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A biochemical and physicochemical comparison of two recombinant enzymes used for enzyme replacement therapies of hunter syndrome

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Abstract Mucopolysaccharidosis II (MPS II, Hunter syndrome; OMIM 309900) is an X-linked lysosomal storage disease caused by a deficiency in the enzyme iduronate-2-sulfatase (IDS), leading to accumulation of glycosaminoglycans (GAGs). For enzyme replacement therapy (ERT) of Hunter syndrome, two recombinant enzymes, idursulfase (Elaprase®, Shire Human Genetic Therapies, Lexington, MA) and idursulfase beta (Hunterase®, Green Cross Corporation, Yongin, Korea), are currently available in Korea. To compare the biochemical and physicochemical differences between idursulfase and idursulfase beta, we examined the formylglycine (FGly) content, specific enzyme activity, mannose-6-phosphate (M6P) content, sialic acid content, and in vitro cell uptake activity of normal human fibroblasts of these two enzymes.

The FGly content, which determines the enzyme activity, of idursulfase beta was significantly higher than that of idursulfase (79.4±0.9 vs. 68.1±2.2 %, $P<0.001$). In accordance with the FGly content, the specific enzyme activity of idursulfase beta was significantly higher than that of idursulfase (42.6±1.1 vs. 27.8±0.9 nmol/min/μg protein, $P<0.001$). The levels of M6P and sialic acid were not significantly different (2.4±0.1 vs 2.4±0.3 mol/mol protein for M6P and 12.3±0.7 vs. 12.4±0.4 mol/mol protein for sialic acid). However, the cellular uptake activity of the normal human fibroblasts *in vitro* showed a significant difference (K_{uptake}, 5.09±0.96 vs. 6.50±1.28 nM protein, $P=0.017$).

In conclusion, idursulfase beta exhibited significantly higher specific enzyme activity than idursulfase, resulting from higher FGly content. These biochemical differences may be partly attributed to clinical efficacy. However, long-term clinical evaluations of Hunter syndrome patients treated with these two enzymes will be needed to demonstrate the clinical implications of significant difference of the enzyme activity and the FGly content.

Keywords Hunter syndrome · MPS II · Iduronate-2-sulfatase · Formylglycine · Mannose-6-phosphate · Enzyme replacement therapy

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Introduction

Mucopolysaccharidosis II (MPS II, Hunter syndrome; OMIM 309900) is an X-linked lysosomal storage disease caused by a deficiency in the enzyme iduronate-2-sulfatase (IDS), leading to accumulation of glycosaminoglycans (GAGs) within lysosomes [1–3]. The incidence is from 0.2 to 1.07 per 100,000

live births [3]. MPS II is progressive and multisystemic, with significant morbidity and early mortality. The clinical spectrum is divided into severe and attenuated forms according to the presence of cognitive impairment [4]. The clinical features include coarse facial features, recurrent ear and respiratory infections, hearing loss, airway obstruction and restriction, cardiac valvular diseases, hepatosplenomegaly, skeletal abnormalities, growth restriction, joint stiffness, and neurological complications, including communicating hydrocephalus, spinal cord compression, carpal tunnel syndrome, and cognitive impairment in patients with a severe form of the disease [5, 6]. Death typically occurs in the second decade in patients with the severe form, whereas patients with the attenuated phenotype remain cognitively intact and may survive into adulthood, although premature mortality occurs [4].

The treatment of MPS II was palliative prior to the introduction of enzyme replacement therapy (ERT). However, successful clinical trials [7–9] have led to the approval of ERT with human recombinant idursulfase (Elaprase®, Shire Human Genetic Therapies, Lexington, MA) by the United States Food and Drug Administration (FDA) in July 2006 and with human recombinant idursulfase beta (Hunterase®, Green Cross Corporation, Yongin, Korea) by the Korea Food and Drug Administration (KFDA) in January 2012. Therefore, these two drugs are currently available for ERT of patients with MPSII in Korea.

Iduronate-2-sulfatase (IDS; EC 3.1.6.13) is a lysosomal enzyme, which cleaves O-linked sulfate from heparan sulfate and dermatan sulfate [8]. The IDS sequence contains eight N-linked glycosylation sites at positions 31, 115, 144, 246, 280, 325, 513, and 537. Expression studies have shown that all these glycosylation sites can be utilized and that no single glycosylation site is essential for IDS stability, although the glycosylation site at position 280 is important for cellular internalization and lysosomal targeting via the mannose-6-phosphate (M6P) receptor pathway. IDS is highly sialyated and this is presumed to prevent both antibody recognition and recapture by the hepatic asialoglycoprotein receptor of the enzyme, resulting in the maintenance of a circulating pool of IDS [10]. Another important co- or post-translational modification of IDS is the conversion of a conserved cysteine-84 residue into a 2-amino-3-oxopropionic acid residue (C α-formylglycine), which is required for the degradation of heparan sulfate and dermatan sulfate [11, 12].

We conducted biochemical and physicochemical studies of these two enzymes to determine the structural and/or functional differences. Because both proteins are derived from the human *IDS* gene and the amino acid sequence of the two recombinant enzymes was 100 % identical, any structural differences are likely due to differences in post-translational modifications, particularly glycosylation, and levels of formylglycine (FGly), M6P, and sialic acid, which can influence enzyme activity, cellular uptake, distribution, stability, and potentially immunogenicity. Our studies focused on the comparison of post-translational modifications and their potential impact on specific enzyme activity and cellular uptake.

Materials and methods

Idursulfase and idursulfase beta

Both idursulfase (Elaprase®, Shire Human Genetic Therapies, Lexington, MA) and idursulfase beta (Hunterase®, Green Cross Corporation, Yongin, Korea) are produced by genetic engineering, which yields a glycosylated protein analogous to the native human enzyme. The idursulfase-producing cell line was generated by transfecting HT-1080 cells. In contrast, the idursulfase beta-producing cell line was generated with CHO cells, with an expression plasmid encoding the 550 amino acids of human iduronate-2-sulfatase, including a 25 amino acid signal sequence, which is cleaved in the secreted protein [13–15].

To perform a valid comparison of representative lots, several lots of idursulfase were purchased from MYODERM (Norristown, PA); DB1106-02, DB1106A01 and DC1101A02 for analyzing FGly, M6P, and sialic acid content; DB1010A08, DC1006-01, DJ1003A05 for analyzing specific activity and the cellular uptake activity. 709R1006, 709R1007 and 709R1008 of idursulfase beta were used for the comparison. Both products are a liquid formulation with a concentration of 2 mg/ml in a vial. A head-to-head analytical comparison was conducted of idursulfase and idursulfase beta with three lots of each product.

Prior to biochemical analysis, the protein concentration was determined. The labeled concentration was used for FGly, M6P, and sialic acid content analysis; The Bicinchoninic acid (BCA) concentration assay kit (Thermo Scientific Waltham, MA) was used for specific activity and cellular uptake activity analysis. The measured concentration of each product was 1.66 mg/ml of idursulfase and 1.75 mg/ml of idursulfase beta.

Formylglycine content

The absolute quantification (AQUA) technique was used to quantify the content of FGly [16, 17]. Briefly, the peptide sequence AQQAVCAPSRVSF, including Cys-84 (Cys-59 if the numbering system excluded 25 signal peptides sequences), was selected from peptide mapping data using a combination of several different digestion conditions with matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS/MS) and liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS). The Cys-AQUA peptide (cysteine-AQUA, AQQAVCA^{+4}PSRVSF) was prepared by synthesizing the chosen peptide, labeled with heavy isotopes (C^{13}/N^{15}) at Ala-7 position, and therefore the peptide mass was

increased by 4 Da. The conversion of the Cys-AQUA peptide to the FGly-AQUA peptide (FGly-AQUA) was carried out with a FGly-generating enzyme (FGE). The Cys-AQUA and FGly-AQUA standard were prepared by the bicinchoninic acid (BCA) concentration assay kit (Thermo Scientific Waltham, MA).

The Cys-peptides and FGly-peptides in each sample were identified using LC-ESI-MS/MS and extracted ion chromatograms (EIC) after the AQUA standards were spiked into the digested samples. The relative amount of FGly-form peptides and Cys-form peptides in the samples were then quantified.

The samples were prepared through polysorbate 20 removal, deglycosylation with peptide-N-glycosidase F, reduction with dithiothreitol and alkylation with iodoacetamide. Each sample was digested with chymotrypsin in three individual triplicates. The digested samples were analyzed by LC-ESI-MS/MS. LC-ESI-MS/MS analyses were used to determine the FGly-form peptide content of both products.

The peaks of the Cys-peptides and FGly-peptides were extracted from the LC-ESI-MS/MS chromatograms using the EIC method.

The relative amount of FGly-form peptides and Cys-form peptides at position 84 was calculated based on the peak areas from the EIC data. All the relative FGly peptide areas were determined for each of the three replicates. The mean and standard deviation were then calculated.

Specific enzyme activity

To perform a valid comparison of the specific enzyme activity, three individual batches were analyzed. The enzyme activity was measured by determining the rate of enzyme-catalyzed hydrolysis of a synthetic substrate, 4-methylumbelliferyl-α-L-iduronide-2-sulfate-Na2 (4-MU-αIdoA-2S), in 50 mM of sodium acetate, 500 μg/ml bovine serum albumin, pH 5.5. The enzyme concentration was determined with a BCA kit (Thermo Scientific). The released 4-methylumbelliferyl (4MU) was quantified by fluorescence reading (excitation 355 nm, emission 460 nm, Victor × 4, PerkinElmer, Waltham, MA) against a 4-MU standard curve [18].

Mannose-6-phosphate content

To perform a valid comparison of M6P content, three individual batches were analyzed. Each sample was hydrolyzed with 6.75 M trifluoroacetic acid (final concentration) at 100 °C for 1.5 h. After hydrolysis the samples were dried using a vacuum centrifuge, reconstituted with water, and purified with 0.22 μm filter units. The separation of M6P was performed with high Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) on ICS 3000 system (Dionex, Sunnyvale, CA). A separation column (Dionex CarboPAC™ PA-10 [4×250 mm]) and guard column (Dionex AminoTrap [3×30 mm]) were used. The eluents were 100 mM NaOH (A) and 100 mM NaOH/1 M sodium acetate (B). M6P was eluted with a gradient from 17 B % to 70 % B within 21 min at 25 °C, with a flow rate of 1.0 ml/min. The calibration (quantification and peak assignment by retention time) and quantification were performed using a defined M6P standard (Sigma, St. Louis, MO) [13].

Sialic acid analysis

To perform a valid comparison of the sialic acid content, three individual batches were analyzed. Each sample was hydrolyzed under mild conditions with 0.1 M hydrochloric acid (final concentration) at 80 °C in a heating block for 1 h. After hydrolysis, the samples were dried in a vacuum centrifuge and reconstituted with pure water. The separation of sialic acid was performed with HPAEC-PAD on an ICS 3000 system (Dionex). A separation column (Dionex CarboPAC™ PA-20 [3×150 mm]) and a guard column (Dionex AminoTrap [3×30 mm]) were used. The eluents were pure water (A), 0.2 M sodium hydroxide (B), and 1 M sodium acetate (C). Sialic acid was eluted at 50 % B with a gradient from 7 % to 30 % C within 10 min at 30 °C, with a flow rate of 0.3 ml/min. The calibration (quantification and peak assignment by retention time) and quantification were performed using a defined sialic acid mixture (Sigma) [14, 15].

Cellular uptake activity

To perform a valid comparison of the cellular uptake activity, three individual batches were analyzed. Normal human fibroblasts (CCD-96Sk, ATCC, Manassas, VA) were grown to confluence in a T-175 flask using Iscove's modified Dulbecco's media/fetal bovine serum (IMDM/FBS) media.

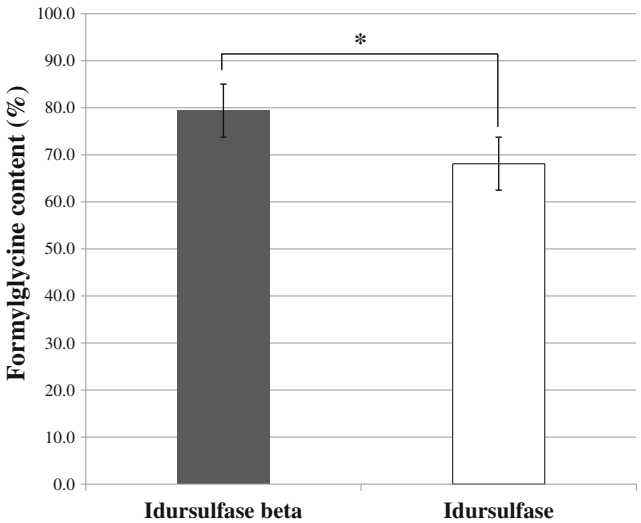

Fig. 1 Mean of three replicate measurements of the relative formylglycine content of idursulfase beta and idursulfase

Table 1 Relative FGly level determined by relative peak areas

Run	Idursulfase beta			Idursulfase		
	709R1006	709R1007	709R1008	DB1106-02	DB1106A01	DC1101A02
1	79.1 %	81.1 %	78.8 %	68.6 %	69.6 %	70.0 %
2	78.4 %	79.2 %	80.1 %	64.3 %	66.1 %	65.9 %
3	78.2 %	79.8 %	79.9 %	71.0 %	69.4 %	68.2 %
Mean ± SD	79.40±0.92 %			68.12±2.22 %		

SD, Standard deviation

The cells were washed with phosphate-buffered saline and plated at 3×10^5 cells/well in a 12-well plate. The plates were incubated overnight at 37 °C. Following incubation, the samples (assayed in duplicate) were diluted in reduced serum media (IMDM/5 % FBS) and added to the cells. Following incubation for 20 h at 37 °C, the cells were washed and lysed by the freezing and thawing method. The cell lysates were frozen at −20 °C for the next analysis. Protein determination (BCA) and quantification of the enzyme uptake (using the ELISA method) were performed on the cell lysates, and the K_{uptake}, the half-maximal rate of uptake of the enzymes into the fibroblasts of each sample was determined [19] .

Statistical analysis

Descriptive statistics were used for the other exploratory efficacy variables. All values were expressed as mean and standard deviation. Statistical analysis for all the experiments was performed using two-sample *t*-tests. *P* values<0.05 were considered significant. If equal variance failed, the Mann–Whitney rank sum test was used. The error bars refer to mean ± standard deviation. All analysis were performed with SigmaPlot version 12.5 (Systat Software Inc., San Jose, CA)

Results

Formylglycine content

The proportion of FGly-form peptides in the samples was determined. The relative FGly level of each sample was very similar in all three replicate measurements. However, idursulfase beta had a significantly higher FGly content than idursulfase (79.40±0.92 vs. 68.12±2.22 %, $P<0.001$), (Table 1, Fig. 1).

Specific enzyme activity

The specific enzyme activities of idursulfase beta and idursulfase using the synthetic substrate (4-MU-αIdoA-2S) are shown in Table 2 and Fig. 2. The specific enzyme activity of idursulfase beta was significantly higher than that of idursulfase (42.58±1.11 vs. 27.76±0.94 nmol/min/μg protein, $P<0.001$). This is in agreement with the findings of the FGly content analysis.

Mannose-6-phosphate content

The M6P molar ratio was calculated by means of the protein concentration and by the molecular weight of the glycosylated protein. The average molar ratio of M6P is summarized in Table 3. The M6P molar ratios of idursulfase beta and idursulfase were not significantly different (2.38±0.07 vs. 2.42±0.28 mol/mol protein, $P=0.647$)

Sialic acid content

The sialic acid content was calculated by means of the protein concentration and by the molecular weight of the glycosylated protein. The average molar ratios of sialic acid are summarized in Table 4. The sialic acid molar ratios of idursulfase beta and idursulfase were not significantly different (12.31±0.65 vs. 12.38±0.36 mol/mol protein, $P=0.767$)

Table 2 Enzyme activity of idursulfase beta and idursulfase

Run	Enzyme activity (nmol/min/μg)					
	Idursulfase beta			Idursulfase		
	709R1006	709R1007	709R1008	DB1106-02	DB1106A01	DC1101A02
1	40.83	43.15	41.86	26.49	26.57	27.18
2	41.35	43.28	43.34	27.78	29.21	28.52
3	42.07	43.04	44.27	27.31	28.46	28.29
Mean ± SD	42.58±1.11			27.76±0.94		

SD, Standard deviation

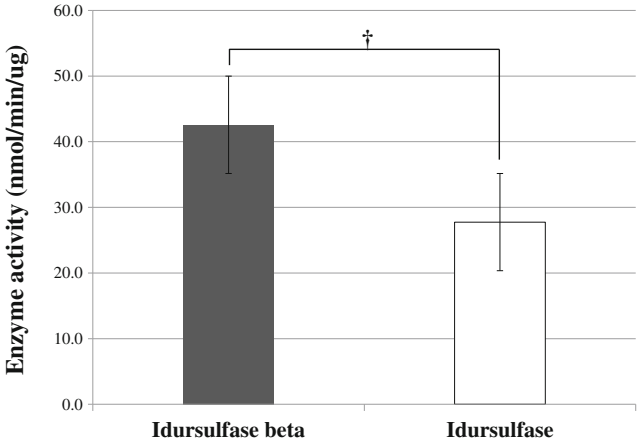

Fig. 2 Results of three independent measurements of the enzyme activity of idursulfase beta and idursulfase

Cell uptake activity

The *in vitro* cell uptake activity of idursulfase and idursulfase beta in normal human fibroblasts is summarized in Table 5. The cellular uptake activity (K_{uptake}) of idursulfase beta was significantly higher than that of idursulfase (K_{uptake}, 5.09± 0.96 vs. 6.50±1.28 nM, P=0.017)

Discussion

For ERT of Hunter syndrome, two recombinant enzymes (idursulfase and idursulfase beta) are currently available in Korea. In this study, we examined the biochemical and physicochemical characteristics of the two recombinant enzymes which can potentially influence to the clinical efficacy.

In general, co- or post-translational modification is necessarily required for catalytic activity of human sulfatase, that is well demonstrated in multiple sulfatase deficiency (MSD), one of the lysosomal storage disorders. In MSD, the deficiency of sulfatases is resulted from the lack of co- or post-translational conversion of cysteine to 2-amino-3-oxopropionic acid (FGly) that is required for their catalytic activity and is common to all sulfatases [20, 21]. Cysteine in the active site of all sulfatases is converted to FGly by formylglycine generating enzyme (FGE), and the stability and the activity of FGE are proportional to the sulfatase activity. MSD patients with drastic impairments in the stability of FGE and residual enzyme activity were found to display the most severe clinical phenotype, and the mildest phenotype was associated with the highest residual FGE activity [22].

For IDS activity, Cys-84 on the highly conserved sequence C^{84}-X-P-S-R^{88} has to be converted into a 2-amino-3-oxopropionic acid. According to a previous study, when this conserved cysteine was replaced either by alanine (Cys84Ala) or threonine (Cys84Thr) using site-directed mutagenesis or not converted into 2-amino-3-oxopropionic acid, IDS activity was abolished [11]. Therefore, FGly is essential for the enzymatic activity of IDS.

In this study, the FGly content of idursulfase beta was significantly higher than that of idursulfase. In addition, the specific activity of idursulfase beta was significantly higher (approximately 1.5 times) than that of idursulfase. This result is in good agreement with the results of the FGly content analysis. Previous studies reported that IDS catalyzes the hydrolysis of sulfate esters and that the key catalytic residue in sulfatase is a unique FGly, which is generated from a cysteine at position 84 [23, 24]. Therefore, the higher percentage of FGly in idursulfase beta may explain the higher specific enzyme activity. Although we could not demonstrate the mechanism of these difference in this study, one of the possible mechanism could be the difference of FGE activity of the two different cell lines; human HT-1080 for idursulfase vs CHO cell lines for idursulfase beta. Because we could not find the published evidence that the CHO cell lines usually express higher level of FGE activity than that in the HT-1080, the further analysis of FGE activity of the two cell lines will be needed. The other possible cause of the difference could be resulted from the different cell culture and purification conditions used in the manufacture of the two enzymes. However, according to the report by Muenzer *et al.* [9], the extent of post-translational modification of Cys-84 to FGly was approximately 50 % for idursulfase, whereas it was 68.1 % in our study. This difference may be due to the analytical methods.

For effective ERT of patients with MPS II, targeting of the enzyme to affected cells is very important. IDS contains eight N-linked glycosylation sites and consists of M6P-containing glycans, which enable high receptor-mediated cell uptake,

Table 3 Results of M6P analysis of idursulfase beta and idursulfase

Run	M6P molar ratio (mol/mol)					
	Idursulfase beta			Idursulfase		
	709R1006	709R1007	709R1008	DB1106-02	DB1106A01	DC1101A02
1	2.5	2.4	2.3	3.1	2.4	2.5
2	2.4	2.4	2.4	2.5	2.3	2.3
3	2.4	2.3	2.3	2.3	2.2	2.2
Mean ± SD	2.38±0.07			2.42±0.28		

SD, Standard deviation

Table 4 Results of sialic acid analysis of idursulfase beta and idursulfase

Run	Sialic acid molar ratio (mol/mol)					
	Idursulfase beta			Idursulfase		
	709R1006	709R1007	709R1008	DB1106-02	DB1106A01	DC1101A02
1	11.93	13.59	11.35	11.87	12.94	12.48
2	12.08	12.52	12.66	12.95	12.09	12.28
3	12.73	11.87	12.06	12.29	12.39	12.17
Mean ± SD	12.31±0.65			12.38±0.36		

SD, Standard deviation

leading to cellular internalization of the enzyme and subsequent targeting to intracellular lysosomes. In addition, IDS contains highly sialylated glycans, which prolong the circulating half-life of the enzyme. Sialylation of IDS was found to reduce the uptake by hepatic asialoglycoprotein receptors [9, 11, 25]. Thus, we hypothesized that successful targeting of IDS is strongly dependent on the M6P and sialic acid content of the sugar chains in enzyme preparations. However, the M6P and sialic acid content of the two products showed no significant difference in this study. In addition, we performed qualitative analysis of monosaccharide composition and glycan pattern to compare oligosaccharide structure of the two enzymes. The two enzymes showed a similar monosaccharide composition and similar patterns of N-glycans (data not shown). Further quantitative analysis is needed to find whether there is difference of oligosaccharide structures of the two enzymes. Nevertheless, the *in vitro* cellular uptake activity of the idursulfase beta by normal human fibroblasts was significantly higher than that of idursulfase. Therefore, we speculated that this difference could have resulted from the difference in the M6P type, rather than the total amount of M6P. A bis-phosphorylated glycan bind to the M6P receptor with higher affinity than a mono-phosphorylated glycan [26]. Therefore, bis-phosphorylated glycans is more effective for cellular uptake of recombinantly produced lysosomal proteins than mono-phosphorylated glycan [26]. Additional experiments of different M6P type and its effects on cell uptake activity in not only normal human fibroblast but MPSII fibroblast in vitro and in vivo will be needed. In terms of turnover rate of the two recombinant enzymes in circulating system, the turnover rate of enzymes would be influenced by not only the sialic acid contents, but also N-glycan structures attached the enzymes. Despite of the similar sialic acid contents of the both enzymes, the N-glycan structures attached to the two enzymes derived from the human HT-1080 and CHO cell lines are different. In vitro pharmacokinetic study will be needed for analyzing the turnover rate.

Furthermore, it is possible that the CHO cell line derived-enzyme may exhibit antigenicity to patients due to the species-specific oligosaccharide structure. However, the immunogenicity of therapeutic proteins is influenced by interactions of disease-, patient-, and product-related factors. Disease- and patient-related factors have the potential to predispose an individual to an immune response. Also, differences of the product- and process-related impurities of two enzyme preparations (*e.g.* break down products, aggregates, host cell proteins and bovine serum residues, *etc.*) may have the different immunogenicity [27]. Therefore, the the immunogenicity also should be considered to interpret the clinical efficacy of the two enzymes. In conclusion, idursulfase beta exhibited significantly higher specific enzyme activity than idursulfase, resulting from higher FGly content. These biochemical differences may be partly attributed to clinical efficacy. However, long-term clinical evaluations of Hunter syndrome patients treated with these two enzymes will be needed to demonstrate the clinical implications of significant difference of the enzyme activity and the FGly content.

Table 5 Results of cell uptake activity analysis of the idursulfase beta and the idursulfase

Run	K_{uptake} (nM)					
	Idursulfase beta			Idursulfase		
	709R1006	709R1007	709R1008	DB1106-02	DB1106A01	DC1101A02
1	3.76	5.69	5.11	6.92	6.33	7.67
2	5.57	4.79	5.12	8.86	6.05	6.46
3	5.57	6.61	3.57	6.18	5.83	4.23
Mean ± SD	5.09±0.96			6.50±1.28		

SD, Standard deviation

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All of the authors have nothing to disclose.

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