THE EFFECTS OF CHONDROITIN/DERMATAN SULPHATE DISACCHARIDES TOWARDS THE ACTIVITY OF IDURONATE-2-SULPHATASE

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ABSTRACT

Mucopolysaccharidoses Type II (MPS II) is a rare inherited disease caused by a mutation in *IDS* gene encoding iduronate-2-sulphatase (IDS) enzyme which is responsible for the degradation pathway of dermatan sulphate (DS) and heparan sulphate (HS). The current treatment for MPS II patients is enzyme replacement therapy (ERT) or haematopoietic stem cell transplantation (HSCT). Recently, pharmacological chaperone (PC) has been an alternative approach for managing MPS II patients. This study described the inhibition and specificity study of chondroitin/dermatan (CD) sulphate disaccharide using recombinant human iduronate-2-sulphatase (rhIDS). Potential PC labelled as ΔUA,2S-GalNAc,4S; ΔUA,2S-GalNAc,6S; ΔUA,2S-GalNAc,4S,6S and ΔUA,2S-GalNAc were diluted into several concentrations before incubated with rhIDS for 10 minutes at 0 °C. Fifty µL of 2 mM ρ-nitrocatechol sulphate was added into 50 µL of each concentration of the respective CD candidates in the microplate and incubated at 37 °C for 24 hours. The reaction was terminated with 100 µL of 0.2 M sodium hydroxide. The liberated ρ-nitrocatechol was measured using a spectrophotometer at 515 nm. The inhibition concentration, IC50 of ΔUA,2S-GalNAc,4S; ΔUA,2S-GalNAc,6S; ΔUA,2S-GalNAc,4S,6S and ΔUA,2S-GalNAc were calculated as 221 µM, 385 µM, 44 µM and 44 µM. The inhibition constant, K_i were 0.6, 3.5, 24.4 and 2.2 for ΔUA,2S-GalNAc,4S; ΔUA,2S-GalNAc,6S; ΔUA,2S-GalNAc,4S,6S and ΔUA,2S-GalNAc, respectively. In conclusion, ΔUA,2S-GalNAc,4S,6S with the lowest IC_{50} and highest K_i may become the potential small molecules as PC. Analysis of thermal stability and cell-based experiments should be the direction for future study.

KEYWORDS: Lysosomal storage diseases; mucopolysaccharidoses type II; pharmacological chaperone; iduronate-2-sulphatase

INTRODUCTION

Iduronate-2-sulphatase (IDS) (EC 3.1.6.13; L-iduronate-2-sulphatase 2-sulphohydrolase) is one of the enzymes involve in the catabolism of heparan sulphate (HS) and dermatan sulphate (DS). During the degradation of HS and DS, IDS catalyses the hydrolysis of the C2-sulphate ester bond at the non-reducing end of 2-O-sulpho-α-L-iduronic acid residues similarly with other sulphatase groups (Demydchuk et al. 2017).

Mutation on the IDS gene is translated into a defective IDS which can cause lysosomal storage disease (LSD) of mucopolysaccharidoses type II (MPS II) also known as Hunter syndrome. Patients with MPS II may present broad ranges of clinical manifestation such as short stature, stiff joints, mental retardation, organomegaly especially hepatomegaly or splenomegaly or both as well as coarse facial features (Muenzer 2011). MPS II is noted to be more common in Asia than MPS type I, which frequently occur among Caucasian populations (Neufeld and Muenzer 2001; Lin et al. 2009; Omar et al. 2019).

The two primary treatments for patients with MPS II are haematopoietic stem cell transplantation (HSCT) and enzyme replacement therapy (ERT). Treatment using HSCT exhibited a positive effect in patients with MPS II especially to the brain, provided no manifestation of developmental delays before transplantation (Tanaka et al. 2012).

Meanwhile, treatment using ERT for patients with MPS II has been commercially available since 2006 and improves the health of MPS II patients significantly (Muenzer et al. 2006; Muenzer et al. 2007; Okuyama et al. 2010). However, this intravenous ERT is unable to ameliorate the neurological complication in MPS II patients. This is due to the large size of recombinant enzymes in ERT which make it unable to cross the blood-brain barrier (BBB) (National MPS Society 2008). Currently, intrathecal or intraventricular form of ERT is being developed (Cho et al. 2015; Seo et al. 2021); however, its progress is still under clinical trial and not ready for treatment yet.

Pharmacological chaperone therapy (PCT) is the latest development in the treatment of MPS II. Known as molecules with low molecular weight, pharmacological chaperone (PC) is target-specific. PC binds to targeted protein which can induce the thermodynamic stabilisation of the protein to correct the folding or changes in folding/unfolding protein. The advantage of PC is that it could be administered orally and able to cross the BBB due to its small size.

The intended role of PC is to facilitate an inactive enzyme with a certain kind of mutation to recover its activity by binding to the active site of the molecule and causing it to fold into the correct three-dimensional structure. Any incorrectly folded enzyme can be saved by PC from degradation system in endoplasmic reticulum, therefore stimulates the trafficking of mutant enzymes to the lysosome. Consequently, these enzymes are still able to perform their function, despite the early phase of misfolding caused by a missense mutation (Fan et al. 1999).

The discovery of PCT was first reported by Fan et al. (1999) involving 1-deoxy–galactonojirimycin (DGJ), a potent competitive inhibitor of α-galactosidase in Fabry lymphoblasts. Though PC has been exploited for other LSD such as Gaucher, Pompe, Tay-Sachs, Sandhoff, GM1 gangliosidosis and Niemann-Pick C, there are still no promising compound has been found that can function as a PC for IDS.

Chondroitin/dermatan (CD) sulphate disaccharide are widely distributed on the cell surface and in the extracellular matrix in the form of proteoglycan where they participate in various biological processes (Wang et al. 2020). Due to its vast role, CD may be a potential candidate of PC for the mutant IDS. Thus, the present study described the inhibition and specificity study of CD sulphate disaccharide toward recombinant human iduronate-2-sulphatase (rhIDS). These studies demonstrated that all four potential PC inhibited rhIDS depending on their catalytic values. This inhibition study could be used as a guideline for selecting potential PC in the heat stability and in vitro cell-based study.

MATERIALS AND METHODS

Chemicals

Small molecules from chondroitin/dermatan (CD) sulphate disaccharide group: ΔUA,2S-GalNAc,4S (CD005); ΔUA,2S-GalNAc,6S (CD006); ΔUA,2S-GalNAc,4S,6S (CD007) and ΔUA,2S-GalNAc (CD008), were purchased from Iduron (Manchester, UK). Substrate 4-nitrocatechol sulphate was purchased from Sigma Aldrich (Milwaukee, USA).

Samples

Recombinant human iduronate-2-sulphatase (rhIDS) was purchased from R&D Systems (Minneapolis, USA). A normal control from commercial fibroblast cells was purchased from American Type Culture Collection

(ATCC, Virginia, USA) and grown until confluent in 1 T-300 flask with MEM containing 200 µM uridine, non-essential amino acids and 100 mL/L fetal bovine serum, before cultivated and trypsinised to obtain cell pellets. The fibroblast cell pellet was resuspended in 20 mmol/L phosphate buffer (pH 7.2) and sonicated on ice for two 5 seconds bursts at 10% power. The cell lysate was centrifuged at 600 g for 10 minutes, and the supernatants were kept on ice until analysis. All samples were subjected to protein quantitation by using modified Lowry protein assay kit (Thermo Fisher Scientific, Watham, MA, USA).

Inhibition Assay

Small molecules of CD (n=4) were challenged in the inhibition assay described in product insert of rhIDS with some modification (R&D Systems 2019). These small molecules were dissolved in deionized water before diluted into several desired concentrations ranging from 25-707 µM. Different series of concentration were used for each small molecules according to their molecular weight. The diluted small molecules were incubated with rhIDS for 10 minutes at 0 oC. A volume of 50 µL of each concentration of the individual small molecules was added into 50 µL of 2 mM ρ-nitrocatechol sulphate (ρNCS) in the 96-well microplates before the plate was incubated for 24 hours at 37 oC. The process was terminated with 100 µL of 0.2 M sodium hydroxide.

The absorbance of the product, ρNC was measured on a Spark 20M multimode microplate reader (Tecan, Mannedorf, Switzerland) with a specific wavelength of 515 nm. The relative absorbances were calculated by subtracting the reading with blanks. The amount of products was calculated by comparing the relative absorbance with absorbance from a series of ρNC calibrator, ranging from 0-300 nmol. Enzymatic activity was expressed as the amount of ρNC (nmol) released from ρNCS in one hour per mg protein.

Specificity Assay

Various concentration of small molecules ranging from 25-707 µM were incubated with commercial fibroblast for 10 minutes at 0 oC. A volume of 50 µL of each concentration of the individual small molecules was added into 50 µL of 2 mM ρ-nitrocatechol sulphate (ρNCS) in the 96-well microplates before the plate was incubated for 24 hours at 37 oC. The process was terminated with 100 µL of 0.2 M sodium hydroxide.

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Statistical Analysis

The values of maximal velocity (V_m), the inhibition concentration (IC_{50}) and inhibition constant (K_i) were obtained from Lineweaver-Burk plots, constructed using Sigmaplot Version 14 (San Jose, CA, USA). Statistical differences between the group of small molecules-rhIDS and small molecules-commercial fibroblast cells were assessed using the non-parametric Mann-Whitney U test. Differences with $p<.05$ were considered statistically significant. All experiments were performed at least in triplicate. Data were expressed as mean ± SD.

RESULTS

Kinetic properties of rhIDS

The kinetic properties were determined with ρNCS at various concentrations as the substrate in 0.05 M sodium acetate buffer with 0.1 M sodium chloride solution (pH 5.0) at 37 oC for 24 hours. K_i and V_m values of rhIDS which were incubated with respective small molecules of CD were determined (Table 1). The IC_{50} values generated from this study indicate the inhibition of serial concentration in CD towards rhIDS. IC50 values for each small molecule were calculated and ΔUA,2S-GalNAc,4S,6S demonstrated the lowest IC50 value among all. The values of IC_{50} in ΔUA,2S-GalNAc,4S, ΔUA,2S-GalNAc,6S and ΔUA,2S-GalNAc were 5-12 times higher compared with ΔUA,2S-GalNAc,4S,6S (Table 1).

The ΔUA,2S-GalNAc,4S was found to have the highest Vm value but has the lowest Ki among all. Meanwhile, Ki value for ΔUA,2S-GalNAc,4S,6S was the highest compared to others with an acceptable Vm value. Figure 1 illustrated the inhibition profiling of each small molecules of CD. Inconsistent IDS inhibition patterns were observed in ΔUA,2S-GalNAc,4S; ΔUA,2S-GalNAc,6S; and ΔUA,2S-GalNAc; whereas ΔUA,2S-GalNAc,4S,6S showed a significant inhibition pattern ($p<.05$). These results suggest that small molecules with the lowest IC50 value and highest Ki are catalytically

Table 1. Kinetic properties of small molecules

Small molecules of CD	IC_{50} (μM)	K_i	V_m
ΔUA,2S-GalNAc, 4S (diB)	221	0.6	4762
ΔUA,2S-GalNAc,6S (diD)	385	3.5	3333
ΔUA,2S-GalNAc,4S,6S (triS)	44	24.4	1429
ΔUA,2S-GalNAc	544	2.2	5000

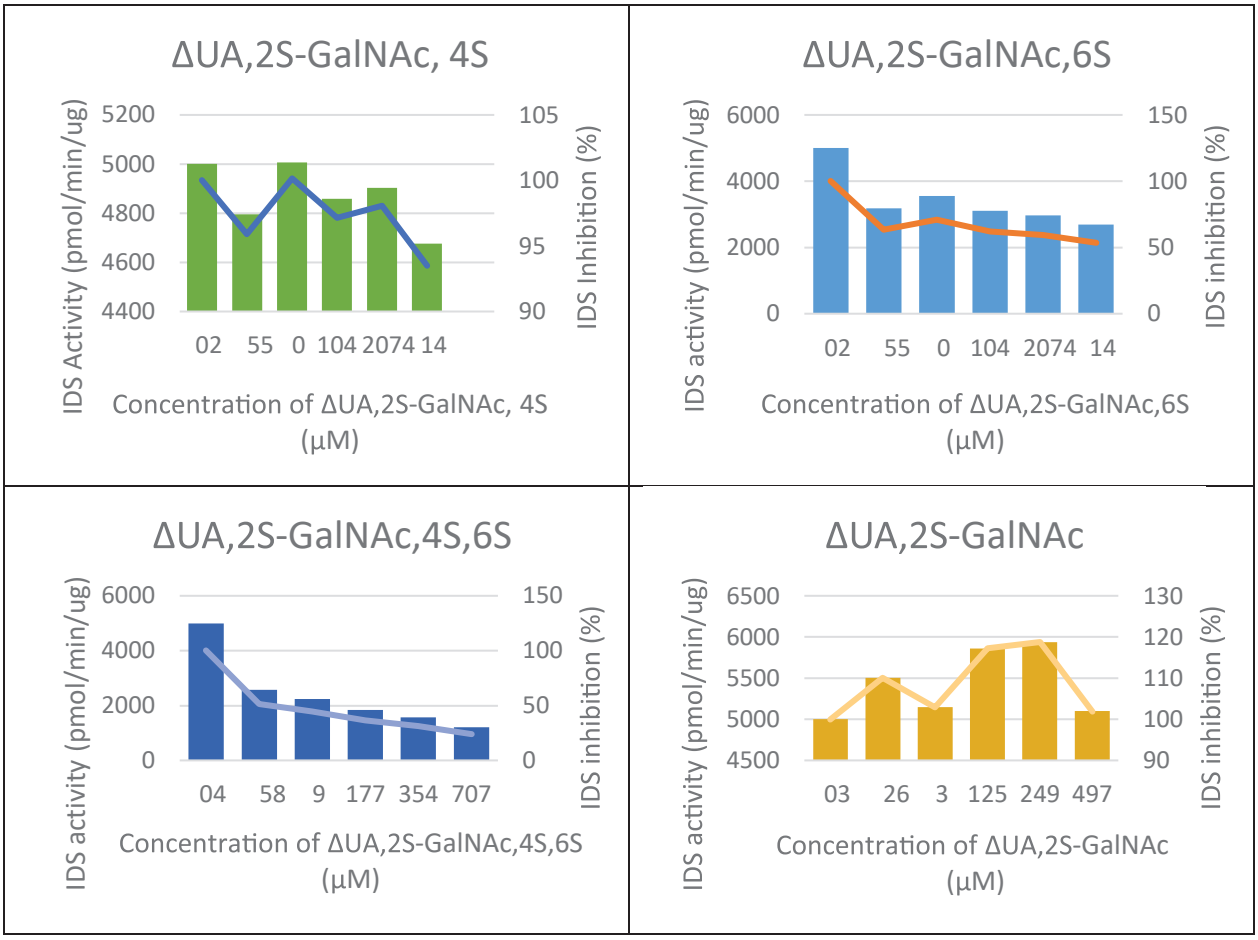

Figure 1: Inhibition profiling of respective CDs

active to the active site of rhIDS.

Inhibitory Potency Level

In this study, rhIDS were incubated with increasing concentration of each small molecule. We found that rhIDS activity incubated with ΔUA,2S-GalNAc,4S; ΔUA,2S-GalNAc,6S; and ΔUA,2S-GalNAc were comparable to the control with tolerable concentration from 25 to 994 μM ($p<.05$). At ΔUA,2S-GalNAc,4S,6S concentration of 45 μM, activity of rhIDS was more than 50% reduced compared to control value (Table 2). These data showed that ΔUA,2S-GalNAc,4S,6S has a high inhibitory potency level, suggesting that it is a safe drug and a good candidate for further drug development.

Specificity of small molecules

We examined the specificity of small molecules by incubating normal control fibroblasts that contained other lysosomal enzyme hydrolysing different glycosidic bond-cleavage (mainly arylsulphatase A, arylsuphatase B and arylsulphatase C) with respective small molecules. All IDS activities in normal control fibroblasts were not

Table 2. Inhibitory potency effects of different small molecules

Small molecules of CD	Average Activity		Inhibitory potency effects (%)
	nmol/hr/mg protein	%	
Control	5638 ± 78.27	100	0
ΔUA,2S-GalNAc, 4S	4873 ± 126.61	86.4	13.6 ± 2.25
ΔUA,2S-GalNAc,6S	3318.46 ± 603.42	58.8	41.2 ± 10.70
ΔUA,2S-GalNAc,4S,6S	1997.18 ± 551.06	35.4	64.6 ± 9.77
ΔUA,2S-GalNAc	5352.05 ± 518.79	94.91	5.09 ± 9.20

inhibited, thus confirming the specificity of these small molecules for IDS (p>.05).

DISCUSSION

There is a possibility of PCT to become an alternative treatment for MPS disorder, especially MPS II. This is based on the positive finding in other LSD disorders with a missense mutation, especially in Fabry disease (Ishii et al. 2007), Krabbe disease (Berardi et al. 2014), Schindler disease (Clark et al. 2012) and GM1 gangliosidosis (Fantur et al. 2010). PC will operate as biological tool which attach to the catalytic site of the mutant enzyme and subsequently assist the enzyme to enter the lysosome. Thus, it is important to understand the characteristics of the most suitable PC that can fulfil those criteria.

To the best of our knowledge, this study provides the first information on the effect of CD on IDS activity. The use of rhIDS in this study is to obtain purified and unlimited stock for determination of the kinetic parameters of each CD compound. Extraction of IDS from cells are not economical as it requires a large number of cells to be harvested just to obtain a concentrated form of IDS. Therefore, it is wise to use a commercial rhIDS that gives the same impact as the natural IDS.

Kinetic parameters, mainly IC_{50} and K_i of each small molecule, were assessed to select the potential candidate from four small molecules. IC_{50} refers to the concentration of small molecule which is required to inhibit 50% of the enzyme activity (Yung-Chi and Prusoff 1973). A lower IC_{50} generally means a more potent inhibitor (Lineweaver and Burk 1934). Inhibition constant (K_i), also known as inhibitor dissociation constant, is a reversible equilibrium constant inhibitor to form a complex with its target enzyme (Cornish-Bowden 1974; Waley 1982).

Our study showed that ΔUA,2S-GalNAc,4S,6S has the highest K_i value with the lowest IC_{50} concentration. It has the highest binding affinity of inhibitors and lower potency, which is preferred for further development of the candidate compound to be therapeutically useful. Specificity study revealed all investigated small molecules were very specific and selective towards IDS.

The important drug candidates elements for evaluating formal animal toxicity studies in pharmacological properties includes (i) selective high-affinity binding to target binding site; (ii) selective and potent functional effect on target receptor molecule in vitro and (iii) effectiveness in an animal model of targeted human indication (Hefti 2008).

The journey to search for a better approach in strengthening any promising therapies is becoming a vital goal of current research apart from overcoming the limitations of available treatment in treating MPS II. Defective protein-coding, which caused by genetic errors involved in genetic disorders may affect the patients.

Biosynthesis of non-functional mutant enzymes can occur from the substitution of critical amino acids, either via nonsense, splicing, frame-shift or missense mutation. Among all these types of mutations, missense mutation or small in-frame deletion/insertion were reported to have slightly less or no significant impact on the biological activity of the mutant enzyme (Zhang et al. 2010; Zhang et al. 2011).

The distribution of missense mutation among MPS II patients were noted to be significantly high. Vafiadaki (Vafiadaki et al.1998) reported that nearly 32% of patients with MPS II who attended the Willink Unit, Royal Manchester Children's Hospital, were diagnosed with a missense mutation. In Tunisia, 40% of MPS II patient were identified to have missense mutation (Chkioua et al. 2020). Patients with missense mutation are usually associated with less severe clinical manifestation and would have limited residual IDS activity.

This low level of IDS activity may result from either (i) normal processing of mutant IDS with its active

site altered or (ii) normal enzyme but with its catalytic activity reduced or (iii) only some of the correctly folded mutant IDS went through the endoplasmic-reticulum-associated protein degradation (ERAD) (Ishii et al. 2007). The existence of PC plays an important role at this stage where it binds to the active site of mutant IDS hence trafficking the misfolded enzyme into lysosome. Once in lysosome, PC would dissociate from the active site of IDS in reversible action, thus allowing the enzyme to facilitate the degradation process of HS and DS. Hence, there is an urgency to speed the PCT discovery for this disorder.

The heat stability study and *in vitro* cell-based study might reveal an understanding between mutant enzyme interaction with PC and vice versa and provide knowledge of defective enzyme nature caused by a specific genotype. More information is needed regarding the thermal stability of the small molecule and its mechanism in the cell environment. Furthermore, the ability of candidate PC to be displaced by the natural substrate of the IDS enzyme is still unknown and need to be explored further in future research.

CONCLUSION

In summary, our study showed that ΔUA,2S-GalNAc,4S,6S has the highest affinity towards IDS with the lowest IC_{50} and the highest K_i. Thus, it may become a potential small molecule as PC. We also anticipate that all CD sulphate disaccharides group in this study are specific for IDS.

ACKNOWLEDGEMENTS

The authors would like to express our gratitude to the Director General of Health Malaysia for permission to publish this paper. The authors would also like to acknowledge the staff of the Inborn Errors of Metabolism & Genetics Unit and staff of the Biochemistry Unit for their technical assistance, and Dr Zabedah Md Yunus for initiating the project. Our special thanks to Muhammad Nor Farhan Saat and Syed Muhamad Asyraf Syed Taha for the critical reading of the manuscript and valuable comments. This research was supported by the Ministry of Health Malaysia Research Grant (NMRR-17-2351-38166).

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