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Cyclophellitol based ABPs for broad-spectrum profiling of retaining exo-α-mannosidases

4.1 Introduction

Retaining exo-α-mannosidases are found in numerous organisms in nature. In humans, five different forms are present: MA2B1, MA2B2, MA2C1, MA2A1 and MA2A2, which are all part of the GH38 family. These glycosidases are capable of hydrolyzing α1-2, α1-3 or α1-6 linkages of high-, hybrid- or complex mannose N-linked glycans. Diverse biological functions have been ascribed to these glycosidases. Some are involved in the biosynthesis assembly of N-linked glycans in the Golgi system (MA2A1 and MA2A2), whereas others are present in the lysosome (MA2B1, MA2B2) or in the cytosol (MA2C1) as part of the catabolic system. Retaining exo-α-mannosidases have been associated with various pathologies. For instance, increased
expression of lysosomal α-mannosidases in larynx cancer cells was observed.\textsuperscript{9} α-Mannosidosis, a lysosomal storage disorder\textsuperscript{10,11}, results from mutations in the MAN2B1 gene. Lack of hydrolysis results in swelling of lysosomes and loss of cellular functions.\textsuperscript{12} The enzyme reaction itinerary of retaining exo-α-mannosidases takes place via a double-displacement mechanism, i.e. with retention of the configuration of the anomeric center. In general, the substrate proceeds according to the $^0S_2 \rightarrow B_{2,5} \rightarrow ^1S_5 \rightarrow B_{2,5} \rightarrow ^0S_2$ catalytic itinerary (Figure 4.1).\textsuperscript{13,14} After the substrates adapts to a $^0S_2$ skew boat conformation in the active site, an aspartic acid residue of the enzyme attacks the anomeric center and forms a covalent intermediate with the substrate after expulsion of the aglycon which is protonated by the acid/base residue. The glycosidic bond of the formed enzyme-substrate complex is then hydrolyzed, resulting in the enzyme and the α-mannose product. The catalytic nucleophile has been trapped with a fluororous substrate and analyzed by the group of Withers, who identified an aspartic acid as the catalytic nucleophile, which supports this mechanism.\textsuperscript{15,16}

\[ \text{Figure 4.1 Proposed mechanism of substrate hydrolysis by exo-α-mannosidases.}^{13,14} \]

(+)−Cyclophellitol is a covalent inhibitor of retaining β-glucosidases.\textsuperscript{17,18} Several configurational isomers have been synthesized and resulted in selective and potent inhibitors of different glycosidases.\textsuperscript{19−21} Based on these precedents, in this chapter the synthesis of α-mannopyranose-configured cyclophellitol and cyclophellitol aziridine is described (Figure 4.2). Cyclophellitol aziridine 2 was used as a starting point in the synthesis of activity-based probes (ABPs) that specifically tag retaining exo-α-mannosidases, which could help to elucidate the biological processes connected with the complex pathophysiology of α-mannosidase linked diseases.\textsuperscript{22}
4.2 Results and discussion

The synthesis of α-mannose-configurated cyclophellitol 1 and α-mannose-configurated cyclophellitol aziridine 2 was previously described. However, a one-step synthesis of aziridine 10 was investigated with the aim to improve on this synthesis, which comprised mesylation of alcohols 8 and 9, followed by α-aziridine formation by treatment with LiAlH₄, (Scheme 4.1). Staudinger reaction with compound 8 and 9 using triphenylphosphine on beads gave aziridine 10 which was easily purified by filtration. Compared to the earlier described method (22%), the yield could be considerably improved in this manner (54%). Debenzylation using Birch reduction conditions gave aziridine 2. ABPs 4 - 6 were obtained by N-alkylation of aziridine 2 with 1-azido-8-iodooctane followed by copper(I)-catalyzed [2+3]-cycloaddition ‘click’
ligation with fluorophore 11 (see Chapter 2), 12\(^{25}\) or biotin-alkyne 13\(^{26}\). As described in Chapter 2, \(N\)-alkyl aziridines are more easily synthesized compared to \(N\)-acyl aziridines. However, in the case of GH29 \(\alpha\)-fucosidases, the appropriately configured \(N\)-alkyl probes proved to considerable less effective than the corresponding \(N\)-acyl probes.\(^{27}\) Therefore and to be on the safe side, \(N\)-acyl probe 7 was synthesized as well.

**Scheme 4.1** Synthesis of \(\alpha\)-mannopyranose-configured aziridine inhibitors and ABPs.

**Reagents and conditions:** a) PPh\(_3\) polymer-bound on styrene-divinylbenzene copolymer, CH\(_3\)CN, reflux, overnight, 54%; b) Li, THF, NH\(_3\) (l), -60 °C, 75 min; c) 1-azido-8-iodooctane (see Chapter 3), K\(_2\)CO\(_3\), DMF, 80 °C, overnight, 38% over 2 steps; d) 11 (see Chapter 2), 12\(^{25}\) or 13\(^{26}\), CuSO\(_4\)·5H\(_2\)O, sodium ascorbate, DMF, rt, 4 35%, 5 8.5%, 6 16%; e) 14 (see Chapter 2), EEDQ, DMF, 0 °C, 2.5 h, 8% over 2 steps.

**Inhibitory potency of \(\alpha\)-mannopyranose-configured cyclophellitol 1 – 7.**

Having compounds 1 – 7 in hand, their inhibition potential was assessed towards GH38 jack bean (\textit{Canavalia ensiformis}) \(\alpha\)-mannosidase. The inhibitor was pre-incubated for 30 min in varying concentrations after which the residual enzymatic
activity was measured using the fluorogenic substrate 4-methylumbelliferyl \( \alpha \)-D-mannoside. The resulting apparent IC\textsubscript{50} values are listed in Table 4.1.

Table 4.1 Apparent IC\textsubscript{50} values of inhibitors 1 - 7 towards \( \alpha \)-mannosidase from GH38 jack bean.

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} ((\mu)M)</th>
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<tbody>
<tr>
<td>1 (TB440)</td>
<td>35.36 ± 6.7</td>
</tr>
<tr>
<td>2 (TB450)</td>
<td>7.19 ± 0.1</td>
</tr>
<tr>
<td>3 (TB481)</td>
<td>3.54 ± 0.5</td>
</tr>
<tr>
<td>4 (TB482)</td>
<td>3.43 ± 0.7</td>
</tr>
<tr>
<td>5 (TB521)</td>
<td>38.69 ± 4.5</td>
</tr>
<tr>
<td>6 (TB484)</td>
<td>2.22 ± 0.2</td>
</tr>
<tr>
<td>7 (TB480)</td>
<td>3.36 ± 1.3</td>
</tr>
</tbody>
</table>

The potency of \( \alpha \)-mannosidase configurated cyclophellitol 1 is in agreement with previous published data.\textsuperscript{21} As observed in Table 4.1, the cyclophellitol aziridine isomers are in general more potent compared to the cyclophellitol isomer. The introduction of a lipophilic tail increases in general the potency even more.

The next research object was to determine whether \( \alpha \)-mannosidase configured cyclophellitols have an irreversible mode of binding – a prerequisite to allow their use in ABPP experiments. Therefore, Golgi \( \alpha \)-mannosidase II from drosophila (GH38) was soaked with aziridine 2. Indeed, the catalytic nucleophile (Asp 204\textsuperscript{13}) opened the aziridine moiety and a covalent enzyme-2 complex was formed. As depicted in Figure 4.3, the cyclohexane adopts a skew boat (\(1S_5\)) as expected according to the proposed itinerary of this class of \( \alpha \)-mannosidases.\textsuperscript{13,14}

Figure 4.3 Crystal structure of Golgi \( \alpha \)-mannosidase II from drosophila (GH38) in complex with aziridine 2 (left). Proposed conformation of the substrate in GH38 retaining exo-\( \alpha \)-mannosidases (right).
Finally, ABPP was performed in commercial available GH38 jack bean α-mannosidase with compound 4 (Figure 4.4). The pH dependent labeling efficiency was investigated by treatment of 4 with the enzyme at various pH followed by analyzing the samples by SDS-PAGE and subsequently fluorescence scanning of the gels. Indeed, labeling of both subunits was observed (66 kDa and 44 kDa). The pH dependent labeling corresponds with the pH dependent activity of jack bean α-mannosidase.

Figure 4.4 ABPP of GH38 jack bean α-mannosidase with 10 μM compound 4 at various pH at 37 °C. After incubation for 2 h, the samples were denatured, resolved by SDS-PAGE and scanned for Cy5 fluorescence followed by Coomassie Brilliant Blue (CBB) staining.

4.3 Conclusion

In this chapter α-mannopyranose-configured cyclophellitol aziridine based inhibitors and probes (4 – 7) were synthesized in order to specifically target retaining exo-α-mannosidases. A fluorogenic substrate activity assay was performed to evaluate the inhibition potency of these probes, which revealed apparent IC₅₀ values in the low micromolar range towards α-mannosidase from jack bean. Next, a covalent enzyme-aziridine 2 complex was obtained, supporting the irreversible mode of binding of this class of compounds in GH38 glycosidases. Finally, ABPP with 4 in jack bean α-mannosidase successfully labelled both subunits in a pH dependent fashion. These results set the stage for the synthesized probes (4 – 7) to perform additional ABPP experiments in vitro and in vivo and to use these ABPs as tools to further examine α-mannosidosis and other α-mannosidase connected diseases such as larynx cancer.
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Experimental

**General:** Chemicals were purchased from Acros, Sigma Aldrich, Biosolve, VWR, Fluka, Merck and Fisher Scientific and used as received unless stated otherwise. Tetrahydrofuran (THF), N,N-dimethylformamide (DMF) and toluene were stored over flame-dried 4 Å molecular sieves before use. Traces of water from reagents were removed by co-evaporation with toluene in reactions that require anhydrous conditions. All reactions were performed under an argon atmosphere unless stated otherwise. TLC analysis was conducted using Merck aluminum sheets (Silica gel 60 F254) with detection by UV absorption (254 nm), by spraying with a solution of (NH4)6Mo7O24·4H2O (25 g/L) and (NH4)4Ce(SO4)4·2H2O (10 g/L) in 10% sulfuric acid or a solution of KMnO4 (20 g/L) and K2CO3 (10 g/L) in water, followed by charring at ~150 °C. Column chromatography was performed using Screening Device b.v. Silica Gel (particle size of 40 – 63 μm, pore diameter of 60 Å) in the indicated solvents. For reversed-phase HPLC purifications an Agilent Technologies 1200 series instrument equipped with a semi-preparative column (Gemini C18, 250 x 10 mm, 5 μm particle size, Phenomenex) was used. LC/MS analysis was performed on a Surveyor HPLC system (Thermo Finnigan) equipped with a C18 column (Gemini, 4.6 mm x 50 mm, 5 μm particle size, Phenomenex), coupled to a LCQ Advantage Max (Thermo Finnigan) ion-trap spectrometer (ESI+). The applied buffers were H2O, MeCN and 1% aqueous TFA. 1H NMR and 13C NMR spectra were recorded on a Bruker AV-400 (400 and 101 MHz respectively) or a Bruker DMX-600 (600 and 151 MHz respectively) spectrometer in the given solvent. Chemical shifts are given in ppm (δ) relative to the residual solvent peak or tetramethylsilane (0 ppm) as internal standard. Coupling constants are given in Hz. High-resolution mass spectrometry (HRMS) analysis was performed with a LTQ Orbitrap mass spectrometer (Thermo Finnigan), equipped with an electrospray ion source in positive mode (source voltage 3.5 kV, sheath gas flow 10 mL/min, capillary temperature 250 °C) with resolution R = 60000 at m/z 400 (mass range m/z = 150 – 2000) and dioctyl phthalate (m/z = 391.28428) as a “lock mass”. The high-resolution mass spectrometer was calibrated prior to measurements with a calibration mixture (Thermo Finnigan).

To a mixture of 8 and 9 (0.193 g, 0.332 mol) in MeCN (3.3 mL) was added PPh3 polymer-bound on styrene-divinylbenzene copolymer (0.188 g, 0.564 mmol, 3 mmol/g). After stirring at reflux conditions overnight, the reaction mixture was concentrated in vacuo. Purification over silica gel column chromatography (1% MeOH in DCM) gave cyclophellitol aziridine 10 (95.6 mg, 0.178 mmol, 54%) as a yellow oil. 1H NMR (400 MHz, CDCl3) δ 7.42 – 7.17 (m, 20H), 4.93 (d, J = 12.3 Hz, 1H), 4.84 (d, J = 11.3 Hz, 1H), 4.76 – 4.57 (m, 3H), 4.55 – 4.34 (m, 3H), 4.21 (t, J = 2.2 Hz, 1H), 3.80 – 3.65 (m, 2H), 3.61 (dd, J = 9.1, 4.0 Hz, 1H), 3.50 (t, J = 8.7 Hz, 1H), 2.42 (dd, J = 5.8, 2.3 Hz, 1H), 2.33 (d, J = 5.8 Hz, 1H), 2.30 – 2.22 (m, 1H). 13C NMR (101 MHz, CDCl3) δ 139.2, 139.0, 139.0, 138.5, 128.4, 128.4, 128.3, 128.1, 127.8, 127.7, 127.6, 127.5, 127.5, 80.8, 75.6, 75.2, 74.7, 73.3, 73.0, 73.0, 70.9, 43.4, 34.5, 31.8. HRMS: calculated for [C32H36NO4]+ 536.27954, found 536.27923.
Liquid NH$_3$ (30 mL) was obtained by condensation of gas NH$_3$ at -60 °C. Lithium (140 mg) was added and the mixture was stirred for 30 min, resulting in a dark blue solution. Aziridine 10 (149 mg, 278 mmol) was dissolved in THF (7 mL) and slowly added to the reaction mixture. After stirring for 75 min, the reaction was quenched with milliQ-H$_2$O (4 mL). The solution was allowed to come to room temperature followed by concentrating in vacuo. The crude mixture was taken up in milliQ-H$_2$O and neutralized with Amberlite IR H$^+$. Product bound on the resin was washed with milliQ-H$_2$O followed by elution with NH$_4$OH (1M). The basic solution was concentrated in vacuo and dissolved again in milliQ-H$_2$O. Addition of Amberlite-NH$_4^+$, followed by elution with milliQ-H$_2$O and concentration in vacuo gave cyclophellitol aziridine 2 as a clear oil which was used without further purification. $^1$H NMR (400 MHz, D$_2$O) $\delta$ 4.34 (s, 2H), 3.89 (dd, $J$ = 11.1, 3.4 Hz, 1H), 3.72 (dd, $J$ = 10.9, 7.6 Hz, 1H), 3.51 – 3.42 (m, 2H), 2.50 (d, $J$ = 5.5 Hz, 1H), 2.31 (d, $J$ = 5.7 Hz, 1H), 1.87 (s, 1H). $^{13}$C NMR (101 MHz, D$_2$O) $\delta$ 70.5, 68.3, 66.5, 62.0, 45.0, 35.2, 31.0.

Cyclophellitol aziridine 2 (0.2 mmol), 1-azido-8-iodooctane (see Chapter 3, 0.24 mmol, 67 mg) and K$_2$CO$_3$ (118 mg, 0.86 mmol) in DMF (2 mL) was stirred at 80 °C. After stirring overnight the reaction mixture was concentrated in vacuo and purification over silica gel column chromatography (12% MeOH in DCM) gave azide-cyclophellitol aziridine 3 (25.2 mg, 76 μmol, 38% over two steps) as a clear oil. $^1$H NMR (400 MHz, MeOD) $\delta$ 4.27 – 4.20 (m, 1H), 3.92 (dd, $J$ = 10.6, 4.2 Hz, 1H), 3.62 (dd, $J$ = 10.6, 8.4 Hz, 1H), 3.44 (dd, $J$ = 10.2, 3.4 Hz, 4H), 3.38 (d, $J$ = 9.2 Hz, 1H), 3.30 (t, $J$ = 3.4 Hz, 2H), 2.38 – 2.23 (m, 2H), 1.89 – 1.81 (m, 2H), 1.73 (d, $J$ = 6.1 Hz, 1H), 1.66 – 1.57 (m, 4H), 1.44 – 1.35 (m, 8H). $^{13}$C NMR (101 MHz, MeOD) $\delta$ 72.8, 69.7, 68.2, 63.9, 61.8, 52.4, 47.0, 45.9, 41.7, 30.6, 30.2, 29.9, 28.3, 27.8. HRMS: calculated for [C$_{15}$H$_{29}$N$_4$O$_4$]$^+$ 329.21833, found 329.21822.

**General click procedure**

To a solution of aziridine-azide (1 equiv.) and desired alkyne (1 equiv.) in DMF (2.0 mL) were added copper(II)-sulfate pentahydrate (0.66 equiv., 1 M in H$_2$O) and sodium ascorbate (0.76 equiv., 1 M in H$_2$O). After stirring overnight at room temperature, the reaction mixture was concentrated in vacuo, purified with HPLC (linear gradient, A: 50 mM NH$_4$HCO$_3$ in H$_2$O, B: MeCN, in 12 min) and lyophilized.

**ABP 4 (TB482)**

Azidirine-azide 3 (7.0 mg, 21 μmol) and Cy5-alkyne 11 (see Chapter 2, 11.7 mg, 21 μmol) were treated according to General Click Procedure. Purification by HPLC (44% → 50%, A
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in B) followed by lyophilizing gave ABP 4 (6.44 mg, 7.3 μmol, 35%) as a blue powder. 1H NMR (500 MHz, MeOD) δ 8.29 – 8.21 (m, 2H), 7.84 (s, 1H), 7.50 (d, J = 7.2 Hz, 2H), 7.46 – 7.37 (m, 2H), 7.33 – 7.23 (m, 4H), 6.62 (s, J = 12 Hz, 1H), 6.28 (d, J = 13.6 Hz, 2H), 4.41 (s, 2H), 4.37 (t, J = 7.0 Hz, 2H), 4.22 – 4.16 (m, 1H), 4.09 (t, J = 7.4 Hz, 2H), 3.89 (dd, d = 10.6, 4.1 Hz, 1H), 3.63 (s, 3H), 3.59 (dd, J = 10.6, 8.4 Hz, 1H), 3.40 (dd, J = 10.2, 3.5 Hz, 1H), 3.35 (t, J = 5.0 Hz, 1H), 2.30 – 2.19 (m, 4H), 1.90 (s, 2H), 1.89 – 1.84 (m, 2H), 1.84 – 1.78 (m, 4H), 1.74 – 1.70 (m, 12H), 1.68 (d, J = 6.5 Hz, 1H), 1.55 – 1.49 (m, 2H), 1.49 – 1.43 (m, 2H), 1.36 – 1.26 (m, 8H). 13C NMR (126 MHz, MeOD) δ 175.7, 175.4, 174.6, 155.6, 146.2, 144.3, 143.5, 142.6, 142.5, 129.8, 129.7, 126.6, 126.3, 126.2, 124.1, 123.4, 123.3, 112.0, 111.9, 104.4, 104.2, 72.8, 69.7, 68.2, 63.9, 61.8, 51.3, 50.6, 49.5, 49.3, 49.2, 49.0, 48.8, 48.7, 48.5, 47.0, 45.9, 44.8, 41.7, 36.5, 35.6, 31.5, 31.3, 30.4, 29.9, 28.2, 28.1, 28.0, 27.8, 27.4, 27.3, 26.4. LC/MS analysis: Rt 6.10 min (linear gradient 10→90% B in 12.5 min), m/z 848.33 [M] +. HRMS: calculated for [C50H70N7O5]+ 848.54329, found 848.54294.

ABP 5 (TB582)

Azidirine-azide 3 (8.0 mg, 24 μmol) and Bodipy 1225 (8.0 mg, 24 μmol) were treated according to General Click Procedure. Purification by HPLC (42% → 48%, A in B) followed by lyophilizing gave ABP 5 (1.34 mg, 2.05 μmol, 8.5%) as an orange powder. 1H NMR (600 MHz, MeOD) δ 7.74 (s, 1H), 6.12 (s, 2H), 4.35 (t, J = 6.9 Hz, 2H), 4.20 (dd, J = 3.3, 1.9 Hz, 1H), 3.62 – 3.59 (m, 1H), 3.58 – 3.51 (m, 1H), 3.49 – 3.44 (m, 1H), 3.41 (dd, J = 10.2, 3.5 Hz, 1H), 3.07 – 3.00 (m, 2H), 2.79 (t, J = 7.2 Hz, 2H), 2.44 (s, 6H), 2.39 (s, 6H), 2.31 – 2.19 (m, 2H), 1.95 – 1.79 (m, 6H), 1.70 – 1.63 (m, 3H), 1.55 – 1.49 (m, 2H), 1.35 – 1.26 (m, 8H). LC/MS analysis: Rt 6.13 min (linear gradient 10→90% B in 12.5 min), m/z 657.00 [M + H] +. HRMS: calculated for [C34H52BF2N6O4]+ 657.41117, found 657.41122.

ABP 6 (TB484)

Azidirine-azide 3 (7.2 mg, 22 μmol) and biotin 13 (9.1 mg, 25 μmol) were treated according to General Click Procedure. Purification by HPLC (18% → 24%, A in B) followed by lyophilizing gave ABP 6 (2.6 mg, 3.6 μmol, 16%, purity > 80%) as a white powder. LC/MS analysis: Rt 3.91 min (linear gradient 10→90% B in 12.5 min), m/z 723.13 [M + H] +. 1H NMR (600 MHz, MeOD) δ 7.84 (s, 1H), 4.49 (dd, J = 7.8, 4.8 Hz, 1H), 4.42 (s, 2H), 4.38 (t, J = 7.0 Hz, 2H), 4.30 (dd, J = 7.8, 4.5 Hz, 1H), 4.20 (dd, J = 3.2, 1.9 Hz, 1H), 3.90 (dd, J = 10.7, 4.1 Hz, 1H), 3.70 – 3.66 (m, 1H), 3.58 – 3.55 (m, 3H), 3.53 (dd, J = 10.7, 6.3 Hz, 1H), 3.46 – 3.39 (m, 1H), 3.37 – 3.34 (m, 1H), 3.21 (dd, J = 8.8, 5.0 Hz, 1H), 3.16 (td, J = 6.9, 2.2 Hz, 2H), 2.92 (dd, J = 12.7, 5.0 Hz, 1H), 2.70 (d, J = 12.7 Hz, 1H), 2.33 – 2.28 (m, 1H), 2.25 – 2.17 (m, 5H), 1.89 (s, 2H), 1.86 – 1.81 (m, 2H), 1.71 – 1.69 (m, 1H), 1.66 – 1.55 (m, 4H), 1.53 – 1.46 (m, 2H), 1.46 – 1.40 (m, 2H), 1.39 – 1.26 (m, 9H). HRMS: calculated for [C34H59N8O7S]+ 723.42219, found 723.42225.
ABP 7 (TB480)

Cy5-acid 14 (see Chapter 2, 63 mg, 85 μmol) was pre-activated with EEDQ (21 mg, 85 μmol) in DMF (2 mL) for 3 h at room temperature. Deprotected aziridine 2 (0.10 mmol) was dissolved in DMF (0.4 mL) and cooled to 0 °C, after which activated ester solution (1.0 mL, 30 μmol, 0.5 equiv.) was added. After stirring at 0 °C for 30 min, another 0.5 equiv. of the activated ester was added. The reaction mixture was stirred for another 2 h at 0 °C before the reaction mixture was quenched with MeOH and concentrated in vacuo. Purification by HPLC under neutral conditions with use of a gradient of 43 → 49% MeCN in H2O in 12 min followed by lyophilizing yielded ABP 7 (7.0 mg, 7.8 μmol, 8% over 2 steps) as a blue powder. 1H NMR (600 MHz, MeOD) δ 8.26 (t, J = 13.0 Hz, 2H), 7.82 (s, 1H), 7.51 (d, J = 7.4 Hz, 2H), 7.49 – 7.42 (m, 2H), 7.34 – 7.25 (m, 4H), 6.64 (t, J = 12.5 Hz, 1H), 6.30 (d, J = 13.7 Hz, 2H), 4.42 (s, 2H), 4.39 (t, J = 7.0 Hz, 2H), 4.31 – 4.27 (m, 1H), 4.11 (t, J = 7.5 Hz, 2H), 3.95 – 3.89 (m, 1H), 3.64 – 3.62 (m, 1H), 3.50 – 3.42 (m, 2H), 2.89 (d, J = 5.6, 2.1 Hz, 1H), 2.78 (d, J = 5.4 Hz, 1H), 2.49 – 2.36 (m, 2H), 2.26 (t, J = 7.3 Hz, 2H), 2.07 – 1.94 (m, 3H), 1.90 – 1.87 (m, 2H), 1.85 – 1.82 (m, 2H), 1.74 (s, 1H), 1.65 – 1.58 (m, 2H), 1.50 – 1.45 (m, 2H), 1.36 – 1.29 (m, 6H). 13C NMR (151 MHz, MeOD) δ 187.9, 180.2, 175.7, 175.4, 174.6, 155.5, 154.4, 146.1, 144.2, 143.5, 142.6, 142.5, 129.7, 129.7, 126.6, 126.3, 126.2, 124.1, 123.4, 123.2, 112.0, 111.8, 104.4, 104.2, 72.3, 69.1, 68.0, 63.3, 51.3, 50.5, 46.9, 44.7, 42.6, 39.0, 37.2, 36.4, 35.5, 31.5, 31.2, 29.9, 29.6, 28.1, 27.9, 27.8, 27.3, 27.2, 26.4, 25.9. LC/MS analysis: R<sub>t</sub> 6.44 min (linear gradient 10→90% B in 12.5 min), m/z 862.33 [M + H]<sup>+</sup>. HRMS: calculated for [C<sub>50</sub>H<sub>68</sub>N<sub>7</sub>O<sub>6</sub>]<sup>+</sup> 862.52256, found 862.52240.

Determination of the apparent IC<sub>50</sub> values

For the determination of apparent IC<sub>50</sub> values of the inhibitors, α-mannosidase from Jack bean, (Canavalia ensiformis, Sigma Aldrich) 4 ng total protein was equilibrated in 12.5 μL McIlvaine buffer (150 mM, pH 4.5) for 5 min on ice. After which it was incubated with 12.5 μL inhibitor dilutions prepared in McIlvaine buffer for 30 min at 37 °C. After addition of 100 μL of 4-methylumbelliferyl-β-D-mannopyranoside substrate mixture (10 mM 4-mu-α-D-mannopyranoside in McIlvaine buffer) and incubation for 30 min at 37 °C, the reaction was quenched by adding 200 μL 1M Glycine-NaOH (pH 10.3) to the reaction mixture. The 4-mu fluorescence was measured in 96-well plates using a LS55 fluorometer (PerkinElmer) at λ<sub>Ex</sub> = 366 nm and λ<sub>Em</sub> = 445 nm. Measured activity values were subtracted with background values, normalized against control values (no inhibitor), and plotted against the inhibitor concentrations. IC<sub>50</sub> values were calculated with Prism 7.0 (Graphpad), using one-phase-exponential decay function. Standard deviations were obtained from 2 sets of calculated IC<sub>50</sub> values, each derived from 3 technical repeats at each inhibitor concentrations.
Cloning and expression of dGMII

A plasmid containing cDNA encoding for the dGMII gene was obtained with kind permission from Dr. Sean Sweeney (University of York). From this plasmid, cDNA encoding for amino acids 76-1108 of dGMII (to remove the N-terminal cytosolic, transmembrane, and stalk domains) was subcloned into the pOMNI-Bac vector (Geneva Biotech), behind a honeybee mellitin secretion peptide, 6xHis tag, and TEV cleavage site.

Recombinant bacmid was produced using the Tn7 transposition method in DH10EMBacY (Geneva Biotech)\(^3\), and purified using the PureLink miniprep kit (Invitrogen) following standard protocols. V1 baculovirus was produced by transfection of bacmid into low passage adherent Sf21 cells (Invitrogen) using FuGENE HD transfection reagent (Promega), at a ratio of 2 \(\mu\)g DNA to 4.5 \(\mu\)L FuGENE. V1 \(\rightarrow\) V2 virus amplification was carried out using suspension Sf21 cells, using the YFP marker present in EMBacY baculovirus to determine optimum amplification prior to harvesting (typically ~60% cells fluorescent). For expression, T. Ni cells (Invitrogen) were infected with V2 baculovirus at a MOI>1, and infection followed using the EMBacY YFP marker to determine optimum timepoint for harvesting (typically 72 h, with > 80 % cells fluorescent). All insect cells used tested negative for mycoplasma contamination.

Purification of dGMII

Harvested High Five cultures were spun for 15 minutes at 200 g to remove cells and spun again at 4000 g for 1 hour to remove insoluble cellular components. AEBSF (to a final concentration of 0.1 mM) and DDT (to a final concentration of 1 mM) were added to the supernatant. Clarified supernatant was loaded on to 2 x 5mL HiTrap Blue HP columns (GE Healthcare) pre-equilibrated in blue agarose buffer A (20 mM HEPES pH 7.4, 100 mM NaCl, 1 mM DTT). Loaded columns were washed with 5 column volumes of blue agarose buffer A and eluted using a linear gradient of blue agarose buffer B (20 mM HEPES pH 7.4, 2 M NaCl, 1 mM DTT) over 20 column volumes.

HiTrap Blue fractions containing dGMII (as determined by SDS-PAGE) were pooled and diluted approximately 5-fold in HisTrap buffer A (50 mM HEPES pH 8.0, 0.5 M NaCl, 30 mM Imidazole, 1 mM DTT) and loaded onto a 1 mL HisTrap FF Crude column (GE Healthcare). The loaded histrap column was washed with 10 column volumes of HisTrap buffer A and dGMII eluted using a linear gradient of HisTrap buffer B (50 mM HEPES pH 8.0, 0.5 M NaCl, 0.5 M imidazole, 1 mM DTT) over 20 column volumes, followed by 10 column volumes of 100 % Buffer B.

HisTrap fractions containing dGMII were pooled and concentrated to less than 2 ml by centrifugation using VivaSpin 30,000 MW concentrator (GE Healthcare). Pooled dGMII was rediluted to ~2 ml in 1x AcTEV (Invitrogen) reaction buffer before addition of 5 \(\mu\)l AcTEV protease to remove the N-His-TEV tag. Digests were typically carried out overnight at ambient temperature, and reaction progress assessed by comparison to positive and negative controls using SDS-PAGE.

Upon completion of the AcTEV digest, as indicated by SDS-PAGE, dGMII was purified using a S200 16/600 size exclusion chromatography (SEC) column equilibrated in SEC buffer (50 mM HEPES pH 7.4, 200 mM NaCl, 1 mM DTT), at 1 ml min\(^{-1}\). SEC fractions containing dGMII were pooled, concentrated to 10 mg ml\(^{-1}\) and stored in 20 \(\mu\)l aliquots at -80 °C.
3D Crystallography of dGMII

Initial crystallization conditions were screened using JCSG+ HT-96, PACT premier HT-96 (both Molecular Dimensions), Index HT and PEG/Ion HT (both Hampton Research) commercial screens. Hits were optimized, scaled up to maxi 48-well plates and a dGMII seed stock generated from crystals grown in 0.1 M imidazole pH 7.0 and 10 % (w/v) PEG 3350 using the Seed Bead protocol (Hampton Research). The above screens were repeated with seeding using an Oryx8 (Douglas Instruments) and additional hits optimised further. Diffraction quality dGMII crystals were grown in maxi 48-well plates using sitting drop vapour diffusion in 0.1 M sodium succinate pH 7.0 and 10 % (w/v) PEG 3350. Crystals were cryoprotected using cryoprotectant solution (mother liquor supplemented with 25% v/v ethylene glycol) prior to flash freezing in liquid N\textsubscript{2} for data collection.

Datasets were collected using beamline I04-1 at the Diamond Light Source, processed via with the xia2 pipeline\textsuperscript{31} of the CCP4 software suite, using XDS\textsuperscript{32} and Aimless\textsuperscript{33} for data processing. Apo dGMII was solved by molecular replacement with PDB model 1HWW\textsuperscript{34} using MolRep\textsuperscript{35}, followed by alternating rounds of manual model building and refinement using Coot and REFMAC5 respectively\textsuperscript{36,37}. CCP4mg was used to produce figures of the crystal structure.

For ligand complexes, dGMII crystals were soaked in solutions of TB450 (1 mM) or TB482 (1 mM) in dGMII cryoprotectant solution for ~3 hours before flash freezing in liquid N\textsubscript{2} for data collection. Complexes were solved by molecular replacement with the apo dGMII structure, followed by rounds of manual model building and refinement using Coot and REFMAC5. Ligand coordinates were built using jLigand.\textsuperscript{38} Diagrams of dGMII-ligand complexes were generated using CCP4mg\textsuperscript{39}.

Labeling of jack bean α-mannosidase with TB482 (4) at varying pH

Jack bean α-mannosidase (Sigma Aldrich, 1 pmol) was equilibrated in 10 μL McIlvaine buffer at varying pH (2.5 – 8.0) at 37 °C for 15 min. After incubation with 10 μM TB482 (4, end concentration; prepared in McIlvaine buffer at pH 2.5 – 8.0) at 37 °C for 2 h, the sample was denatured, resolved by SDS-PAGE and scanned for Cy5 fluorescence with a Typhoon FLA 9500 imager (GE Healthcare).

References


Cyclophellitol based ABPs for broad-spectrum profiling of retaining exo-α-mannosidases


