, Kim GH, Kim YW. *J Chem Soc Perkin Trans*. 1996; 1:803–808.
- [21]. Mercer TB, Jenkinson SF, Bartholomew B, Nash RJ, Miyauchi S, Kato A, Fleet GWJ. *Tetrahedron-Asymmetry*. 2009; 20:2368–2373.
- [22]. Corsi M, Bonanni M, Catelani G, D'Andrea F, Gragnani T, Bianchini R. *Int J Org Chem*. 2013; 3:41–48.
- [23]. Vallee F, Yip P, Lipari F, Sleno B, Romero P, Karaveg K, Moremen K, Herscovics A, Howell PL. *Glycobiology*. 2000; 10:1141–1141.
- [24]. a) Legler G. Inimosugars as Glycosidase Inhibitors: Nojirimycin and beyond. Stütz AE, editor Wiley-VCH Verlag GmbH; Weinheim: 1999. 31–67. b) Lopez O, Qing FL, Pedersen CM, Bols M. *Bioorg Med Chem*. 2013; 21:4755–4761. [PubMed: 23583693]

- [25]. De-Freitas-Junior JCM, Bastos LG, Freire-Neto CA, Du Rocher B, Abdelhay ESFW, Morgado-Diaz JA. *J Cell Biochem.* 2012; 113:2957–296. [PubMed: 22552949]
- [26]. Paschinger K, Hykollari A, Razzazi-Fazeli E, Greenwell P, Leitsch D, Walochnik J, Wilson IBH. *Glycobiology.* 2012; 22:300–313. [PubMed: 21983210]
- [27]. a) Nosková V, Džubák P, Kuzmina G, Ludková A, Stehlik D, Trojanec R, Janoš áková A, Ko ínková G, Mihal V, Hajduch M. *Neoplasma.* 2002; 49:418–425. [PubMed: 12584592] b) Perlíková P, Rylová G, Nauš P, Elbert T, Tlouš ová E, Bourderieux A, Slav ínská LP, Motyka K, Doležal D, Znojek P, Nová A, et al. *Mol Cancer Ther.* 2016; 15:922–937. [PubMed: 26819331]
- [28]. a) Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK, Shaw DE, et al. *J Med Chem.* 2004; 47:1739–1749. [PubMed: 15027865] b) Glide, version 7.0. Schrödinger, LLC; New York, NY: 2016.
- [29]. Schrödinger Suite 2016 Protein Preparation Wizard; Epik v. 3.5; Impact v. 7.0, Schrödinger; Maestro v.10.5, LLC: New York, NY, 2016
- [30]. Zhao Y, Truhlar DG. *Theor Chem Res.* 2008; 120:215–241.
- [31]. a) Murphy RB, Philipp DM, Friesner RA. *J Comput Chem.* 2000; 21:1442–1457. b) Qsite. Schrödinger, LLC; New York, NY: 2016.
- [32]. Jaguar, version 9.1. Schrödinger, LLC; New York, NY: 2016.
- [33]. Maestro, version 10.5. Schrödinger, LLC; New York, NY: 2016.
- [34]. Klicic JJ, Friesner RA, Liu SY, Guida WC. *J Phys Chem A.* 2002; 106:1327–1335.
- [35]. a) Li H, Robertson AD, Jensen JH. *Proteins.* 2005; 61:704–721. [PubMed: 16231289] b) Bas DC, Rogers DM, Jensen JH. *Proteins.* 2008; 73:765–783. [PubMed: 18498103]
- [36]. a) Wadt WR, Hay PJ. *J Chem Phys.* 1985; 82:284–298. b) Hay PJ, Wadt WR. *J Chem Phys.* 1985; 82:270–283.
- [37]. Kaminski GA, Friesner RA, Tirado-Rives J, Jorgensen WL. *J Phys Chem B.* 2001; 105:6474–6487.
- [38]. a) Fedorov DG, Nagata T, Kitaura K. *Phys Chem Chem Phys.* 2012; 14:7562–7577. [PubMed: 22410762] b) Tanaka S, Mochizuki Y, Komeiji Y, Okiyama Y, Fukuzawa K. *Phys Chem Chem Phys.* 2014; 16:10310–10344. [PubMed: 24740821] c) Fedorov DG, Kitaura K. *The Fragment Molecular Orbital Method - Practical Applications to Large Molecular Systems.* CRC Press, Taylor and Francis Group; 2009. d) Gordon MS, Fedorov DG, Pruitt SR, Slipchenko LV. *Chem Rev.* 2012; 112:632–672. [PubMed: 21866983]
- [39]. a) Arulmozhiraja S, Matsuo N, Ishitsubo E, Okazaki S, Shimano H, Tokiwa H. *PLoS One.* 2016; 11. b) Sriwilaijaroen N, Magesh S, Imamura A, Ando H, Ishida H, Sakai M, Ishitsubo E, Hori T, Moriya S, Ishikawa T, Kuwata K, et al. *J Med Chem.* 2016; 59:4563–4577. [PubMed: 27095056]
- [40]. Boys SF, Bernardi F. *Mol Phys.* 1970; 19:553–566.
- [41]. Sheng XW, Mentel L, Gritsenko OV, Baerends EJ. *J Comput Chem.* 2011; 32:2896–2901. [PubMed: 21735451]
- [42]. Fukuzawa K, Watanabe C, Kurisaki I, Taguchi N, Mochizuki Y, Nakano T, Tanaka S, Komeiji Y. *Comput Theor Chem.* 2014; 1034:7–16.
- [43]. a) Schmidt MW, Baldrige KK, Boatz JA, Elbert ST, Gordon MS, Jensen JH, Koseki S, Matsunaga N, Nguyen KA, Su SJ, Windus TL, et al. *J Comput Chem.* 1993; 14:1347–1363. b) Gordon MS, Schmidt MW. *Theory and Applications of Computational Chemistry.* Elsevier; 2005.
- [44]. a) Weigend F. *Phys Chem Chem Phys.* 2006; 8:1057–1065. [PubMed: 16633586] b) Weigend F, Ahlrichs R. *Phys Chem Chem Phys.* 2005; 7:3297–3305. [PubMed: 16240044]
- [45]. Ren XG, Rinke P, Blum V, Wieferink J, Tkatchenko A, Sanfilippo A, Reuter K, Scheffler M. *New J Phys.* 2012; 14:053020.
- [46]. a) Green MC, Fedorov DG, Kitaura K, Francisco JS, Slipchenko LV. *J Chem Phys.* 2013; 138b) Fedorov DG, Kitaura K. *J Comput Chem.* 2007; 28:222–237. [PubMed: 17109433]
- [47]. Sladek V, Tvaroška I. *J Phys Chem B.* 2017; 121:6148–6162. [PubMed: 28617600]

Figure 1.

(a) A superposition of the bound swainsonine (PDB ID: 3BLB) [5f, 17] and computationally docked compound **21** into the active site of dGMII. Due to a steric effect of hydroxymethyl moiety of **21** its pyrrolidine ring (in grey) is shift away from the catalytic nucleophile Asp204 compared with the swainsonine ring (in green) (distances between oxygen of Asp204 and nitrogen of the inhibitors are in Å). (b) A superposition of the docked compound **21** in the active site of dGMII (PDB ID: 3BLB)[5f, 17] (**21** with carbons in grey)

and bLMan (PDB ID: 1O7D)[8a] (**21** with carbons in green) indicates similar binding mode of both the pyrrolidine core and the benzyl linker.

Scheme 1.

Synthesis of the pyrrolidines **10** and **11**. *Reagents and conditions:* a) NaBH_4 , EtOH, rt, 2 h, 90% for **4**, 98% for **5**; b) MsCl , Et_3N , CH_2Cl_2 , rt, overnight, 98% for **6**, 95% for **7**; c) BnNH_2 , 120°C , 7-8 h, 87% for **8**, 97% for **9**; d) 6M HCl/MeOH 1:2 (v/v), rt, overnight, 62% from **8**, 68% from **9**; e) 1. H_2 , 10% Pd-C , MeOH, rt, overnight, 2. 10% HCl , 89%.

Scheme 2.

Synthesis of target pyrrolidines **15-17**. *Reagents and conditions:* a) RNH₂, 120 °C, 7-8 h, 87% for **12**, 78% for **13**, 77% for **14**; b) 6M HCl/MeOH 1:2 (v/v), rt, overnight, 52% for **15**, 54% for **16**, 62% for **17**.

Scheme 3.

Synthesis of target pyrrolidines **21** and **22**. *Reagents and conditions:* a) H₂, 10% Pd-C, MeOH, rt, 4 h, 80%; b) RBr, K₂CO₃, DMF, 40 °C, 4h, 89% for **19**, 74% for **20**; c) 6M HCl/MeOH 1:2 (v/v), rt, overnight, 73% for **21**, 66% for **22**.

Table 1

Measured IC_{50} (and K_i) values [M] for class II (GMIb, LManII and JbMan) and class I (AspMan) α -mannosidases (in M). For comparison representative inhibitors with a known selectivity index to GMII (as appropriate to either GMII, dGMII or GMIb) are also included.[6a, 6e, 6r]

Compound	GMIb IC_{50} [K_i]	LManII IC_{50} (Inhibition in %)[a]	JbMan IC_{50} (Inhibition in %)[a]	AspMan IC_{50} (Inhibition in %)[b]	Selectivity index $IC_{50}(\text{LManII})/IC_{50}$ (GMIb)
11 (R = H)	$(2.7 \pm 0.75) \times 10^{-4}$ [(2.2 $\pm$ 0.15) $\times 10^{-4}$]	7.5×10^{-3}	0.7×10^{-3}	$(1.0 \pm 0.2) \times 10^{-3}$ (50)	28
10 (R = Bn)	$(8.8 \pm 0.06) \times 10^{-5}$ [(7.6 $\pm$ 0.13) $\times 10^{-5}$]	7.0×10^{-3}	n.d. (1)	n.i.	80
15 (R = EtPh)	$(1.9 \pm 0.23) \times 10^{-4}$ (n.d.)	1.2×10^{-3}	2.7×10^{-3}	n.m.	6
16 (R = 4-MeO-Bn)	$(1.4 \pm 0.17) \times 10^{-4}$ (n.d.)	n.d. (9)	n.d. (4)	n.i.	n.d.
17 (R = 4-Py-Bn)	$(7.7 \pm 0.40) \times 10^{-4}$ [n.d.]	n.d. (2)	n.d. (9)	n.m.	n.d.
21 (R = 4-I-Bn)	$(5.2 \pm 0.12) \times 10^{-5}$ [(5.0 $\pm$ 0.12) $\times 10^{-5}$]	6.1×10^{-3} (19)	n.d. (9)	n.i.	117
22 (R = 4-Br-Bn)	$(5.5 \pm 0.15) \times 10^{-5}$ [(5.8 $\pm$ 0.06) $\times 10^{-5}$]	7.5×10^{-3} (16)	n.d. (8)	n.i.	136
1	5.0×10^{-8} [c] [3 $\times 10^{-9}$] [d]	11.0×10^{-8} [c]	2.0×10^{-7} [c]	$>10.0 \times 10^{-3}$ [h]	2
23	2.9×10^{-8} [d]	3.1×10^{-7} [d]	2.5×10^{-7} [d]	n.m.	11
24	1.0×10^{-4} [e]	1.3×10^{-5} [e]	n.m.	n.m.	<0
25	6.9×10^{-4} [e,f]	1.5×10^{-3} [e,f]	n.m.	n.m.	2
26	2.0×10^{-3} [g]	n.i. ^g	2.0×10^{-3} [c]	n.i. [b,c]	>2

n.m. - not measured. n.d. - not determined (the inhibition <50% at 8 mM). n.i. - no inhibition or inhibition <10% at 2 mM.

[a]. inhibition in the presence of 2 mM of the inhibitor calculated towards control activity without inhibitor.

[b] inhibition in the presence of 1 mM of the inhibitor calculated towards control activity without inhibitor.

[c] IC_{50} values measured for GMIIb, LManII, JBMan and AspMan by Poláková et al.[9q]

[d] IC_{50} values measured for dGMII, dLMan and JBMan by Kuntz et al. [5f]

[e] IC_{50} values measured for GMII and LMan by Winchester et al. [9a]

[f] calculated IC_{50} values by Bobovská et al.[18] based on measured in vitro assays with human α -mannosidases. [9a]

[g] IC_{50} values measured for GMIIb and LManII by Poláková et al.[9p]

[h] K_i value measured for hERManI by Vallée et al.[23]

Table 2

Cytotoxic activity of the selected pyrrolidines **10**, **15**, **16**, **21**, **22** and swainsonine **1** against eight tumor and two non-malignant cell lines (IC₅₀ in μ M).

Comp	A549	CCRF-CEM	CEM-DNR	K562	K562-TAX	HCT116	HCT116p53-/-	U2OS	BJ	MRC-5
10	> 200	101 $\pm$ 12	93 $\pm$ 11	194 $\pm$ 8	107 $\pm$ 13	> 200	> 200	> 200	> 200	> 200
15	> 200	95 $\pm$ 7	99 $\pm$ 11	189 $\pm$ 12	117 $\pm$ 13	> 200	> 200	> 200	> 200	> 200
16	> 200	105 $\pm$ 12	94 $\pm$ 10	192 $\pm$ 9	110 $\pm$ 11	> 200	> 200	200 $\pm$ 0.3	> 200	> 200
21	> 200	102 $\pm$ 11	87 $\pm$ 11	156 $\pm$ 10	103 $\pm$ 13	> 200	> 200	169 $\pm$ 21	> 200	> 200
22	> 200	94 $\pm$ 24	122 $\pm$ 22	> 200	125 $\pm$ 13	> 200	> 200	189 $\pm$ 18	> 200	> 200
1	> 200	113 $\pm$ 12	132 $\pm$ 20	> 200	137 $\pm$ 13	> 200	> 200	> 200	> 200	> 200

IC₅₀ is the lowest concentration that kills 50 % of cells. The standard deviation in cytotoxicity assays is typically up to 15 % of the average value. Compounds with IC₅₀ > 200 μ M are considered inactive.

Table 3Calculated pK_a values for ligands and selected Asp residues in water and in the enzymes.

pK_a					
	Water pH=7		dGMII pH=5.9		bLMan pH=4.5	
Compound	ligand	ligand	Asp341	Asp340	ligand	Asp319
11 (R=H)	7.9	5.9	2.2	5.8	6.9	2.1
10 (R=Bn)	6.3	7.3	2.4	5.9	7.5	2.2
15 (R=EtPh)	6.9	6.8	4.4	4.4	7.8	2.0
16 (R=4-MeO-Bn)	7.5	7.3	2.5	5.9	7.7	2.2
17 (R=4-Py-Bn)	6.4	7.5	2.3	5.9	7.5	1.8
21 (R=4-I-Bn)	6.9	7.4	2.4	5.9	7.3	2.3
22 (R=4-Br-Bn)	7.3	7.3	2.5	5.9	7.3	2.3
1	7.8 [a] (7.5)[b]	5.0 [a]	5.6	4.3	8.2	3.0
24	6.6 [a] (7.4)[b]	4.7 [a]	6.8	4.4	8.1	1.9
Holoenzyme			5.8			3.9

[a] calculated pK_a value by Sladek et al.[13][b] experimentally measured pK_a values.[2a, 9b]