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PII: S0045-2068(14)00031-5
DOI: http://dx.doi.org/10.1016/j.bioorg.2014.04.007
Reference: YBIOO 1718

To appear in: Bioorganic Chemistry

Received Date: 20 February 2014

Please cite this article as: E.P. Carreiro, P. Louro, G. Adriano, R.A. Guedes, N. Vannuchi, A.R. Costa, C.M.M. Antunes, R.C. Guedes, A.J. Burke, 3-Hydroxypyrrolidine and (3,4)-Dihydroxypyrrolidine Derivatives: Inhibition of rat intestinal α-Glucosidase, Bioorganic Chemistry (2014), doi: http://dx.doi.org/10.1016/j.bioorg.2014.04.007

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Graphical Abstract

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**ABSTRACT**

Thirteen pyrrolidine-based iminosugar derivatives have been synthetized and evaluated for inhibition of α-glucosidase from rat intestine. The compounds studied were the non-hydroxy, mono-hydroxy and dihydroxypyrrolidines. All the compounds were N-benzylated apart from one. Four of the compounds had a carbonyl group in the 2,5-position of the pyrrolidine ring. The most promising iminosugar was the trans-3,4-dihydroxypyrrolidine giving an IC_{50} of 2.97±0.046 and a K_{i} of 1.18 mM. Kinetic studies showed that the inhibition was of the mixed type, but predominantly competitive for all the compounds tested. Toxicological assay results showed that the compounds have low toxicity. Docking studies showed that all the compounds occupy the same region as the DNJ inhibitor on the enzyme binding site with the most active compounds establishing similar interactions with key residues. Our studies suggest that a rotation of ~90º of some compounds inside the binding pocket is responsible for the complete loss of inhibitory activity.

Despite the fact that activity was found only in the mM range, these compounds have served as simple molecular tools for probing the structural features of the enzyme, so that inhibition can be improved in further studies.

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1. Introduction

Over the last two decades, glycosidase inhibitors have been a key target for academic researchers, because of their role in a variety of ailments [1], such as: diabetes mellitus type II [2], cancer [3], hepatitis [4], HIV [5] and Gaucher disease [6]. One of the most studied classes of inhibitor are the polyhydroxylated pyrrolidines and piperidines [7]. Before the 90s [8] most glycosidase inhibitors studied were obtained from plants and micro-organisms, and three important examples are: deoxynojirimycin (DNJ), 2R,5R-dihydroxymethyl-3R,4R-dihydroxypyrrolidine (DMDP) and 1,4-dideoxy-1,4-imino-d-arabinitol (DAB) (Figure 1). These three molecules since the 90s have been the prototypes for the synthesis of a wide range of potential glycosidase inhibitors [1]. For example, miglitol, an N-alkyl derivative of DNJ used in the treatment of diabetes [2]. Curiously, Miglustat (N-butyl DNI, Zavesca) is structurally similar to miglitol and is licensed for substrate reduction therapy in Gauchers disease. In the case of DAB, it shows strong inhibition of α-glucosidases, but its enantiomer, LAB is more potent and specific than DAB [9]. In the literature there exists an extensive variety of iminosugars with structures based on the pyrrolidine unit, which exhibit α-glucosidase activity. Several derivatives of DAB were synthetized and tested in different types of α-glucosidases [10]. Most of the structures were functionalized with alkyl [11], aryl [12], amide [13], amine [13], or alcohols [14] units in the 2,5-positions of the pyrrolidine ring. In 2012 Kato et al. [11a] described a new family of potent pyrrolidine α-glucosidase inhibitors; this family was basically a set of analogues of the LAB structure, having alkyl chains of different lengths in the 2 and 4 positions. The most promising α-glucosidase inhibitor was the C-butyldihydroxypyrrolidine (C-butyldihydroxy-2,4-dihydroxypyrrolidine) giving an IC_{50} of 2.97±0.046 and a K_{i} of 1.18 mM. Kinetic studies showed that the inhibition was of the mixed type, but predominantly competitive for all the compounds tested. Toxicological assay results showed that the compounds have low toxicity. Docking studies showed that all the compounds occupy the same region as the DNJ inhibitor on the enzyme binding site with the most active compounds establishing similar interactions with key residues. Our studies suggest that a rotation of ~90º of some compounds inside the binding pocket is responsible for the complete loss of inhibitory activity.

Despite the fact that activity was found only in the mM range, these compounds have served as simple molecular tools for probing the structural features of the enzyme, so that inhibition can be improved in further studies.

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insight into the mechanism of inhibition of glycosidases by polyhydroxylated pyrrolidines. These compounds were assayed for baker’s yeast α-glucosidase inhibition using acarbose as a reference and it was the non-benzylated diol 7 which showed the highest inhibition (IC_{50} = 10.9 mM). It seems that the presence of a free NH group and free hydroxyl groups in the 3 and 4 positions are important for favorable interaction with the enzyme active site. There were no substituents in the 2,5-position of the pyrrolidine ring in these compounds, but it appears quite obvious now that the presence of substituents in these positions is important for inhibition [7]. Our results are similar to those obtained with the iminosugars reported by Bols [15] and Lundt [16] (Figure 1), although in their case the 3,4-hydroxy groups had a cis relative configuration.

On the basis of our last publication we observed that O-acetylated dihydroxypyrrolidines - despite showing good docking simulations [7a] - seem to suffer hydrolysis during the biological assays, and for this reason we decided to substitute the acetyl group for the more robust benzyl group. Besides this the possibility of the benzyl groups establishing significant non-covalent π-π interactions with the active site residues was very likely, and worth investigating.

2. Materials and Methods

2.1. General Chemical

All reagents were obtained from Aldrich, Fluka, Alfa Aesar or Acros. Solvents were dried using common laboratory methods. Compounds 5 and its respective enantiomer 5′ were synthesized using the precursors: (3S,4S)-N-benzyl-3,4-dihydroxy-2,5-dioxopyrrolidine and (3R,4R)-N-benzyl-3,4-dihydroxy-2,5-dioxopyrrolidine, respectively, both enantiomers had an enantiomeric purity of 99% ee. TLC was carried out on aluminium backed Kiselgel 60 F254 plates (Merck) and the plates were visualized either by UV light or with phosphomolybdic acid in ethanol. The ^1H NMR and ^13C NMR spectra were recorded on a Bruker Avance instrument (^1H: 400 MHz and ^13C: 100 MHz) using CDCl3 as solvent and the signal from residual CHCl3 as an internal standard (for the measurements made with the Bruker Avance instrument). Specific rotations were measured on a Perkin-Elmer 241 polarimeter.

2.2. General Biological assays

2.2.1 Enzymatic assays with rat α-glucosidase

The enterocytes were isolated from Wistar rat small intestine according to Watford et al., 1979 [19a] with few modifications. Briefly, the α-glucosidase rich homogenates were obtained after mucus removal with calcium free Krebs-Henseilt buffer (in mM: 120 NaCl, 2 KCl, 26 NaHCO3, 10 MgSO4, 1.18 KH2PO4, 11 glucose, 5 EDTA; supplemented with 0.25% (p/v) BSA; at 37°C during 20 min under mild stirring – 60 rpm) and detachment of mucosa epithelial cells using Krebs-Henseilt buffer (as previously described without EDTA and with 0.5 mM CaCl2, supplemented with 2.5% (p/v) BSA). The total protein
concentration was determined using the Bradford dye-binding method [19b].

The α-glucosidase activity was determined by monitoring the p-nitrophenol (p-NP) released from p-nitrophenyl-α-D-glycopyranoside spectrophotometrically at 405 nm, over 60 min. The assay mixture had the following composition: 0.1M phosphate buffer (pH 7), 12 mM p-nitrophenyl-α-D-glycopyranoside (the method was optimized giving Kᵣₗ and Vₘₐₓ values of 0.961 mM and 2.51x10⁻⁵ mmol p-NP.min⁻¹.mg⁻¹, respectively) and 30 μg/mL rat enterocyte α-glucosidase rich homogenates. The test compounds were dissolved in 2% DMSO in 0.1M phosphate buffer (pH 7) and solutions with concentration in the range of 0.01 x 10⁻³ and 92 mM were used. All experiments were performed in 5 replicates. The IC₅₀ values were obtained from the inhibition curves.

2.2.2 Toxicity assays

The toxicity of the compounds was measured using two methods. The first method described consists in the determination of the cell viability of the cellular line BRIN-BD 11 from pancreatic beta cells. The second method was the evaluation of the half maximal lethal concentration (LC₅₀) using Artemia salina.

2.2.2.1 Cytotoxicity Assay

BRIN BD-11 cells were cultured in 96 well micro plates (2x10⁴ cells/well) in an incubator at 37°C with 5% CO₂ and 95% O₂. The cells were exposed to the test compounds in the concentration range of 3 – 50 mM for 24 h. A negative control (without inhibitors) and a positive control (1% sodium dodecyl sulfate) tests were performed. Cell viability was determined using the cell counting kit-8 (CCK-8, Sigma-Aldrich) as established by the supplier.

2.2.2.2 Artemia salina lethal toxicity assay

The percentage of dyed nauplii of Artemia salina, grown in the presence of variable concentrations of the inhibitor compounds, was measured to determine the LC₅₀ values. The Artoxkit M was used.

2.2.3 Statistical analysis

Results are presented as mean ± sd for a given number of observations. Statistical analyses were done using one-way ANOVA for enzymatic activity. In the case of the toxicological results Paired samples t-Test was used for comparison with samples showing negative control.

2.3. Molecular Modeling

2.3.1 Molecular docking calculations

Molecular docking calculations of the synthesized compounds with human and a homology model of rat α-glucosidase were performed with GOLD 5.1.0 (Genetic Optimization Ligand Docking) [20] software using the Goldscore scoring function. This software uses an evolutionary genetic algorithm to optimize the docked conformation of the flexible inhibitor within the enzyme. The docking calculations were performed using the homology model described in the next section for the rat α-glucosidase and the available human α-glucosidase crystallographic structure. The experimental x-ray structure was obtained from the RCSB Protein Data Bank (PDB ID 3L4U, resolution 1.9 Å). The preparation of the human α-glucosidase structure involved the removal of the original crystallographic ligand (kotalanol) as well as the crystallographic waters. The protonation and tautomeric states of Asp, Glu, Arg, Lys, and His were adjusted to match a pH of 7 using the Protonate 3D algorithm within the Molecular Operating Environment (MOE) 2012.10 program (www.chemcomp.com). These enzyme structures and the proposed docking protocol were previously validated by re-docking the co-crystallized ligand. The molecular structures of the synthesized compounds were built and optimized with MOE package (2012.10) with the MMFF94x forcefield as implemented in this software. These compounds were docked into the homology model of rat intestinal glucosidase developed previously by this group and to human glucosidase active sites. For each compound, 500 docking runs were performed. The following genetic algorithm parameters were used: population size = 100; selected pressure = 1.1; number of operations = 1000; number of islands = 5; niche size = 2; migrate = 10; mutate = 95; crossover = 95. Each conformation was ranked according to its goldscore scoring function. The top solutions (the ones with the highest goldscore) were visually inspected and critically evaluated and, for each inhibitor, the highest scoring conformation was chosen as the actual binding conformation (Figure 5).

2.4. Homology modelling of the Rat intestinal glucosidase:

To investigate the inhibitory activity of the synthesized compounds against rat intestinal glucosidase, a homology modeling of α-glucosidase from Rattus norvegicus was carried out to predict its 3D-structure (the 3D structure has never been resolved experimentally). The amino acid sequence of α-glucosidase from Rattus norvegicus comprises 953 amino acid residues and was retrieved from the UniProt protein resource data bank (http://www.uniprot.org/), under the access code Q6P7A9. Using the Molecular Operating Environment program (MOE) version 2012.10 (http://www.chemcomp.com/software) we searched for proper structural templates on the PDB database of protein structures and sequences and aligned the results obtained with the MOE-Align feature. MOE-Align implement a modified version of the alignment methodology originally introduced by Needleman and Wunsch. All the default settings in the MOE-Align panel were used for the sequence alignment. Our search identified the Homo sapiens intestinal maltase-glucoamylase (in complex with O-sulfonated kotalanol) crystallographic structure (PDB code 3L4U, 1.90 Å resolution)[21] sharing 45.7% of sequence identity (calculated with BLAST) with α-glucosidase enzyme from Rattus norvegicus as the most suitable template. The catalytic site is highly conserved in both structures. The crystallographic structure and the homology model were superposed with MOE software and the RMSD values obtained were 1.04 Å for the Rattus norvegicus homology model. The 3D homology models were built with Molecular Operating Environment (MOE) software using only a single template and a set of 10 intermediate models were generated and refined with Amber99 forcefield, resulting in the corresponding homology model. The stereochemical quality of the enzyme backbone and side chains was validated by Ramachandran plots. To validate the model we first docked kotalanol into the structure’s active site and compared the final docked complexes with the crystallographic structure obtained by Sim et al.[21] We confirmed that kotalanol was placed in an identical position to that adopted in the crystallographic structure (PDB ID: 3L4U) having similar active site residue interactions.
Besides that, acarbose (a known α-glucosidase inhibitor widely used in the treatment of diabetes type 2) was also docked into the homology model active site, and was correctly placed.

2.5. Synthesis of Pyrrolidine Iminocyclitol Inhibitors

Synthesis of (3R)-1-benzyl-3-hydroxy-yrrrolidine-2,5-dione (1): (3R)-1-benzyl-3-pyrrolidine-2,5-dione 1 was synthesized according to the thermal condensation method [22a,23]. The enantioselectivity was determined by chiral HPLC (column AD-H, (60:40) n-hexane:ethanol, 1 mL/min: $t_{R}$ = 15.8 min, 98% ee.

Synthesis of (3S)-1-benzyl-3-hydroxy-yrrrolidine-2,5-dione (1’): (3S)-1-benzyl-3-hydroxy-yrrrolidine-2,5-dione 1 was synthesized according to the thermal condensation method [22a,23]. The enantioselectivity was determined by chiral HPLC (AD-H, (60:40) n-hexane:ethanol, 1 mL/min: $t_{R}$ = 20.6 min, 100% ee.

**Synthesis of (3R)-1-benzyl-3-acetatepyrrolidine-2,5-dione (2):** (3R)-1-benzyl-3-hydroxy-yrrrolidine-2,5-dione 1 (300 mg, 1.5 mmol) was dissolved in pyridine (0.78 mL) and acetic anhydride (1.23 mL) was then added. The solution was stirred for 23h at rt. The solvents were removed in vacuo, and the crude product was purified by silica gel column chromatography (2:1 to (1:1) Hex:EtOAc) to give the title compound as a light brown solid (0.19 g, 52%).

$^1$H NMR (CDCl$_3$, 400 MHz): $\delta$: 2.14 (s, 3H, CH$_3$) ppm, 2.64 (dd, J = 4, 20 Hz, 1H, CH$_2$CO), 3.13 (dd, J = 8, 20 Hz, 1H, CH$_2$CO), 4.6 (d, J = 20 Hz, 1H, ABX system, CH$_2$Ph), 4.70 (d, J = 12, 1H, ABX system, CH$_2$Ph), 5.42 (dd, J = 8, 12 Hz, 1H, CHOAc), 7.33 (m, 5H, Ph). $^1$C NMR (100.6 MHz, CDCl$_3$): $\delta$: 20.5 ppm, 35.7, 42.6, 67.5, 128.1, 128.2, 128.4, 135.1, 169.8, 172.9, 173.2. $[\alpha]^{28}_{D}$ = +20.6 ($c$ 1.45, CHCl$_3$). $[\alpha]^{20}_{D}$ = +39 (1%, w/v MeOH) [24].

Synthesis of (3S)-1-benzyl-3-acetatepyrrolidine-2,5-dione (2’): (3S)-1-benzyl-3-acetatepyrrolidine-2,5-dione 2 was synthesized according the procedure described previously. The title compound was obtained as a brown solid (0.29 g, 78%).

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$: 2.14 (s, 3H, CH$_3$) ppm, 2.64 (dd, J = 4, 16 Hz, 1H, CH$_2$CO), 3.14 (dd, J = 8, 20 Hz, 1H, CH$_2$CO), 4.65 (d, J = 16 Hz, 1H, ABX system, CH$_2$Ph), 4.70 (d, J = 16 Hz, 1H, ABX system, CH$_2$Ph), 5.25 (d, J = 4, 8 Hz, 1H, CHOAc), 7.34 (m, 5H, Ph). $^1$C NMR (100.6 MHz, CDCl$_3$): $\delta$: 20.5 ppm, 35.7, 42.7, 67.5, 128.2, 128.4, 135.2, 169.8, 172.9, 173.2. $[\alpha]^{28}_{D}$ = -21.5 ($c$ 1.14, CHCl$_3$). $[\alpha]^{20}_{D}$ = -40.6 (1%, w/v MeOH) [22].

Synthesis of (3R)-1-benzyl-3-hydroxy-yrrrolidine (3): (3R)-1-benzyl-3-hydroxy-yrrrolidine 3 was synthesized according to
the method described by Zheng et al.\[22a\] $[\alpha]_D^{20} = +56.3$ (c 0.045, CHCl$_3$).

Synthesis of (3S)-1-benzyl-3-hydroxy-2-pyrrolidine (3'): (3S)-1-benzyl-3-hydroxy-2-pyrrolidine-2,5 3' was synthesized according to the method described by Zheng et al.\[22a\] $[\alpha]_D^{20} = -3.14$ (c 1.08, CHCl$_3$). $[\alpha]_D^{20} = -3.145$ (c 1.2, CHCl$_3$).\[22a\]

Synthesis of (3R)-1-benzyl-3-acetatepyrrolidine (4): (3R)-1-benzyl-3-acetatepyrrolidine 4 was synthesized according to the method described for compound 2, the title compound was obtained as a brown solid (0.14 g, 63%). $\delta_{1}^{1}H NMR$ (400 MHz, CDCl$_3$): 2.25 (m, 3H, CH$_3$), 2.46 (s, 3H, CH$_3$), 2.66 (m, 1H, CH$_2$), 2.8 (m, 2H, CH$_2$), 3.60 (d, J 12 Hz, 1H, ABX system, CH$_2$Ph), 3.79 (d, J 12 Hz, 1H, ABX system, CH$_2$Ph), 4.16 (d, J 4 Hz, 2H), 4.41 (s, 2H, CHO), 4.57 (q, 4H, 2x CH$_2$). 3.73 (d, J 8 Hz, 2H), 3.80 (s, 3H, CH$_3$), 3.93 (d, J 8 Hz, 2H).

To a round bottom flask (100 mL) with a magnetic stir bar was added pyrrolidine (1g, 14 mmol), THF (2 mL) and washed with water (2 mL). The organic phase was dried with MgSO$_4$, filtered and concentrated giving 8 (1.0 g, 46%) as yellow oil.\[27\]

In the case of the synthesis of 1-benzyl-3-hydroxy-2-pyrrolidine derivatives \[22\] 1- or 3-malic acid were used as substrates (Scheme 1). In the case of compounds 1 and 1', two methods were used, in the first method only 89% ee was obtained (1st step formation of hydroxyamide; 2nd step cyclization to form the malimide). The first method was originally reported by Joullié et al.\[22\]. It seems that the second step which involved heating at high temperature without solvent gave some racemization. In the second method using a direct thermally induced condensation between the malic acid and the amine, in xylene, an enantiopurity of 99%ee was obtained for the product. For the synthesis of 3 and 3' the method described by Zheng et al.\[22a\] was used and the yields were approximately 50%. The acetylation of 1, 1', 3, and 3' with pyridine and acetic anhydride formed the products 2, 2', 4, and 4', respectively.\[7\]

The non-hydroxylated 1-benzyl pyrrolidine 8 was synthesized by alkylation of pyrrolidine using triethylamine and benzyl bromide, furnishing the product in a yield of 46% without any purification (Scheme 2). This compound was used as a reference. (3R,4R)-1-Benzyl-3,4-dihydroxypyrrolidine 5 and the respective enantiomer 5' were synthesized using the method of Nagel.\[23\] The dibenzylated derivative 6 was obtained by protecting the hydroxyl groups of 5 using NaH in DMF and benzylbromide, the yield was good.

(3R,4R)-Pyrrrolidine-3,4-diol 7 (Scheme 3) was synthesized using supported palladium (0) - Pd-EnCat - and this was used in order to avoid leaching of the metal into the product, which is a problem with these systems.\[28\]
very poor inhibitory action (with an IC\textsubscript{50} of 72 mM), suggesting the requirement of hydroxyl groups in the pyrrolidine ring. Both enantiomers 2 and 2', have an acetyl group in the 3-position of the pyrrolidine ring. In this case, however, it was not possible to determine an exact IC\textsubscript{50} value; instead a value in the range of 10-20 mM was inferred. The main reason for this result was that probably the acetylated compounds suffered partial hydrolysis during the inhibition period leading to a mixture of acetylated and deacetylated products. In the case of the monohydroxylated compounds 3 and 3', the IC\textsubscript{50} were 4.6±0.1 and 4.2±0.3 mM, respectively. These compounds are better inhibitors than the respective acetylated compounds (2 and 2'), perhaps as a result of better interactions with the active site of the enzyme. The same was the case for compound 6, with a lower IC\textsubscript{50} value compared to that of compound 5. Another problem with compound 6 was its poor solubility in the assay solution hence deviations in the solution concentration might occur. In the case of the enantiomers 5 and 5', the IC\textsubscript{50} values were 2.97±0.046 and 5.82±0.034 mM, respectively; Interestingly 5' is more active than 5. In an attempt to achieve better inhibition we decided to synthetize compound 7, based on the modification of the structure of enantiomer 5, making it more polar, with a free NH group for interaction by hydrogen bonding, with the active site of the enzyme. Unfortunately, the IC\textsubscript{50} values increased slightly to 4.07±0.042 mM. On comparing the monohydroxyl with the dihydroxyl compound series, the inhibition is quite similar. Based on these results, our best inhibitor was compound 5.

In order to understand the molecular interactions between the inhibitors and the enzyme, we carried out some kinetic studies basically to determine the type of inhibition and the (K\textsubscript{s}) value Figure 4 and Table 1.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC\textsubscript{50} (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNJ</td>
<td>1.67 x 10\textsuperscript{-4}±0.163x10\textsuperscript{-4}</td>
</tr>
<tr>
<td>1</td>
<td>NI</td>
</tr>
<tr>
<td>1'</td>
<td>NI</td>
</tr>
<tr>
<td>2</td>
<td>NI</td>
</tr>
<tr>
<td>2'</td>
<td>NI</td>
</tr>
<tr>
<td>3</td>
<td>4.6 ±0.1</td>
</tr>
<tr>
<td>3'</td>
<td>4.2±0.3</td>
</tr>
<tr>
<td>4</td>
<td>10-20</td>
</tr>
<tr>
<td>4'</td>
<td>10-20</td>
</tr>
<tr>
<td>5</td>
<td>2.97±0.046 (K\textsubscript{s}= 1.18 mM)</td>
</tr>
<tr>
<td>5'</td>
<td>5.82±0.034 (K\textsubscript{s}= 0.27±0.25 mM)</td>
</tr>
<tr>
<td>6</td>
<td>7.94</td>
</tr>
<tr>
<td>7</td>
<td>4.07±0.042(K\textsubscript{s}= 0.44±0.164 mM)</td>
</tr>
<tr>
<td>8</td>
<td>72.0±0.3</td>
</tr>
<tr>
<td>NI</td>
<td>no inhibition</td>
</tr>
</tbody>
</table>

Only three compounds were selected, 5, 5' and 7, which were the most promising inhibitors. On the basis of the kinetic results, all the compounds showed a mixed type of inhibition (Figure 4 (B) and (C)). Based on the Dixon plot, the mechanism of inhibition observed is predominately a mixed competitive type. The K\textsubscript{s} values that were determined were different from the respective IC\textsubscript{50} values, again indicating competitive inhibition.

3.2. Biological screening and Toxicological studies

The synthetized compounds were assayed for the inhibition of rat intestine α-glucosidase using inhibitor concentrations between 0 and 46 mM, with the exception of compound 8 which was used at concentrations of between 0 and 92 mM. DNJ was used as the positive control. The IC\textsubscript{50} for DNJ inhibition of rat α-glucosidase was found to be in the nM range (IC\textsubscript{50}=0.167 ± 0.016 µM) (Fig. 3(A)) and consistent with other reports [29]. Table 1 shows the IC\textsubscript{50} of the studied iminosugars, determined by the inhibition curves (Fig. 4 (A)). Based on the results shown in the table 1, all the compounds containing a 2,5- carbonyl group (1, 1', 2, 2') failed to evoke any inhibition; on the contrary all the examples lacking this group have shown inhibition in the concentration range 0.01 – 72 mM. As expected 1-benzylpyrrolidine 8, showed...
Despite the fact that these compounds were moderate α-glucosidase inhibitors, it still was considered of interest to evaluate their toxicological proprieties, particularly as they could be used to address other therapeutic targets. The methods used were (i) BRIN-BD11 cell viability assay and (ii) Artemia Salina toxicity assay. In the case of the first method used, Figure 5(A), it was observed that compounds 5, 5' and 7 had low toxicity, compound 5 is the most toxic affecting the cellular viability in concentration above 12.5 mM and compound 5' affected the cellular viability for concentrations above 25 mM whereas-7 did not affect the cellular viability in the range of concentrations studied, up to 50 mM. It is noteworthy that these concentration values are 4 and 10 times higher than the IC$_{50}$ of compounds 5/5' and 7, respectively. In the case of the tests with Artemia Salina only the enantiomers 5 and 5' were evaluated. The results obtained show that the LC$_{50}$ values were 15.53 and 38.56 mM, respectively (Figure 5(B)). For both compounds the LC$_{50}$ is 5 times higher than the IC$_{50}$. These results are in agreement with the cellular viability test and show that 5' is less toxic than 5. All together, these results suggest that, although moderate inhibitors of mammalian α-glucosidase, these compounds might have some potential as pharmacological agents for other therapeutic targets. In addition, enantiomers 5 and 5' are more toxic than compound 7, suggesting that benzylated pyrrolidine are more deleterious than debenzylated ones.

3.3. Molecular Modeling Studies

In order to rationalize the experimental inhibitory activity for the compounds with different types of substitution in positions 2-5, the compounds were docked into the two enzymes active site. All the compounds were protonated when docked at the α-glucosidase active site. The results obtained for both enzymes were very similar, thus for simplicity, only the docking results using human α-glucosidase will be discussed.

As stated in our previous paper, the docking results show that all the tested compounds occupy the same region of the binding pocket as the amino group and the corresponding cyclohexenyl ring of acarbose. The obtained docking pose show all the compounds lying inside the binding cavity and interacting with the most important residues, notably, Asp203, Asp542, Asp443, Asp327 Arg526 and His600. We also docked DNJ into the α-glucosidase active site and this showed that the most active compounds show a very similar pose when compared with the ones obtained for DNJ (Figure 3).

Compound 5, the most potent compound in the series, is predicted to form an H-bond between the pyrrolidine nitrogen and Asp443 (1.5 Å), as well having interactions between the oxygen atoms from the free hydroxyl groups, with Asp327, Asp542 and His600 (catalytic site) stabilizing the enzyme ligand complex, in a similar fashion to acarbose and DNJ. A very similar pose is observed for compound 7, however, contrary to the former, this compound cannot establish interactions with Asp203 due to lack of the N-benzyl group. Compounds 3, 3', 6 and 8 show an inverted pose inside the binding pocket compared with compounds 5 and 7. However, it was shown that they can establish key interactions with Asp203, Arg526 and Asp542. When we compare, for example compounds 5 and 3 (differing in the mono and dihydroxy-pyrrolidine substitution at positions 3 and 4) we observed an opposed pose inside the binding cavity bearing testimony to the importance of disubstitution for stabilizing interactions that can improve inhibitory activity. Compounds 1, 1', 2 and 2' showed a rotated pose (~90°) inside the binding pocket when compared with compound 5. Despite the fact, that these compounds occupy the same region as the active ones, their lack of activity might be due to the fact that they have carboxylic oxygen H-bond acceptors at positions 2 and 5, apparently forcing them to rotate and lose the important H-bonding interaction with Asp542.

4. Conclusions

Compounds with carbonyl groups in the 2,5-positions of the pyrrolidine ring did not show any α-glucosidase inhibition. Compounds with protected hydroxyl groups and without this group were shown to be poor inhibitors.

On comparing 3-hydroxylpyrrolidine with (3,4)-dihydroxy-pyrrolidine, analogous IC$_{50}$ values were observed. However, compound 5, a (3R,4R)-dihydroxy-pyrrolidine, was the best inhibitor with a IC$_{50}$ value of 2.97±0.046 mM. Compound 5 was more potent than its respective enantiomer.

Kinetic studies on compounds 5, 5' and 7 revealed a mechanism of inhibition predominately of the mixed competitive type. Toxicological evaluation has shown that the compounds studied have, in general, low toxicity, only presenting deleterious effects for concentrations that are 4 to 10 times higher than the IC$_{50}$. Finally, the N-benzylated compounds are more toxic than the debenzylated compound 7.

Our docking studies suggest that a rotation of ~90° of some compounds inside the binding pocket is responsible for the complete loss of inhibitory activity.

We are currently looking at the synthesis of libraries of novel sugar-pyrrolidine-diols for glucosidase inhibition.
Figure 4 - (A) Inhibitory effects of compounds DNJ (●), 3 (△), 3’ (■), 5 (□), 5’ (■), 6 (▽) and 7 (○) against rat α-glucosidase activity, using p-nitrophenol-α-D-glucopyranoside as a substrate. Values are expressed as the mean±sd (obtained in two independent experiments performed in quintuplicate).
DNJ served as positive control. (B) Michaelis-Menten Plot and Dixon Plot for compound 5 in the presence of several concentrations for determination of $K_i$. (C) Michaelis-Menten Plot and Dixon Plot for compound 7 in the presence of several concentrations for determination of $K_i$. The values are expressed as mean±sd (5 replicates).

Figure 5. (A) Viability of BRIN BD-11 cells with different concentrations of compounds 5, 5' and 7. Batches of cells without inhibitors (control) and with 1% SDS, a cell membrane disrupter, were used as negative and positive control, respectively. *indicates statistical significance relative to controls (p<0.05). (B) Mortality (%) of *artemia salina* vs log[iminosugar 5 or 5'] – dose-response curve.

ACKNOWLEDGEMENTS

EPC thanks the Fundação para a Ciência e a Tecnologia (FCT) for a post-doctoral research fellowship (SFRH/BPD/72182/2010). We are very grateful to Dr. Olivia Furtado Burke of LNEG, Lisbon for the optical activity measurements. We acknowledge LabRMN at FCT-UNL for the acquisition of the NMR spectra; the NMR spectrometers are part of the National NMR Network and were purchased within the framework of the National Programme for Scientific Equipment (contract REDE/1517/RMN/2005), with funds from POCI 2010 (FEDER) and PEst-C/SAU/LA0001/2011 (CNC) and PEst-C/QUI/UI0062/2011 (ICAAM). The University of Vigo (Spain) is gratefully acknowledged for MS analysis.

References


  1) Protein-ligand hydrogen bond energy (external H-bond);
  2) Protein-ligand van der Waals energy (external vdw);
  3) Ligand internal van der Waals energy (internal vdw);
  4) Ligand intramolecular hydrogen bond energy (internal- H-bond).

- The synthesis of a library of 13 chiral pyrrolidine derivatives.
- Enzyme kinetics implied a mixed inhibition mode, corroborating our molecular docking studies.
- Toxicological studies showed that they were non-toxic.
- Molecular docking studies indicated the mechanism of competitive inhibition.