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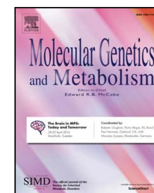

Chaperone effect of sulfated disaccharide from heparin on mutant iduronate-2-sulfatase in mucopolysaccharidosis type II

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ABSTRACT

Small molecules called pharmacological chaperones have been shown to improve the stability, intracellular localization, and function of mutated enzymes in several lysosomal storage diseases, and proposed as promising therapeutic agents for them. However, a chaperone compound for mucopolysaccharidosis type II (MPS II), which is an X-linked lysosomal storage disorder characterized by a deficiency of iduronate-2-sulfatase (IDS) and the accumulation of glycosaminoglycans (GAGs), has still not been developed. Here we focused on the Δ-unsaturated 2-sulfouronic acid-*N*-sulfoglucosamine (D2S0), which is a sulfated disaccharide derived from heparin, as a candidate compound for a pharmacological chaperone for MPS II, and analyzed the chaperone effect of the saccharide on IDS by using recombinant protein and cells expressing mutated enzyme. When D2S0 was incubated with recombinant human IDS (rhIDS) *in vitro*, the disaccharide attenuated the thermal degeneration of the enzyme. This effect of D2S0 on the thermal degeneration of rhIDS was enhanced in a dose-dependent manner. D2S0 also increased the residual activity of mutant IDS in patient fibroblasts. Furthermore, D2S0 improved the enzyme activity of IDS mutants derived from six out of seven different mutations in HEK293T cells transiently expressing them. These results indicate that D2S0 is a potential pharmacological chaperone for MPS II.

1. Introduction

Mucopolysaccharidosis type II (MPS II, OMIM 309900), also known as Hunter syndrome, is an X-linked recessive disorder caused by a deficiency of iduronate 2-sulfatase (IDS) [1]. MPS II is characterized by progressive lysosomal accumulation of glycosaminoglycans (GAGs) comprised of heparan sulfate and dermatan sulfate, and presents systemic manifestations such as skeletal deformities, mental retardation, valvular heart disease, hepatosplenomegaly, and skin abnormality. Enzyme replacement therapy (ERT) with recombinant human IDS (rhIDS) is currently the standard treatment for MPS II patients, and has been shown to improve the accumulation of GAGs in urine and some clinical symptoms including hepatosplenomegaly, the six minute walk distance, and percentage of predicted forced vital capacity [2–4]. Hematopoietic stem cell transplantation (HSCT) is a potential option for MPS II treatment, which may have similar effects to ERT, namely, reducing accumulated GAGs and improving visceral disease [5]. However, these therapies have several limitations: no or little effect on brain, bone, and heart valves [6,7]; need of weekly intravenous administration (ERT) [3]; and risk of mortality due to the conditioning regimen using chemotherapy agents (HSCT) [8].

To date, > 550 mutations in the IDS gene have been reported, approximately 45% of which are missense mutations (Human Gene Mutation Database; http://www.hgmd.org). Many of these mutations produce a misfolded protein, which is prone to being degraded by the protein quality control system in the endoplasmic reticulum, leading to a reduction of intracellular IDS activity followed by the accumulation of GAGs. Pharmacological chaperone (PC) therapy has been investigated as a potential treatment for several lysosomal storage diseases, including Fabry, Gaucher, G_{M1}-gangliosidosis, and Pompe diseases caused by missense mutations; in particular, the therapy for Fabry disease was approved by the European Medicines Agency in 2016 [9–12]. PCs are small molecules that have an affinity to mutant enzymes, directly bind to them, and improve their folding, stability, and enzymatic activity [13–15]. Some compounds can be oral administration and have been shown to provide positive effect to various tissues including brain in

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model animals and/or patients with several diseases containing G_{M1}-gangliosidosis, Gaucher, and Fabry diseases [9,11,12]. However, no promising compound that functions as a PC for IDS has been found.

In this study, we focused on Δ-unsaturated 2-sulfouronic acid-*N*-sulfoglucosamine (D2S0), which is a sulfated disaccharide derived from heparin and has a similar structure with natural substrate of IDS, as a PC candidate molecule, and analyzed its chaperone effect on IDS by using recombinant enzyme and cell-based experiments.

2. Materials and methods

2.1. Chemicals

4-Methylumbelliferyl-α-L-iduronide-2-sulfate (4MU-ido-2S) was obtained from Moscerdam Substrates (Rotterdam, Netherlands). Δ-Unsaturated 2-sulfouronic acid-*N*-sulfoglucosamine (D2S0) derived from heparin was purchased from Iduron (Manchester, UK). rhIDS was a gift from Sanofi-Genzyme Japan (Tokyo, Japan).

2.2. Construction of expression vector

Synthesized cDNA coding full-length human wild-type IDS was purchased from Eurofins Genomics (Tokyo, Japan). Point mutations were introduced into the IDS by using site-directed mutagenesis, as described previously [16]. All of the seven mutants were confirmed by DNA sequencing. Wild-type IDS and mutated IDS were subcloned into the expression vector pCMV-Script (Stratagene, Cedar Creek, TX). These experiments were approved by The Recombinant Gene Research Safety Committee of the Jikei University School of Medicine.

2.3. Cell culture and transfection of expression plasmid

Skin fibroblasts from an MPS II patient and a normal control were obtained from Coriell Institute for Medical Research (Camden, NJ). The fibroblasts and HEK293T cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2. HEK293T cells were transiently transfected with 1 μg of expression plasmid DNA using X-tremeGENE 9, in accordance with the manufacturer's instructions. At 24 h after transfection, the medium of the cells was replaced with fresh medium with or without D2S0.

2.4. Analysis of IDS thermal stability

To analyze IDS thermal stability, 1.3 μM rhIDS was dissolved in phosphate citrate buffer (200 mM Na_2HPO_4 and 200 mM citric acid, pH 4.0) supplemented with 13 μM D2S0. Subsequently, rhIDS solutions were heated at several temperatures, namely, 37, 47, 57, or 67 °C, for 1 h followed by the analysis of IDS activity. To assay the dose-dependent effect of D2S0 on the thermal stability of IDS, the enzymatic activity in phosphate citrate buffer containing 1.3 μM rhIDS and several concentrations of D2S0 was also measured after 1 h of incubation at 67 °C.

2.5. Analysis of IDS activity and IDS inhibition

To analyze IDS inhibition by D2S0, 0.5 nM rhIDS was incubated with 1 μM 4MU-ido-2S in the absence or presence of several concentrations of D2S0. To draw the Lineweaver-Burk plot, rhIDS solutions containing 0.5 nM enzyme and several concentrations of 4MU-ido-2S (5, 1, 0.5, 0.25, and 0.125 μM) were incubated with or without 1 μM D2S0. IDS activity in rhIDS solutions was measured using a microplate reader (Perkin Elmer, Waltham, MA), and that in cell lysates was assayed as described previously [17].

2.6. LC-MS/MS analysis of pathological iduronic acid (PIA)

PIA derived from non-reducing end of GAGs was extracted and purified from cell lysate as previously described [18]. Briefly, the GAGs in cell lysates were purified by using an anion-exchange column followed by digestion with recombinant human iduronidase and iduronate-2-sulfatase. The PIA was analyzed by high-performance liquid chromatography equipped with a triple quadrupole mass spectrometer (LCMS-8040, Shimadzu, Kyoto, Japan). Mobile phase A was ultrapure water (Wako Pure Chemicals, Tokyo, Japan) containing 1% formic acid and mobile phase B consisted of 100% acetonitrile. The PIA was quantified by multiple reaction monitoring (MRM) in negative ion mode. Transitions and collision energies (CE) are listed below. m/z 193.0 > 89; CE 11 V, m/z 193.0 > 113.05; CE 12 V, m/z 193.0 > 59.10; CE 19 V, m/z 193.0 > 73.05; CE 14 V, 193.0 > 85.0; CE 17 V. The concentration of PIA was calculated by using a calibration curve of iduronic acid (Toronto Research Chemicals Inc., North York, Canada).

2.7. Alcian blue staining

Alcian blue staining was performed following a slightly modified version of a previously described procedure [19]. Briefly, patient fibroblasts were fixed with 4% paraformaldehyde, permeabilized using phosphate buffer saline containing 0.1% Triton X-100, and incubated with Alcian blue solution for 1 h.

3. Results

3.1. D2S0 increases thermal stability of rhIDS in vitro

To investigate whether D2S0 directly binds to IDS, we incubated the disaccharide with rhIDS *in vitro* and determined the IC_{50} value by assaying enzymatic activity. The IC_{50} value of D2S0 was calculated to be 30.1 μM (Fig. 1A). In addition, Lineweaver-Burk plots suggested that D2S0 inhibited the enzymatic activity of rhIDS in a competitive manner (Fig. 1B). Next, we determined the effect of D2S0 on the thermal stability of rhIDS. Whereas decreased activity of rhIDS was observed upon incubation at 67 °C, D2S0 significantly attenuated the reduction of enzymatic activity (Fig. 2A). Moreover, the preventive effect of D2S0 on the thermal degeneration of rhIDS was shown to be dependent on the concentration of the disaccharides (Fig. 2B). These results indicate that D2S0 functions as a pharmacological chaperone for rhIDS *in vitro*.

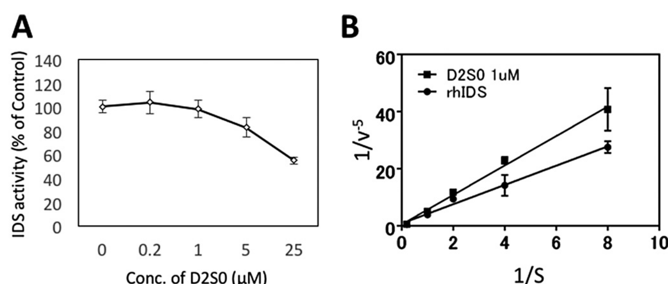

Fig. 1. Δ-Unsaturated 2-sulfouronic acid-*N*-sulfoglucosamine (D2S0) binds to recombinant human IDS (rhIDS) and inhibits their activity. A: Enzymatic activity in rhIDS solution with several concentrations of D2S0. rhIDS solutions were supplemented with or without the indicated concentrations of D2S0 and their enzymatic activity was measured. Data is expressed as a percentage relative to the D2S0-untreated IDS activity. B: Lineweaver-Burk plot of IDS inhibition by D2S0. rhIDS solutions were incubated with several concentration of 4-Methylumbelliferyl-α-L-iduronide-2-sulfate in the absence or presence of 1 μM D2S0 and their enzymatic activity was assayed. Each value represents mean ± standard error of the mean (SEM) of triplicate samples.

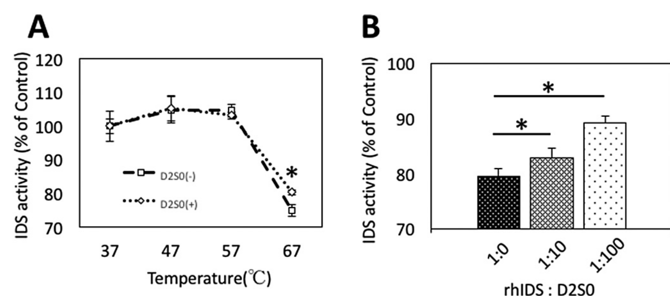

Fig. 2. D2S0 increases the thermal stability of rhIDS. A: Thermal stability of rhIDS in the presence or absence of D2S0. rhIDS solutions were incubated with or without D2S0 (ten-fold excess molar of enzymes) at several temperatures (37, 47, 57, or 67 °C) for 1 h. IDS activity was measured in triplicate. B: Dose-dependent effect of D2S0 on the thermal stability of rhIDS. rhIDS solutions were incubated with or without two concentrations of D2S0 (molar rhIDS: D2S0 ratio = 1:10 or 1:100) at 67 °C for 1 h and the IDS activity was measured in triplicate. Each value represents mean ± SEM of triplicate samples and is expressed as a percentage relative to the D2S0-untreated IDS activity obtained from incubation at 37 °C. Statistical analysis of the data was performed by Student's *t*-test (*: $p < 0.05$ compared with D2S0-untreated samples).

3.2. D2S0 improves the IDS activity and GAG accumulation in fibroblasts from a patient with MPS II

To investigate the effect of D2S0 on endogenous IDS mutants, we incubated fibroblasts from an MPS II patient carrying the p.P231L mutation with 10 μM D2S0 for 4 days, and analyzed the enzymatic activity and PIA levels in lysates from the cells. IDS activity in D2S0-treated patient fibroblasts was approximately double that of untreated cells (Fig. 3A). Although the statistically significant difference was not observed between treated and untreated cells, mean values of PIA were decreased in D2S0-treated fibroblasts (approx. 75.6% of untreated one) (Fig. 3B). When we analyzed the levels of GAG accumulation in patient fibroblasts treated with D2S0 for 8 days by using Alcian blue, significant reduction of Alcian blue-positive granules was observed in them (Fig. 3C). These results suggest that D2S0 can contribute to improve the activity of endogenous IDS mutants in patient fibroblasts.

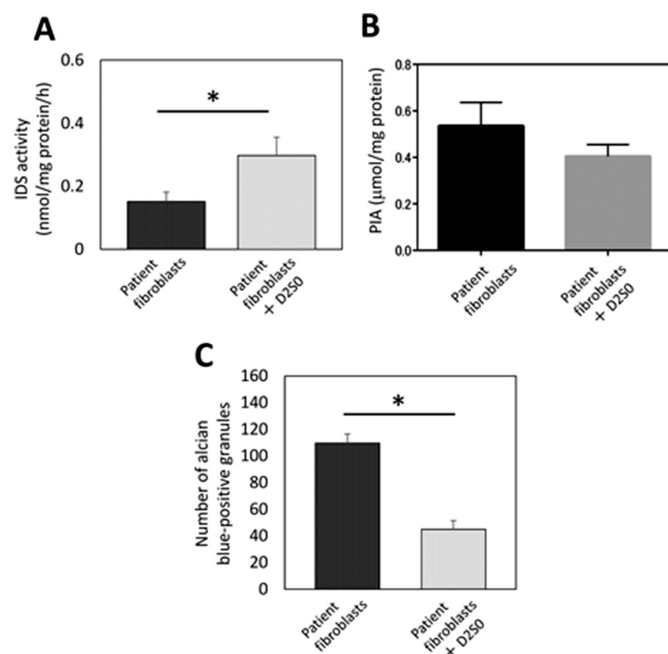

Fig. 3. Effect of D2S0 on endogenous IDS in fibroblast from patient with MPS II. A: Enzymatic activity of IDS in MPS II patient fibroblasts treated with D2S0. Patient fibroblasts carrying p.P231L mutation were cultured for 4 days with or without 10 μM D2S0. The IDS activity in cell lysates was measured and is expressed as mean ± SEM (n = 3). B: PIA assay of patient fibroblasts treated with D2S0. Patient fibroblasts were incubated with or without 10 μM D2S0 for 4 days. PIA derived from non-reducing end of GAGs were purified from cell lysates and analyzed by LC-MS/MS. Data are expressed as mean ± SEM (n = 5–6). C: Number of Alcian blue-positive granules in patient fibroblasts treated with D2S0. Patient fibroblasts were incubated with or without 10 μM D2S0 for 8 days. Cells were fixed and stained with Alcian blue. Alcian blue-positive dots were counted in independent triplicate samples. Statistical analysis of data was performed by Student's *t*-test (*: $p < 0.05$).

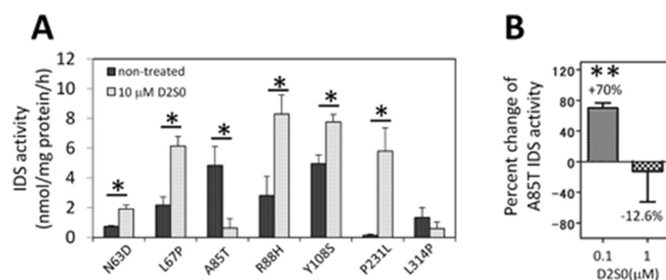

Fig. 4. D2S0 improves the several types of mutant IDS in HEK293T cells. A: Effect of D2S0 on IDS activity in HEK203T cells expressing mutant enyzme. HEK293T cells were transiently transfected with mutant IDS and incubated with or without 10 μM D2S0. B: Enzymatic activity of IDS in p.A85T mutant expressing HEK293T cells treated with several concentrations of D2S0. The IDS activity in cell lysates was measured and subtracted the enzymatic activity obtained from untransfected cells. Data are presented as mean ± SEM (A) and expressed as percent-change of D2S0-untreated p.A85T mutant IDS activity (B) (n = 3, respectively). Enzyme activity of wild-type IDS was 114.1 ± 9.3 nmol/mg protein/h (A). Statistical analysis of data was performed by Student's t-test (*: $p < 0.05$, **: $p < 0.01$ compared with D2S0-untreated samples).

3.3. D2S0 increases the activity of several types of mutated IDS in HEK293T cells

To characterize the responsivity of mutant IDS to D2S0, we generated seven different IDS mutants (p.N63D, p.L67P, p.A85T, p.R88H, p.Y108S, p.P231L, and p.L314P) that have been identified from patients with phenotypes ranging from severe to mild [20–23]. We transiently transfected the mutant forms of IDS into HEK293T cells, and analyzed the enzymatic activity in them after treatment with D2S0. Six IDS mutants responded to 10 μM D2S0; five mutants (p.N63D, p.L67P, p.R88H, p.Y108S, and p.P231L) showed increases in enzymatic activity ranging from 1.6-fold to 39.6-fold, and one mutant (p.A85T) exhibited a 7.7-fold decrease in activity (Fig. 4A). When p.A85T mutant expressing HEK293T cells were treated with 0.1 μM D2S0, improved IDS activity was detected in the cells compared to D2S0-untreated cells (1.7-fold increase) (Fig.4B). Four of the responsive mutants (p.L67P, p.A85T, p.R88H, and p.Y108S) had initial activity that was higher than approximately 2% of that of wild-type IDS (Fig. 4A). Other responsive mutants (p.N63D and p.P231L) showed original activity that was lower than 1% of that of the wild-type enzyme. No significant difference in enzymatic activity was observed between D2S0-treated cells expressing wild-type IDS and untreated ones (data not shown). These results indicate that D2S0 has a positive effect on the enzymatic activity of several IDS mutants, regardless of the level of initial enzymatic activity.

4. Discussion

In the current study, we demonstrated that D2S0 attenuates the thermal degeneration of rhIDS *in vitro* and improves the function of intracellular IDS in patient fibroblasts and HEK293T cells expressing mutated IDS (Figs. 2, 3, and 4).

D2S0 is a sulfated disaccharide produced by bacterial heparinase from heparin, which is one of the GAGs [24]. When heparinase cleaves the non-reducing end of uronic acid in heparin, this enzyme generates a double bond between C4 and C5 in sugar acids such as iduronic acid and glucuronic acid [25]. Therefore, the structure of D2S0 is similar to that of 2-O-sulfated iduronic acid-linked N-sulfated glucosamine, which

is a natural substrate of IDS. Some chaperone compounds for lysosomal storage diseases are known as substrate analogs and act as competitive inhibitors in a dose-dependent manner [13,15,26]. Indeed, our results showed that D2S0 competes with the artificial substrate in the enzyme reaction of IDS (Fig. 1). It thus appears that the chaperone effect of D2S0 is due to direct binding to IDS, similar to chaperone compounds for other lysosomal storage diseases.

Our results demonstrated that D2S0 improves the activity of several types of mutated IDS containing p.N63D, p.L67P, p.A85T, p.R88H, p.Y108S, and p.P231L mutations (Fig. 4). In particular, four mutants (p.L67P, p.R88H, p.Y108S, and p.P231L) displayed enzyme activity that was higher than 5% of that of wild-type IDS irrespective of the levels of initial activity after 10 μM D2S0 treatment (Fig. 4A). These responsive mutations have been identified in MPS II patients with not only a mild clinical phenotype (p.N63D, p.A85T, p.Y108S, and p.P231L) but also a severe one (p.L67P, p.A85T, and p.R88H) [20–23,27], suggesting that the classification of disease severity of MPS II patients may not be useful to predict the responsivity of mutated IDS to D2S0.

According to the human IDS structural model established using homology modeling methods based on the crystal structures of arylsulfatases A and B [27–29], the positions of A85, R88, Y108, and P231 are located adjacent to the putative active site region, which consists of the D45, D46, C84, K135, and D334 residues [29]. On the other hand, the D2S0-unresponsive L314 residue is located furthest away from the active site among the seven mutations that we analyzed in this study. It thus seems that mutations around the active site of IDS may be more responsive to D2S0 than those in other regions.

We demonstrated that whereas 10 μM D2S0 decreased the enzymatic activity in HEK293T cells expressing p.A85T mutant IDS, exhibiting higher residual activity than other mutants, 0.1 μM D2S0 enhanced the IDS activity in them (Fig.4); this suggests that A85T-substituted IDS is susceptible to competitive inhibition by D2S0 in the enzymatic reaction using an artificial substrate. Because the position of A85 is located in the vicinity of C84, which is converted to the catalytic core formylglycine, the substitution to threonine is suggested to affect the shape of the active site [27]. In addition, Saito et al. showed that the p.A85T mutant has a minor change of three-dimensional structure by calculating the root-mean-square distance value [29]. Our observations provide new enzymological insight into the p.A85T mutation, which induces a change of the substrate preference of IDS from 2-O-sulfated iduronic acid to Δ-unsaturated 2-sulfouronic acid.

In summary, we found the pharmacological chaperone effect of D2S0 for IDS, and demonstrated the responsivity of several IDS mutations to the disaccharide. Our results provide evidence that D2S0 may be useful for treating MPS II.

Conflict of interest disclosure

H. H., Y. S., T. H., and H. K. have no conflicts of interest. H. I. and T. O. have received research support from Sanofi-Genzyme. These activities have been fully disclosed and are managed under a Memorandum of Understanding with the Conflict of Interest Resolution Board of The Jikei University School of Medicine.

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