In vitro pharmacological profiling of selected small molecules compound using recombinant human iduronate-2-sulphatase


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Abstract

Background: Mucopolysaccharidoses type II (MPS II) is an X-linked lysosomal storage disease (LSD). It is due to mutation in IDS gene encoding iduronate-2-sulphatase (IDS) involved in the catabolism of dermatan sulphate and heparan sulphate. Currently, the treatments for MPS II patients are enzyme replacement therapy (ERT) and bone marrow transplantation (BMT). However, ERT is not effectively reducing the central nervous system manifestation and finding the suitable donor maybe quite challenging in BMT. Over the past decades, pharmacological chaperone has been an alternative approach for management of MPS II patient. Here, we described the in vitro profiling of small molecules in group of chondroitin/dermatan (CD) sulphate disaccharide, heparin oligosaccharides, unsaturated heparin disaccharides and 6-O-desulphated heparin oligosaccharide, using recombinant human iduronate-2-sulphatase (rhIDS). Twenty-one small molecule compounds with several concentrations were each screened by inhibition and thermal stability assays.

Results: Our study revealed that condroitin dermatan trisulphate (CD3S), heparin tetrasaccharide (H4Sac), heparin octasaccharide (H8Sac) and heparin octadecasaccharide (H18Sac) showed high inhibition constant, K_i and low inhibition concentration, IC_50 in comparison to others. In the thermal stability study, only rhIDS incubated with CD3S was found to preserve enzyme activity (20%) after incubated at 67°C.

Conclusion: Overall, our experiments discovered that CD3S was able to bind, inhibit and chaperone rhIDS. These features suggest a potential pharmacological chaperone for MPS II.

Keywords: Lysosomal storage diseases, Mucopolysaccharidoses Type II, pharmacological chaperone, iduronate-2-sulphatase.
Introduction

Mucopolysaccharidoses (MPS) is a group of rare genetic disease in lysosomal storage disease (LSD) which is characterised by accumulation of glycosaminoglycans (GAGs) in the lysosome. It is caused by enzymes defect during the degradation of GAGs pathway. Each type of MPS has a broad heterogeneity of manifestations which depends on underlying gene defect. Gene rearrangements or frame shift/terminus mutations presented as severe MPS whereas missense mutations presented as mild [1,2,3,4]. MPS can be divided into several types: MPS type I, type II, type III (subtype IIIA, IIIB, IIIC and IIID), type IV (subtype IVA and IVB), type VI and type VII.

MPS type II (MPS II), also known as Hunter syndrome is inherited in an X-linked recessive pattern. This is because of a defective enzyme called iduronate-2-sulfatase (IDS), a lysosomal enzyme (EC 3.1.6.13). Belong to the sulfatase family, IDS catalyses hydrolysis of the C2-sulfate ester bond at the non-reducing end of 2-O-sulpho-L-iduronic acid residues in the degradation of GAGs' heparan sulphate (HS) and dermatan sulphate (DS) [1].

More dominant in male, MPS II is more common in Asian countries compared to MPS I which is more common in Caucasian population [7,10,11]. Like other types of MPS, patients with MPS II can present with wide range of clinical presentation. Stiff joint, short stature, mental retardation, hepatomegaly, splenomegaly as well as coarse facial features may be observed in severe form of MPS II, and death mostly occurred before 15 years of age. Patients with mild presentation, however, may survive longer due to the slow progression of the disease [10].

Currently, the treatments for MPS II are enzyme replacement therapy (ERT) and bone marrow transplantation (BMT). In MPS I, the approach of using BMT or hematopoietic stem cell transplantation (HSCT) is widely used. However, its effectiveness in MPS II is still controversial due to high morbidity rate caused by limited numbers of matched donors [11].

ERT, on the other hand, can be seen as curative treatment to the patients. Using recombinant human IDS (Elaprase, Shire Pharmaceuticals) as ERT is effective in reducing peripheral symptoms in mild disease form. Recipients were reported of showing improved excretion, liver and spleen size reduction and some bone remodelling [12]. However, in severe form, ERT cannot prevent or reverse the characteristics of cardiac and neurological deterioration. This is due to the inability of ERT molecules to cross the blood brain barriers and therefore the treatment remains palliative for many patients with severe presentation [13].

Recently, pharmacological chaperone (PC) therapies have been suggested and considered as a potential treatment for many genetic disorders caused by misfolded and/or unstable proteins [14]. PCs are low-molecular-weight molecules which intended to selectively bind and stabilize mutant proteins by folding into active site of the misfolded mutant proteins, thus liberating it from the endoplasmic reticulum-associated degradation. Most of PCs have the potential to be orally available with broad biodistribution especially central nervous systems (CNS) due to their small size.

The aim of our study was to assess the inhibition properties of small molecules compound towards the activity of IDS in order to evaluate the therapeutic potential of a pharmacological chaperone therapy (PCT) as drugs for the treatment of MPS II. In the present study, we described the in vitro profiling of small molecules in group of chondroitin/dermatan (CD) sulphate disaccharides, heparin oligosaccharides, unsaturated heparin disaccharides standard and 6-O-desulphated heparin oligosaccharide, using recombinant human iduronate-2-sulfatase (rhIDS). The approach used for identification and characterisation of the small molecules was based on cellular biochemical studies and in silico analysis of the interaction between the compounds and the protein.

Methodology

Chemicals

Small molecules chondroitin/dermatan sulphate disaccharide group (ΔUA,2S – GalNAc,4S (ΔDi – diSB), ΔUA,2S – GalNAc,6S (ΔDi – diSD), ΔUA,2S – GalNAc,4S,6S (ΔDi – triS) and ΔUA,2S – GalNAc (ΔDi – UA2S)), heparin oligosaccharides group (heparin tetrasaccharide, heparin octasaccharide, heparin decasaccharide, heparin dodecasaccharide, heparin tetradecasaccharide, heparin hexadecasaccharide, heparin octadecasaccharide and heparin icosasaccharide), unsaturated heparin disaccharides standard group (heparin disaccharide standard 1, heparin disaccharide standard 2, heparin disaccharide standard 3 and heparin disaccharide standard 7) and 6-O-desulphated heparin oligosaccharides group (desulphated 4S heparin oligosaccharide, desulphated 6S heparin oligosaccharide, desulphated 8S heparin oligosaccharide, desulphated 10S heparin oligosaccharide and desulphated 12S heparin oligosaccharide) were purchased from Iduron (Manchester, UK). Recombinant human iduronate-2-sulfatase (rhIDS) was purchased from R&D Systems (Minneapolis, USA). Substrate 4-nitrocatechol sulphate was purchased from Sigma Aldrich (Milwaukee, USA).

Inhibition Assay

All 21 small molecules were diluted into several desired concentrations before incubated with rhIDS for 10 minutes at 0°C. A volume of 50 µL of 2 mM p-nitrocatechol sulphate (pNCS) was added into 50 µL of each concentration of the respective small molecules in the microplate. The plate was incubated at 37°C for 24 hours before the reaction was terminated with 100 µL of 0.2 M sodium hydroxide. Liberated product of p-nitrocatechol (pNC) was measured using spectrophotometer at 515nm.

Thermal Stability Assay

To analyse IDS thermal stability, 1 µM rhIDS was dissolved in phosphate citrate buffer (200 mM Na2HPO4 and 200 mM citric acid, pH 4.0) supplemented with 19 µM heparin tetrasaccharides (H4Sac), 96 µM heparin octasaccharides (H8Sac), 7 µM heparin octadecasaccharides (H18Sac) and 44 µM chondroitin dermanat tri saccharide (CD3S). Subsequently, rhIDS

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solutions were heated at 37, 47, 57 and 67°C respectively, for 1 hour. A volume of 50 µL each of respective small molecules were heated at various temperatures and added into 50 µL of 2 mM pNCS and lastly, incubated at 37°C for 24 hours before the reaction was terminated with 100 µL of 0.2 M sodium hydroxide. Liberated product of pNC was measured using spectrophotometer at 515nm.

Statistical Analysis

All experiments were performed at least in triplicate. Data are expressed as mean ± SD. Kinetic parameters were determined by double-reciprocal plot method of Lineweaver-Burk to determine the inhibition concentration, IC$_{50}$ and inhibition constant, K$_i$ (Sigmaplot Version 14, San Jose, CA, USA).

Results

Selection of potential candidate

The effectiveness of PC is based on a general principle of enzyme inhibition potency. The inhibitory activities of all 21 small molecules against IDS were evaluated through biology assay to investigate the IC$_{50}$ and K$_i$ with different concentrations into rhIDS. The results of IC$_{50}$ and K$_i$ were tabulated in Table 1. Our study showed that the ten lowest IC$_{50}$ values of the investigated small molecules heparin octadecasaccharide, heparin icosasaccharide, heparin hexadecasaccharide, desulphated 10S heparin oligosaccharide, heparin tetradecasaccharide, desulphated 6S heparin oligosaccharide and heparin dodecasaccharide ranged from 7 µM to 21 µM (Table 1).

<table>
<thead>
<tr>
<th>Small Molecule Compounds</th>
<th>Molecular weight</th>
<th>IC$_{50}$ (µM)$^a$</th>
<th>K$_i$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condroitin/Dermatan Sulphate Disaccharide, ∆UA,2S – GalNAc,4S (∆Di – diSB)</td>
<td>605</td>
<td>221.9 ± 19.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Condroitin/Dermatan Sulphate Disaccharide, ∆UA,2S – GalNAc,6S (∆Di – diSD)</td>
<td>605</td>
<td>395.3 ± 74.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Condroitin/Dermatan Sulphate Disaccharide, ∆UA,2S – GalNAc,4S,6S (CD3S)</td>
<td>707</td>
<td>44.0 ± 3.7</td>
<td>24.4</td>
</tr>
<tr>
<td>Condroitin/Dermatan Sulphate Disaccharide, ∆UA,2S – GalNAc (ADi – UA2S)</td>
<td>503</td>
<td>544.2 ± 21.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Heparin Disaccharide Standard 1</td>
<td>665</td>
<td>344.0 ± 50.7</td>
<td>16.6</td>
</tr>
<tr>
<td>Heparin Disaccharide Standard 2</td>
<td>563</td>
<td>117.6 ± 10.4</td>
<td>6.5</td>
</tr>
<tr>
<td>Heparin Disaccharide Standard 3</td>
<td>605</td>
<td>800.2 ± 141.3</td>
<td>4.2</td>
</tr>
<tr>
<td>Heparin Disaccharide Standard 7</td>
<td>503</td>
<td>471.0 ± 76.2</td>
<td>7.0</td>
</tr>
<tr>
<td>Heparin tetrasaccharide (H4Sac)</td>
<td>1200</td>
<td>19.5 ± 1.5</td>
<td>59.3</td>
</tr>
<tr>
<td>Heparin octasaccharide (H8Sac)</td>
<td>2400</td>
<td>65.6 ± 0.4</td>
<td>34.4</td>
</tr>
<tr>
<td>Heparin decasaccharide</td>
<td>3000</td>
<td>24.3 ± 2.4</td>
<td>11.4</td>
</tr>
<tr>
<td>Heparin dodecasaccharide</td>
<td>3550</td>
<td>21.0 ± 0.7</td>
<td>9.3</td>
</tr>
<tr>
<td>Heparin tetradecasaccharide</td>
<td>4100</td>
<td>13.6 ± 0.7</td>
<td>8.4</td>
</tr>
<tr>
<td>Heparin hexadecasaccharide</td>
<td>4650</td>
<td>10.6 ± 4.8</td>
<td>7.3</td>
</tr>
<tr>
<td>Heparin octadecasaccharide (H18Sac)</td>
<td>5200</td>
<td>8.0 ± 3.2</td>
<td>11.6</td>
</tr>
<tr>
<td>Heparin icosaaccharide</td>
<td>5750</td>
<td>9.2 ± 4.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Desulphated 4S Heparin Oligosaccharide</td>
<td>1060</td>
<td>141.5 ± 27.1</td>
<td>15.6</td>
</tr>
<tr>
<td>Desulphated 6S Heparin Oligosaccharide</td>
<td>1590</td>
<td>19.6 ± 2.2</td>
<td>11.2</td>
</tr>
<tr>
<td>Desulphated 8S Heparin Oligosaccharide</td>
<td>2120</td>
<td>18.2 ± 3.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Desulphated 10S Heparin Oligosaccharide</td>
<td>2650</td>
<td>13.2 ± 1.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Desulphated 12S Heparin Oligosaccharide</td>
<td>3180</td>
<td>17.6 ± 3.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

$^a$IC$_{50}$ values were measured in triplicate experiments

The top ten rhIDS’ inhibitors with the highest K$_i$ were heparin tetrasaccharide; heparin octasaccharide; condroitin/dermatan sulphate disaccharide; heparin heptasaccharide, ∆UA,2S – GalNAc,4S,6S (∆Di – triS); heparin disaccharide standard 1; desulphated 4S heparin oligosaccharide; heparin octadecasaccharide; heparin decasaccharide; desulphated
6S heparin oligosaccharide; heparin dodecasaccharide and heparin tetradecasaccharide which ranged from 59 to 8 respectively.

Four potential small molecules eventually were chosen as the good candidates for PC in MPS II patients were condroitin/dermatan sulphate disaccharide AU(A,2S – GalNAc,4S,6S (ΔDi – triS) (CD3S), heparin tetrascaccharide (H4Sac), heparin octacaccharide (H8Sac) and heparin octadecasaccharide (H18Sac). The K_i and IC_50 for CD3S, H4Sac, H8Sac and H18Sac were 44 µM and 24; 19.5 µM and 59.3; 65.6 µM and 34.4; and 8.0 µM and 11.6, respectively. Since, they were conformed to both criteria of IC_50 and K_i, these selected four small molecules were subjected for thermal stability of rhIDS study.

**Thermal stability of small molecules**

The thermal stability of rhIDS experiment was conducted to determine which small molecules can protect the enzyme from heat-induced inactivation. After incubation with H004, H008 and H018, the activities of rhIDS were totally suppressed and denatured when reaching 67°C with PC’s concentration ranging from 7 to 95 µM (Figure 1). Nevertheless, activity of rhIDS was observed to present within 20% when incubated with CD3S (43 µM) at 67°C which demonstrated that CD3S was able to stabilise the rhIDS during heat-induced inactivation process.

**Discussion**

In this study, we show that specific compounds that are likely to act as PC can enable the binding into active site of IDS and thus may provide a potential therapy for MPS II. The selection of 21 small molecules compound were based on their catalytic properties towards the active site of IDS [9].

IC_50 is referring to concentration required to produce 50% inhibition of enzyme activity. Generally, higher inhibition activity presented in a lower IC_50 value [9]. K_i also defined as inhibitor dissociation constant, is an equilibrium constant of a reversible inhibitor for complexation with its target enzyme [9]. In choosing a suitable PC, a potential candidate should possess the lowest IC_50 with the highest K_i. From the top ten of each IC_50 and K_i results, we found that most of the small molecules which have high K_i also tend to have a high IC_50 or vice versa suggests are not suitable candidates for PC. For example, the K_i for HD001 was 16.6 which is high but the IC_50 was 338.2 µM which is also relatively high indicating that this particular small molecule has a very poor inhibitory potency.

The conformational stability of rhIDS also can be assessed by thermo-denaturation process. Characteristically, a stable conformation of a protein resists denaturation as compared with fragile conformational structure which often intolerant to thermo denaturation [9]. A study by Parkinson-Lawrence et al. [10] reported that the thermal denaturation of IDS was 63°C and anything above this temperature point meant a significant loss of enzyme activity.

It has been postulated that N-linked oligosaccharides played an important role in the folding, function and stability of glycoprotein [9]. The IDS sequence contains eight putative N-linked glycosylation sites [9]. In our case, we hypothesized that the N-terminus in the structure of CD3S that may interact with any of the eight N-linked glycosylation sites in the rhIDS to stabilise the folding of the enzyme during the heat-induced activation process. The importance of N-glycosylation for folding, catalytic activity and processing of IDS has been demonstrated [9]. N-glycosylation is required for processing and enzyme activity as unglycosylated IDS precursors are inactive when being synthesized in the presence of tunicamycin.

In this study, we used ρNCS as substrate for rhIDS activity instead of 4-methylumbelliferyl-α-idurionate-2-sulphate (4-MU-Ido-2-S). ρNCS was relatively cheaper compared to 4-MU-Ido-2-S and cost-effectiveness is important as far as a large-scale screening of small molecule is concerned.

**Conclusion**

In summary, our experiments point to CD3S as a reasonable candidate PC for MPSII which met the affinity criterion suggests for developing new PC. Additionally, we showed that CD3S is able to retain enzyme activity of rhIDS during thermal stability assessment. To support these findings, our future research will investigate further the mechanism involved by using MPS II patients’ cell-based assays.

**Abbreviations**

MPS II: Mucopolysaccharidoses Type II; LSD: Lysosomal Storage Diseases; IDS: Iduronate-2-sulphatase; ERT: Enzyme Replacement Therapy; BMT: Bone Marrow Transplantation; CD: Chondroitin/Dermatan; rhIDS: Recombinant Human Iduronate sulphatase; H4Sac: Heparin Tetrascaccharide; H8Sac: Heparin Octascaccharide; H18Sac: Heparin Octadecasaccharide; GAGs: Glycosaminoglycans; MPS: Mucopolysaccharidases; HS: Heparan Sulphate; DS: Dermatan Sulphate; HSCT: Hematopoietic Stem Cell Transplantation (HSCT); PC: Pharmacological Chaperone; PCT: Pharmacological Chaperone Therapy; CNS: Central Nervous Systems; ρNCS: ρ-Nitro catechol Sulphate; ρNC: ρ-Nitrocatechol; IC_50: Inhibition Concentration of 50%; K_i: Inhibition Constant

**Declarations**

**Ethics approval and consent to participate**
All study protocol has been approved by the Medical Research and Ethics Committee (MREC), Ministry of Health Malaysia. Since this study was using commercial recombinant enzyme, the study was exempted from MREC board approval and informed consent was not needed.

Consent for publication
Not Applicable.

Availability of data and material
All data and material are available upon request.

Competing Interests
No competing financial interests exist. The authors declare no conflict of interest. The work embodied in this manuscript has not been published previously or is under consideration for publication in any other journal.

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Authors’ contribution
AO, DPAK and JAJ contributed to the study design and data interpretation. SAR, RM and FDAN contributed to data collection and analysis. All authors contributed to the final manuscript. All authors have read and approved the final manuscript.

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References
1. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol. 1973;22(23):3099-3108. doi:10.1016/0006-2952(73)90196-2

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