

Original Article

Toward reducing immunogenicity of enzyme replacement therapy: altering the specificity of human β -glucuronidase to compensate for α -iduronidase deficiency

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Abstract

Enzyme replacement therapy (ERT) is an effective treatment for many patients with lysosomal storage disorders caused by deficiency in enzymes involved in cell metabolism. However, immune responses that develop against the administered enzyme in some patients can hinder therapeutic efficacy and cause serious side effects. Here we investigated the feasibility of a general approach to decrease ERT immunogenicity by altering the specificity of a normal endogenous enzyme to compensate for a defective enzyme. We sought to identify human β -glucuronidase variants that display α -iduronidase activity, which is defective in mucopolysaccharidosis (MPS) type I patients. A human β -glucuronidase library was screened by the Enzyme Cleavable Surface-Tethered All-purpose Screen sYstem to isolate variants that exhibited 100–290-fold greater activity against the α -iduronidase substrate 4-methylumbelliferyl α -L-iduronide and 7900–24 500-fold enzymatic specificity shift when compared with wild-type β -glucuronidase. *In vitro* treatment of MPS I cells with the β -glucuronidase variants significantly restored lysosome appearance similar to treatment with α -iduronidase. Our study suggests that β -glucuronidase variants can be isolated to display α -iduronidase activity and produce significant phenotype improvement of MPS I cells. This strategy may represent a possible approach to produce enzymes that display therapeutic benefits with potentially less immunogenicity.

Key words: α -iduronidase, β -glucuronidase, enzyme replacement therapy, mucopolysaccharidosis (MPS), protein evolution

Introduction

Lysosomal storage disorders (LSDs) comprise over 50 different diseases with a wide range of clinical phenotypes and a combined incidence of 1 in 7000 live births (Fletcher, 2006). LSDs are caused by a genetic deficiency of lysosomal enzymes, resulting in the accumulation of unprocessed glycosaminoglycans (GAG) and progressive tissue damage. Enzyme replacement therapy (ERT) provides an effective treatment for many LSDs and is approved by the US Food and Drug Administration for treatment of mucopolysaccharidosis (MPS) types I, II, IV, type I

Gaucher and Fabry diseases (Staretz-Chacham *et al.*, 2009). ERT involves intravenous injection of therapeutic enzymes to replenish absent or defective enzymes and thus clear accumulating metabolites.

Administration of recombinant enzymes can induce immune responses in patients that lack or possess truncated endogenous enzymes. In fact, antibodies against therapeutic enzymes are found in the serum of ERT patients with frequencies ranging from 15% for Gaucher, 55–80% for Fabry, 91% for MPS I, 97% for MPS IV and 100% for Pompe diseases (Pastores *et al.*, 1993; Harnatz *et al.*, 2006;

Kishnani *et al.*, 2006; Wang *et al.*, 2008; Zarate and Hopkin, 2008). There is increasing evidence that antibody responses in patients can hinder ERT efficacy. For example, in Pompe disease, there are clear relationships between protein levels, antibody responses and therapeutic outcomes (Amalfitano *et al.*, 2001; Kishnani *et al.*, 2006, 2009). Patients with complete absence of acid α -glucosidase had high antibody titers against this enzyme and showed inhibition of enzyme uptake and activity during ERT (Kishnani *et al.*, 2009). Other studies also suggest similar results in Fabry and Pompe diseases (Linthorst *et al.*, 2004; Sun *et al.*, 2007; Ohashi *et al.*, 2008; Banugaria *et al.*, 2012; Ohashi *et al.*, 2012; Patel *et al.*, 2012). In a MPS I animal model, it was also reported that α -L-iduronidase-specific antibodies reduce ERT therapeutic efficacy (Dickson *et al.*, 2008). Induction of immune-tolerance by treatment of immunosuppressive drugs increased tissue enzyme levels and reduced GAG levels. Antibody-mediated inhibition of enzyme uptake in MPS I patients also strongly correlated to poorer biomarker responses which may suggest an important role in clinical outcomes (Langereis *et al.*, 2014). Life-threatening anaphylactic reactions have also occurred in patients receiving recombinant human α -iduronidase, laronidase (ALDURAZYME[®], BioMarin Pharmaceutical/Genzyme Corporation) (www.aldurazyme.com) (Lin *et al.*, 2005; Ensina *et al.*, 2014). These studies highlight the importance of immune responses in ERT clinical use.

Current strategies to overcome the antibody responses to recombinant enzymes are largely focused on inducing immune-tolerance (Dickson *et al.*, 2008; Peroni *et al.*, 2009; Banugaria *et al.*, 2013). However, this may be harmful to patients because the regimen is usually coupled with high doses of immunosuppressive drugs. Increased risk of infection and malignancy is also of concern.

Here, we investigated an alternative strategy, in which the enzymatic specificity of a normal endogenous enzyme is altered to compensate for the defective enzyme to help alleviate the antibody response (Fig. 1A). Because most of the modified enzyme should appear as a normal endogenous protein, we anticipate that this enzyme will be less immunogenic in LSD patients. We employed human β -glucuronidase as a template to generate α -iduronidase analogs as a proof of principle of this strategy. The expression of β -glucuronidase is normal in MPS I patients, so recombinant β -glucuronidase should be well tolerated and nonimmunogenic. β -Glucuronidase and α -iduronidase share a similar TIM (β/α)₈-barrel structure in their catalytic domains and belong to the same clan of glycoside hydrolase (Durand *et al.*, 2000). They also have similar catalytic mechanism that hydrolyze substrates via a pair of glutamic acid residues, E451 and E540 for β -glucuronidase (Islam *et al.*, 1999), and E182 and E299 for α -iduronidase (Nieman *et al.*, 2003) in a retentive fashion. In addition, in common with α -iduronidase, β -glucuronidase can be targeted to lysosomes by receptor-mediated endocytosis via mannose-6-phosphate receptors present on the surface of deficient cells (Natowicz *et al.*, 1979; Tsukimura *et al.*, 2008). Thus, the similar 3D structure of their active domains, mechanism of catalysis, cellular sorting and function suggests that β -glucuronidase is a good candidate for specificity switching. Based on this concept, we constructed a β -glucuronidase library and screened for α -iduronidase activity using the Enzyme Cleavable Surface-Tethered All-purpose Screen sYstem (ECSTASY) previously developed in our laboratory (Fig. 1B) (Chen *et al.*, 2012). The β -glucuronidase library was surface expressed by fusion to a C-terminal GPI-anchor signal sequence from human decay accelerating factor (DAF) (Caras *et al.*, 1987). The membrane-bound β -glucuronidase library cells were screened by high through-put fluorescence-activated cell sorting (FACS) followed by phosphatidylinositol-specific phospholipase C (PI-PLC) cleavage to facilitate screening for α -iduronidase activity

under defined conditions. After screening, the *in vitro* effect of the β -glucuronidase variants in α -iduronidase deficient cells was investigated.

Materials and methods

Reagents and antibodies

PI-PLC, LysoTracker-Red DND-99 dye and Hoechst 33342 nuclear dye were from Invitrogen (Carlsbad, CA, USA). 4-Methylumbelliferyl β -D-glucuronide (MUG) was from Sigma-Aldrich (St Louis, MO, USA). 4-Methylumbelliferyl α -L-iduronide (MUI) was from USB Corporation (Cleveland, OH, USA). Trace MUG contamination (~2%) in commercial MUI was removed by solid-phase extraction/high-performance liquid chromatography on a LiChroprep RP18 (40–63 μ m) column equilibrated with 20% methanol (pH 4). MUI was eluted with 25% methanol in double-distilled water (pH 4) and condensed in a rotavapor. Mouse anti-human β -glucuronidase monoclonal antibody (mAb) 7G8 was directly labeled with FITC or biotin as described (Goding, 1976; Chen *et al.*, 2007). Streptavidin-horseradish peroxidase (HRP) was from Jackson ImmunoResearch (West Grove, PA, USA).

Cell culture

GP293V cells (derived from human embryonic kidney 293 cells) were kindly provided by Dr Andre Lieber, University of Washington, Seattle, WA, USA. The 34/2000 cells (human α -iduronidase deficient fibroblasts derived from an MPS type I patient) were a kind gift from Dr Mirella Filocamo, Istituto G. Gaslini, Genova, Italy. BALB/3T3 fibroblasts and HEK293 cells were obtained from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2.98 g/l HEPES, 2 g/l NaHCO₃, 100 U/ml penicillin and 100 μ g/ml streptomycin.

Synthetic library construction

3D structures of human β -glucuronidase (PDB ID: 1BHG (Jain *et al.*, 1996)) and human α -iduronidase (PDB ID: 3W82 (Maita *et al.*, 2013)) reveal a similar TIM (β/α)₈-barrel structure in their catalytic pockets. Fifteen residues (S447, N484, N502, S503, Y504, Y508, H509, G542, W587, F592, T594, E595, R600, N604 and K606) in the β -glucuronidase catalytic domain were identified as surrounding the active pocket in which substrates were accommodated. Previous research (Matsumura and Ellington, 2001; Geddie and Matsumura, 2004; Rempel *et al.*, 2005; Chen, *et al.*, 2012) also reported several β -glucuronidase residues associated with enzyme activity and specificity (N484, S503, S506, H509, T545, L565, E595, P598, N604 and K606). A total of 19 amino acid residues were mutated (Supplementary Fig. S1A). The six underlined residues (N484, S503, H509, E595, N604 and K606) were considered as hot spots because they were identified by both structure analysis and a review of the literature. The six hot spots were mutated to variable amino acids to enrich the library diversity. For example, we employed degenerate primers at S503 to mutate serine into amino acids with side chains which are positively charged (histidine), negatively charged (aspartic acid), aromatic (tyrosine and phenylalanine), hydrophobic (alanine, leucine and valine) and special in conformation (proline). The other residues were mutated to the corresponding amino acids that were identified from the structural comparison or in previous studies (Supplementary Fig. S1A). Primer assembly was used to generate the human β -glucuronidase library (Supplementary Fig. S2). Briefly, the full-length β -glucuronidase sequence was divided into 5 fragments with 18 overlapping nucleotides

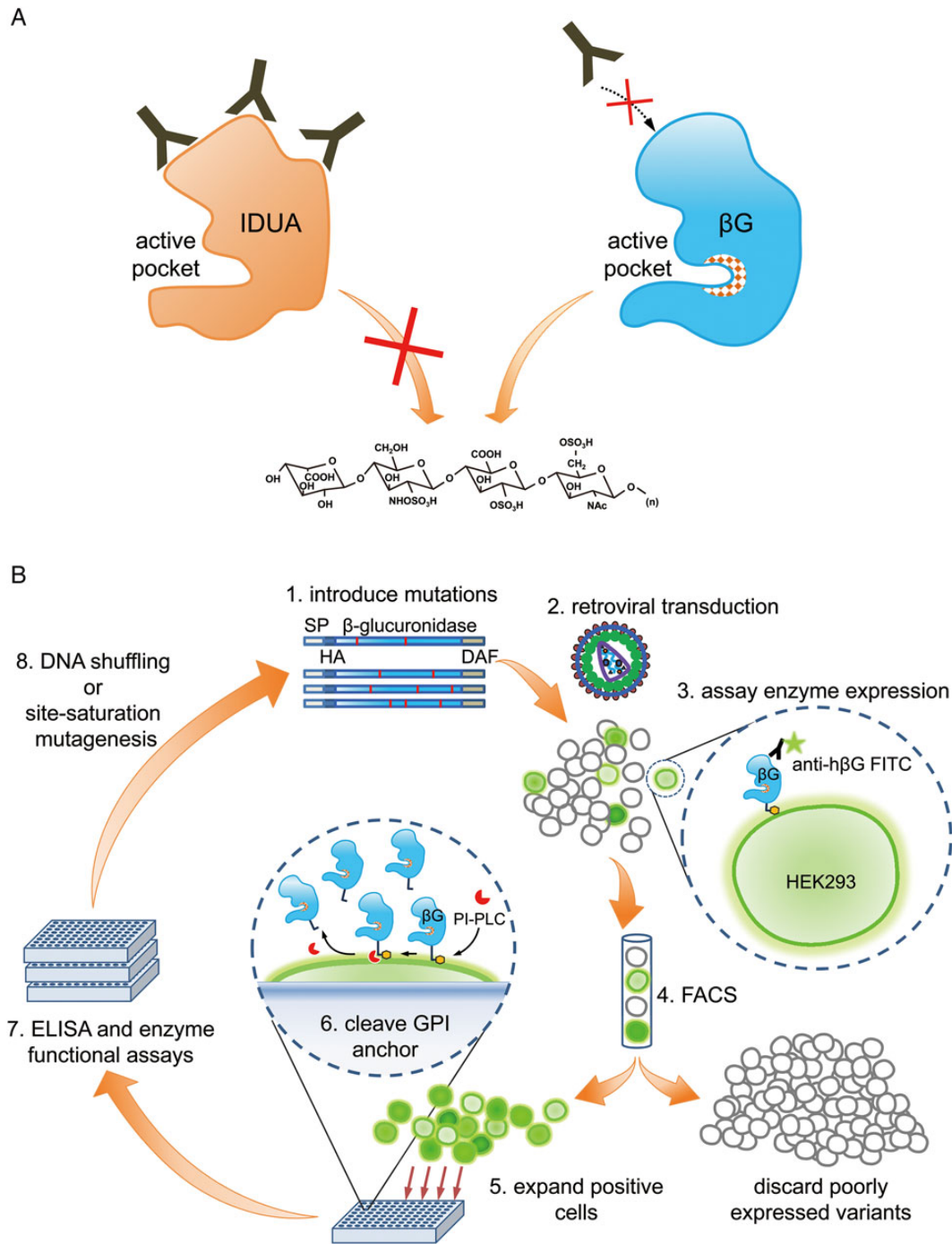


Fig. 1 (A) Illustration of the concept of 'cloaked stealth' enzymes for ERT. Wild-type α -iduronidase (IDUA) can generate specific antibodies in MPS type I patients which can inhibit the therapeutic efficacy and even cause life-threatening anaphylactic reactions. β -Glucuronidase (β G) variants which have been altered in the active pocket to display α -iduronidase activity may appear as normal immunotolerant self-proteins and thus be able to prevent antibody responses, thereby retaining therapeutic activity and reducing side effects. The sizes of human immunoglobulin G (150 kDa), α -iduronidase (83 kDa) and β -glucuronidase (78 kDa) are not drawn to scale. (B) Outline of ECSTASY. (1) A library of β -glucuronidase variants is cloned into a retroviral vector, which codes for a signal peptide, an HA tag, a 6xHis tag, an enzyme variant, a myc tag and the C-terminal 37 amino acids of human DAF. (2) Library cells expressing one GPI-anchored β -glucuronidase variant each are generated by retroviral transduction of HEK293 cells at a low MOI. (3 and 4) The cells are immunofluorescence stained with an anti- β -glucuronidase-FITC conjugate to identify cells expressing β -glucuronidase on their surface. FACS is employed to rapidly discard cells expressing misfolded or unstable enzyme variants as determined by low immunofluorescence staining. (5) Positive cells are individually sorted into 96-well microtiter plates and allowed to grow to full confluence. (6) Surface-tethered enzyme variants are released from the cells by PI-PLC cleavage. (7) The supernatant containing soluble enzyme variants are assayed for enzyme concentration by ELISA and enzyme activity by flourometric assays. (8) Selected mutants with higher activity can be used as templates for a new generation library by DNA shuffling or site-saturation mutagenesis.

between each other (F0–4). The F0 fragment contained an *Apa* I cloning site as well as a silent mutation (nucleotide G741A) to remove a unique *Bgl* II cutting site in the human β -glucuronidase gene to remove wild-type DNA contamination after library construction. The wild-type fragment F1 simply served as a bridge between the F0 and F2 fragments. The F2 and F3 fragments, which contained variable amino acids at 19 positions, were constructed by primer assembly followed by PCR amplification. The wild-type F4 fragment contained a *Sal* I cloning site. All fragments were mixed in the same molar ratio and amplified by PCR to obtain a full-length β -glucuronidase library which was digested with *Apa* I and *Sal* I and ligated into the same sites in pLNCX-h β G-DAF (Chen, *et al.*, 2012) to append a sequence coding for a GPI anchor to the C-terminus of the enzyme variants. The DNA library was digested by *Bgl* II to remove any wild-type DNA contamination and then transformed into DH5 α competent cells by electroporation. Transformed bacteria were selected on 15-cm carbenicillin-containing LB agar plates for 16 h at 37°C. Plasmid DNA was purified from single colonies and sequenced to determine the mutation rates at selected residues. All expected mutations were present in the synthetic library (Supplementary Fig. S1A). Colonies from multiple plates were collected and expanded in carbenicillin-containing LB medium. The plasmid was amplified by addition of chloramphenicol to a final concentration 170 μ g/ml when OD₆₀₀ was 0.5. After overnight culture, plasmid DNA was purified by centrifugation in a CsCl-ethidium bromide density gradient at 60 000 rpm in a Ti 70.1 rotor for 24 h at 4°C using a Beckman Optima L-90K ultracentrifuge (Beckman Coulter, Fullerton, CA, USA).

Generation of stable library cells

To generate stable cell libraries, library plasmid DNA was co-transfected with pVSV-G (Clontech, Mountain View, CA, USA) into GP293V cells to produce recombinant retroviral particles. Two days after transfection, the culture medium was filtered, mixed with 8 μ g/ml polybrene, and incubated with 293 cells at a multiplicity of infection of 0.1. Stable cell lines were selected in medium containing 0.5 mg/ml G418 (Calbiochem, San Diego, CA, USA). The resulting synthetic library cells were denoted as 293/L1 cells.

Flow cytometer analysis and library cell selection

Human β -glucuronidase surface expression was determined by staining 293/L1 cells with 7G8-FITC, which binds to human β -glucuronidase, and measuring immunofluorescence of viable cells with a FACScaliber flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Generally, 2×10^7 cells were washed and suspended in 1 ml HBSS (5.4 mM KCl, 0.3 mM Na₂HPO₄, 0.4 mM KH₂PO₄, 4.2 mM NaHCO₃, 1.3 mM CaCl₂, 0.5 mM MgCl₂, 0.6 mM MgSO₄, 137 mM NaCl, 5.6 mM D-glucose, pH 7.4) containing 0.5% BSA and 20 μ g/ml 7G8-FITC for 30 min at 4°C. The cells were washed with ice-cold HBSS containing 0.5% BSA and suspended in 0.5% BSA/HBSS containing 5 μ g/ml propidium iodide. Cells were sorted on a FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). Dead cells (propidium iodide positive, high FL3 fluorescence) were gated out before 7G8-FITC immunofluorescence was detected at excitation/emission wavelengths of 488/515 nm (FL1). Single cells expressing surface human β -glucuronidase were sorted into 96-well microplates in DMEM supplemented with 10% bovine serum.

Surface enzyme release and enzyme activity screening

293/L1 cells in 96-well microplates were washed once with phosphate-buffered saline (PBS) and incubated with 100 μ l PBS containing 50 mU/ml PI-PLC at 37°C for 1 h to cleave GPI-anchored β -glucuronidase

variants from surface of the cells. α -Iduronidase activity of the released β -glucuronidase was assayed by mixing 20 μ l samples of cleaved enzyme with 80 μ l of 25 μ M MUI in 0.2 M formate buffer, pH 3.5 for 17 h at 37°C. The reaction was stopped by adding 100 μ l stop buffer (1 M glycine, 0.5 M sodium bicarbonate, pH 10.7) and the 4-methylumbelliferone (4-MU) fluorescence in the wells was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. To reduce the systematic error of manual volumetric transfers during large scale MUI assay and sandwich ELISA, an automated liquid handling system, MicroLab MPH-96 (Hamilton Robotics, Reno, NV, USA), was employed. Kinetic parameters against MUI were determined by hydrolysis of serial diluted substrate (400 μ M) with defined concentrations of enzymes. The reaction was terminated at various time points and the fluorescence was measured. The acquired fluorescence was converted to product concentration by comparison with a 4-MU standard curve. Lineweaver–Burk plots were used to determine K_M and k_{cat} .

Sandwich enzyme-linked immunosorbent assay

The concentration of soluble β -glucuronidase generated by PI-PLC cleavage of surface enzyme from individual colonies of the sorted 293-L1 cells was measured by sandwich enzyme-linked immunosorbent assay (ELISA). A 0.1 μ g mAb 7G8 in 50 μ l coating buffer (50 mM Na₂CO₃, 50 mM NaHCO₃, pH 8) was incubated in each well of 96-well ELISA plates at room temperature for 1 h. The plates were washed three times with PBS and then blocked with 2.5% skim milk in PBS at room temperature for 1 h. The plates were washed three times with PBS and 20 μ l human β -glucuronidase variant sample diluted to 50 μ l with PBS was transferred to each well for 1 h at room temperature. The plates were washed three times with PBS containing 0.05% Tween 20 before 20 ng 7G8-biotin and 50 ng streptavidin-HRP in 50 μ l PBS containing 2.5% skim milk were each subsequently added at room temperature for 1 h. After each step, the plates were washed three times with PBS containing 0.05% Tween 20. One hundred and fifty microliters freshly prepared 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) substrate was added at room temperature for 30 min and the absorbance of each well was measured at 405 nm.

Lysosome staining and image acquisition

A lysosomal staining method was employed to visualize the effect of the enzymes in MPS I cells (Wang *et al.*, 2013; Xu *et al.*, 2014). Briefly, MPS I cells were plated in 96-well microplates and incubated with 5.0 μ g/ml of recombinant enzymes for 72 h. Cells were washed with PBS and live stained with 100 μ l medium containing 100 nM LysoTracker-red DND-99 dye and 1 μ g/ml Hoechst 33342 for 30 min at 37°C. The cells were washed twice with PBS, replenished with 200 μ l DMEM without phenol red and live imaged on an ImageXpress Micro XL High-Content Screening System (Molecular Devices, CA, USA). The LysoTracker and Hoechst staining were visualized using TRITC (Em = 545 \pm 20, Ex = 593 \pm 20 nm) and DAPI (Ex = 350 \pm 50, Em = 455 \pm 50 nm) filters, respectively. Nine sites of images per well were recorded and analyzed by MetaXpress High-Content Image Acquisition & Analysis Software (Molecular Devices, CA, USA).

Cellular GAG accumulation assay

A ³⁵SO₄ incorporation assay was employed to measure the effect of the enzymes on cellular GAG accumulation of MPS I cells (Unger *et al.*, 1994). Briefly, MPS I cells were plated in 10-cm dishes and incubated with 10 μ Ci/ml of Na₂³⁵SO₄ (American Radiolabeled Chemicals, St Louis, MO, USA) in SO₄-free medium containing

10% dialyzed FBS for 7 days to radiolabel GAG. The cells were then washed with PBS three times and incubated with 5 $\mu\text{g}/\text{ml}$ of recombinant enzymes for 72 h. Supernatants were collected and the cells were washed with PBS three times followed by lysis with RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS). The differences in radioactivity of cell lysates and supernatants when compared with untreated cells were represented as counts per minute (cpm) per mg total protein.

Statistical analysis

The two-tailed Student's *t*-test was used to calculate the significant differences between wild-type and β -glucuronidase variants by *Graphpad Prism 5.0* (GraphPad Software Inc., San Diego, CA, USA). Data were considered significant at *P* values <0.05.

Results

Wild-type human β -glucuronidase displays detectable α -iduronidase activity

Owing to the similarity between human β -glucuronidase and α -iduronidase, we sought to determine if human β -glucuronidase displayed endogenous α -iduronidase activity. We expressed and purified recombinant human β -glucuronidase from human α -iduronidase deficient fibroblasts derived from MPS type I patients to eliminate possible contamination of the recombinant β -glucuronidase with endogenous α -iduronidase. Recombinant β -glucuronidase bearing a polyhistidine (6xHis) tag was purified by ammonium sulfate precipitation and Ni^{2+} -nitrilotriacetic acid affinity chromatography (Wu *et al.*, 2004). The α -iduronidase activity assay was performed by incubation of recombinant β -glucuronidase with α -iduronidase substrate, MUI at pH 3.5, 37°C for 17 h (Kakkis *et al.*, 1996). The substrate MUI was relatively stable at low concentrations (25 μM) and the background could be ignored at high concentrations (250 μM) when compared with sample groups during incubation (Supplementary Fig. S3A and B). *In vitro* assay showed that human β -glucuronidase exhibited measurable activity against MUI corresponding to $\sim 0.002\%$ of the activity of wild-type human α -iduronidase. The hydrolysis of MUI was proportional to human β -glucuronidase amount and incubation time (Supplementary Fig. S3B). This activity was also confirmed by HPLC which showed the hydrolysis of MUI and an increase in the product 4-MU (Supplementary Fig. S3C and D). Note that commercial MUI contains MUG contamination ranging from 0.1 to 2% in the datasheet provided as well as indicated by HPLC (Supplementary Fig. S3E and F). Thus, the commercial MUI was purified as described in the 'Materials and methods' section before assaying to avoid signals from hydrolysis of MUG.

Identification of human β -glucuronidase variants displaying elevated α -iduronidase activity

We screened human β -glucuronidase variants for clones with higher α -iduronidase activity by ECSTASY (Fig. 1B). Briefly, a human β -glucuronidase library with mutations at 19 residues for a total diversity of 3×10^9 was designed by structural analysis and a review of the literature (Supplementary Fig. S1A). Mutations were introduced at 19 positions in the human β -glucuronidase gene by primer assembly followed by PCR amplification (Supplementary Fig. S2), resulting in 1.2×10^7 bacterial colonies. Compared with the initial library diversity, the number of *Escherichia coli* transformants was ~ 300 -fold lower. However, sequencing of *E. coli* single transformants revealed that all expected mutations were present with acceptable bias (Supplementary Fig. S1A). Retroviral

transduction of 293 cells with library DNA at a multiplicity of infection of 0.1 to ensure only a single β -glucuronidase variant gene in each cell resulted in 3×10^6 stable clones (293/L1 cells). The 4-fold loss of diversity from *E. coli* transformants to stable mammalian library cells may have little effect to the library coverage because sequencing results of nine surface expression negative variants (Supplementary Fig. S1B) and nine high activity variants (Supplementary Fig. S1C) indicated that almost all expected mutations were represented. To remove the human β -glucuronidase variants, which cannot properly fold or be expressed on the surface of cells, we first stained live 293/L1 cells with mAb 7G8-FITC that binds to human β -glucuronidase, and collected the cells that displayed relatively high levels of human β -glucuronidase protein on their surface. Flow cytometry results indicated that 16% of 293/L1 cells expressed GPI-anchored human β -glucuronidase on their surface (dashed gate, Fig. 2A). Cells exhibiting the highest human β -glucuronidase expression level (solid gate, 6.8% of the population) were sorted as single cells into 96-well microplates (Fig. 2A) and allowed to expand to near confluence. Each colony was then treated with PI-PLC to cut the GPI anchor and the concentration and activity of each solubilized β -glucuronidase variant was assayed as described in the 'Materials and methods' section. For example, screening of several 96-well microtiter plates revealed three β -glucuronidase variants that exhibited significantly higher α -iduronidase activity than wild-type β -glucuronidase (Fig. 2B and C). In total, we screened 50 96-well plates of sorted 293/L1 library cells and identified 1.3% (73/4800) of β -glucuronidase variants with elevated α -iduronidase activity when compared with wild-type β -glucuronidase.

Characterization of human β -glucuronidase variants displaying α -iduronidase activity

Nine human β -glucuronidase variants which exhibited high α -iduronidase activity were randomly selected and cloned into a mammalian expression vector to produce greater amounts of recombinant soluble β -glucuronidase from BALB/3T3 fibroblasts and 34/2000 cells (human α -iduronidase deficient fibroblasts derived from a MPS type I patient). All soluble human β -glucuronidase variants displayed enhanced α -iduronidase activity when compared with wild-type β -glucuronidase (Supplementary Fig. S4) and the sequences were analyzed (Supplementary Table SI). Three β -glucuronidase variants (102H1, 101C7 and 70H1) were further characterized. Amino acid sequences of the three selected clones are shown in Table I. The recombinant human β -glucuronidase variants showed similar molecular weights as wild-type human β -glucuronidase as determined by immunoblotting with anti-6xHis tag antibody (Fig. 3A) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Supplementary Fig. S5). The β -glucuronidase variants also exhibited increased activity against MUI when compared with wild-type human β -glucuronidase (Fig. 3B). The kinetic properties of human β -glucuronidase variants against MUI were measured and analyzed (Table II). The substrate affinity K_M to MUI of the human β -glucuronidase variants 102H1, 101C7 and 70H1 was 36.9 ± 3.2 , 28.2 ± 2.4 and 24.5 ± 1.6 μM , respectively. Compared with wild-type human β -glucuronidase, these variants showed 19-, 25- and 29-fold enhanced affinity to MUI, respectively. The enzyme turnover number k_{cat} of the human β -glucuronidase variants 102H1, 101C7 and 70H1 was 0.0099 ± 0.0009 , 0.013 ± 0.0011 and 0.0039 ± 0.0003 , which corresponds to 11-, 14- and 4-fold improvement when compared with wild-type β -glucuronidase, respectively. The overall α -iduronidase activity of the three β -glucuronidase variants was increased from 100- to 290-fold when compared with wild-type β -glucuronidase (Table III).

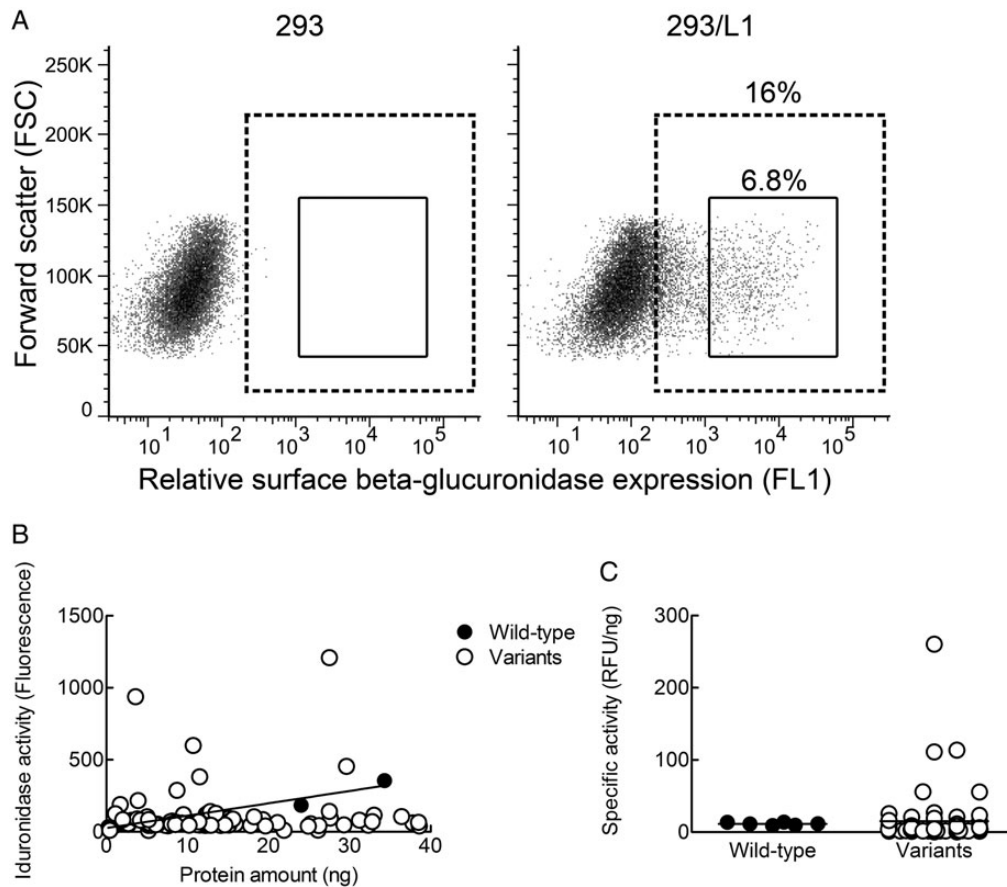


Fig. 2 Example of screening the human β -glucuronidase synthetic library by ECSTASY: one example of the screening. (A) 293 and 293/L1 cells are stained with 7G8-FITC and analyzed on a flow cytometer for surface human β -glucuronidase expression (dashed gate, 16%). Cells exhibiting the highest human β -glucuronidase expression level (solid gate, 6.8%) are sorted into 96-well microplates as single cells for subsequent screening. (B) GPI-anchored human β -glucuronidase variants are cleaved from individual 293/L1 clones by PI-PLC. The supernatant is assayed for hydrolysis of MUI at pH 3.5. Protein amounts are quantitated by sandwich ELISA. Wild-type and β -glucuronidase variants are shown as closed and open circles, respectively. (C) Specific activity of each β -glucuronidase variant is presented as relative fluorescence unit (RFU) per ng protein.

Table I. Amino acid sequences of high α -iduronidase activity variants

Amino acid residue											
Clones	279	447	484	502	503	504	506	508	509	542	
Wild-type	Q	S	N	N	S	Y	S	Y	H	G	
102H1			S	D	A		G		A	D	
101C7	H		C	K	Y	G	G		P		
70H1			D	K	Y		G	D	P		
Clones	545	565	587	592	594	595	598	600	604	606	636
Wild-type	T	L	W	F	T	E	P	R	N	K	P
102H1	A			Y		V			S	F	
101C7	A	A			L	Q			A	F	
70H1	A			Y	L	G	D		T	F	S

The enzyme specificity was shifted from β -glucuronidase to α -iduronidase by a factor ranging from 7900- to 24 500-fold. The β -glucuronidase variants exhibited low but significant α -iduronidase activity ranging from 0.3 to 0.9% of wild-type α -iduronidase.

To address whether recombinant human β -glucuronidase variants could alter the phenotype of MPS I cells, cellular GAG accumulation was measured by a ^{35}S incorporation assay (Unger *et al.*, 1994). MPS I cells were incubated with Na_2^{35}S to radiolabel GAG before the cells were exposed to 5 $\mu\text{g}/\text{ml}$ recombinant enzyme for 72 h (Tsukimura, *et al.*, 2008). Cell lysates and culture medium were then

collected and the ^{35}S radioactivity was measured. Cells treated with wild-type α -iduronidase or the β -glucuronidase variants 102H1 and 70H1 exhibited significantly reduced radioactivity in cell lysates when compared with untreated cells (Fig. 3C). The culture medium of MPS I cells that were treated with wild-type α -iduronidase and the three β -glucuronidase variants exhibited significantly increased radioactivity when compared with cells treated with wild-type β -glucuronidase (Fig. 3D), indicative of enhanced digestion and excretion of cellular GAG product. In summary, when compared with untreated cells and cells treated with wild-type β -glucuronidase, cells

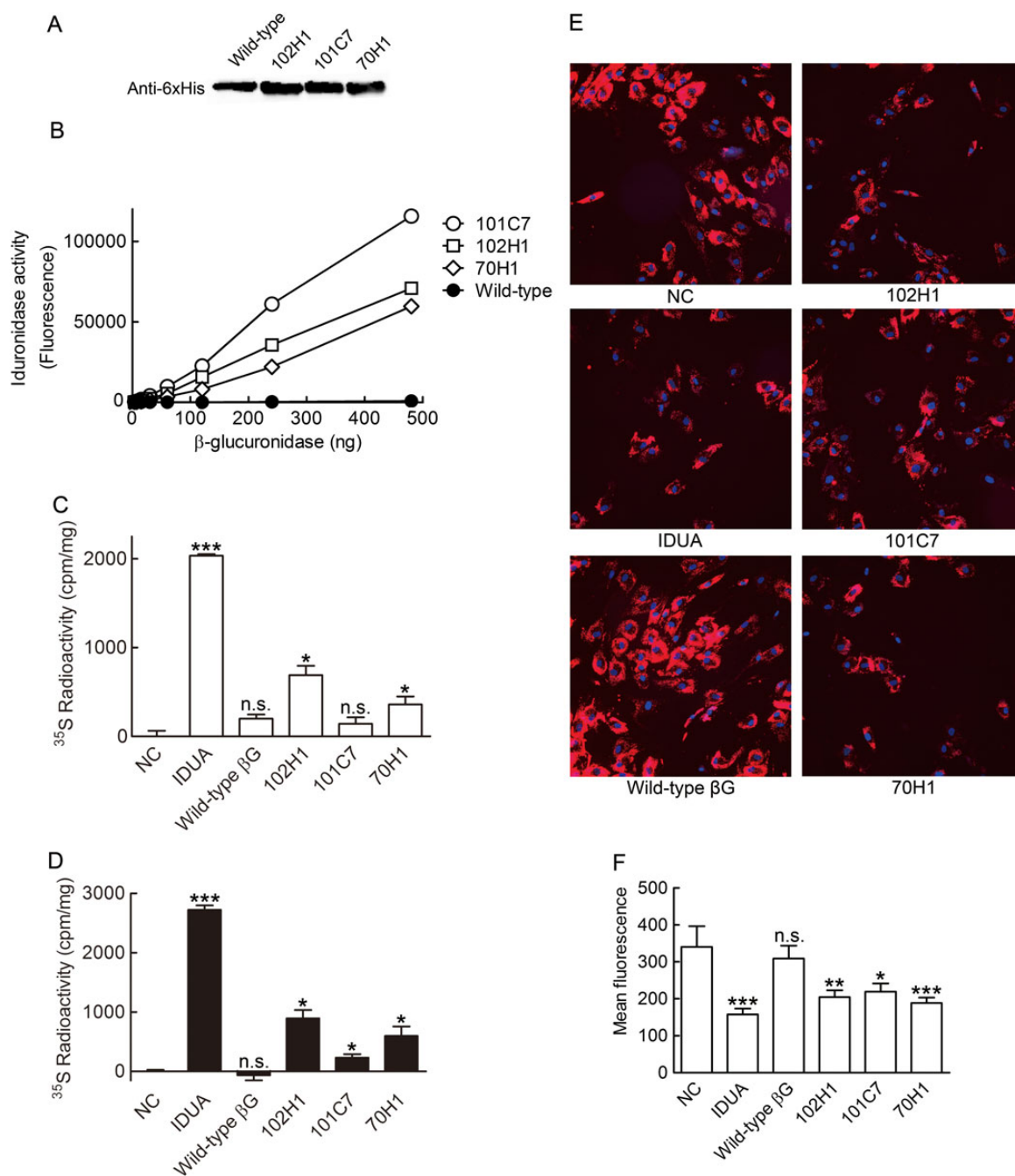


Fig. 3 Characterization of β -glucuronidase variants displaying α -iduronidase activity. (A) 0.1 μ g of recombinant wild-type and β -glucuronidase variants (as determined by ELISA) are electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and immunoblotted with anti-6xHis antibody. (B) Recombinant wild-type and β -glucuronidase variants are incubated with MUI at pH 3.5 and the relative fluorescence is detected. (C and D) Human α -iduronidase deficient cells (34/2000, derived from an MPS type I patient) are incubated with Na_2^{35}S to radiolabel the GAG. Cells are then treated with 5 μ g/ml of recombinant enzymes for 72 h. The reduced radioactivity of cell lysates when compared with untreated cells is represented as mean \pm SD (white bars). The increased radioactivity of supernatants when compared with untreated cells is represented as mean \pm SD (black bars). (E) Human α -iduronidase deficient cells are treated with 5 μ g/ml of recombinant enzymes for 72 h and stained with Lysotracker-Red DND-99 dye for lysosomes and Hoechst 33342 for nuclei. Images are analyzed by MetaXpress High-Content Image Acquisition & Analysis Software. (F) Lysosomal fluorescence per cell is presented as mean \pm SEM. The significance differences to untreated cells in two-tailed *t*-test are indicated. **P* < 0.05; ***P* < 0.01; ****P* < 0.001; n.s.: nonsignificant (*n* = 3).

treated with β -glucuronidase variants 102H1 and 70H1 revealed significantly reduced cellular GAG and increased GAG excretion. Cells treated with β -glucuronidase variant 101C7 displayed a nonstatistically significant decrease of cellular GAG but significantly increased GAG excretion. Although the β -glucuronidase variants were not as

effective as wild-type α -iduronidase, these results indicate partial correction of GAG storage in MPS I cells.

We also employed a qualitative lysosomal staining method to visualize the phenotypic change in MPS I cells (Wang, *et al.*, 2013; Xu, *et al.*, 2014). MPS I cells were incubated with 5 μ g/ml of recombinant

Table II. Kinetic parameters of wild-type α -iduronidase (IDUA), β -glucuronidase (β G) and β -glucuronidase variants for hydrolysis of MUI at pH 3.5

	K_M (μ M)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} M^{-1}$)
IDUA	203 \pm 21	10.2 \pm 0.4	50 100 \pm 3100
Wild-type β G	705 \pm 7.0	0.0009 \pm 0.0002	1.22 \pm 0.35
102H1	36.9 \pm 3.2	0.0099 \pm 0.0009	270 \pm 24
101C7	28.2 \pm 2.4	0.013 \pm 0.0011	470 \pm 40
70H1	24.5 \pm 1.6	0.0039 \pm 0.0003	160 \pm 11

Results are presented as mean value \pm SD of triplicate determinations.

Table III. Relative enzyme activity and specificity change of wild-type and β -glucuronidase (β G) variants

	Relative IDUA activity	Relative β G activity	Specificity shift	Activity relative to wild-type IDUA (%)
Wild-type	1	1	1	0.002
102H1	170	0.02	7900	0.5
101C7	290	0.02	19 200	0.9
70H1	100	0.004	24 500	0.3

The α -iduronidase (IDUA) and β -glucuronidase (β G) activities are assayed with MUI and MUG, respectively. The relative enzyme activity is presented in fold increases of k_{cat}/K_M when compared with wild-type α -iduronidase and β -glucuronidase. The specificity shift is presented in fold change of IDUA activity over β G activity compared with wild-type β -glucuronidase.

enzymes for 72 h and then stained with LysoTracker-red DND-99 dye (Invitrogen, Carlsbad, CA, USA) to visualize the lysosomes (Fig. 3E). The lysosome fluorescence was quantitated and shown as mean fluorescence intensity per cell. High lysosomal staining was observed in nontreated MPS I cells (Fig. 3E, NC). As expected, treatment of the cells with wild-type β -glucuronidase did not affect lysosome fluorescence (Fig. 3E, wild-type β G). In contrast, the cells treated with α -iduronidase (Fig. 3E, IDUA) or β -glucuronidase variants (Fig. 3E, 102H1, 101C7 and 70H1) displayed significantly reduced lysosomal staining when compared with nontreated MPS I cells (Fig. 3F), indicating at least partial normalization of lysosomes in the deficient cells. In summary, these results revealed that β -glucuronidase variants displayed beneficial effects toward correction of the phenotype of MPS I cells.

Discussion

We investigated a new strategy aimed toward reducing ERT immunogenicity in which we modify an endogenously expressed enzyme to compensate for a defective enzyme (Fig. 1A). A human β -glucuronidase library was screened by ECSTASY, and 73 β -glucuronidase variants that displayed increased α -iduronidase activity were identified from 4800 clones (Chen *et al.*, 2012). Three β -glucuronidase variants (102H1, 101C7 and 70H1) which displayed elevated α -iduronidase activity when compared with wild-type β -glucuronidase were purified from human α -iduronidase deficient fibroblasts derived from an MPS type I patient to eliminate possible contamination with α -iduronidase (Fig. 3A). The selected β -glucuronidase variants displayed enhanced α -iduronidase activity with 100–290-fold increased k_{cat}/K_M values when compared with wild-type β -glucuronidase (Fig. 3B, Tables II and III). In addition, MPS I cells treated with the β -glucuronidase

variants exhibited significantly reduced the cellular GAG storage (Fig. 3C and D) and lysosomal staining when compared with nontreated cells (Fig. 3E and F), suggesting at least partial correction of the MPS I phenotype.

The substrate affinity K_M to MUI of the human β -glucuronidase variants 102H1, 101C7 and 70H1 was enhanced by 19-, 25- and 29-fold when compared with wild-type β -glucuronidase, respectively. On the other hand, the enzyme turnover number k_{cat} exhibited an increase of 11-, 14- and 4-fold improvement when compared with wild-type β -glucuronidase. These results suggest that the reduced K_M contributes more to the increased α -iduronidase activity of the selected human β -glucuronidase variants rather than increased k_{cat} . Although the activity of the variants is much lower than wild-type α -iduronidase, ranging from 0.3 to 0.9% (Table III), these variants significantly altered the phenotype of MPS I cells as measured by a cellular GAG storage assay (Fig. 3C and D) and observation of fluorescence stained lysosomes (Fig. 3E and F). This is in agreement with previous studies in which \sim 1% residual α -iduronidase activity can show significant phenotypic effects (Di Domenico *et al.*, 2005; Di Domenico *et al.*, 2006; Herati *et al.*, 2008; El-Amouri *et al.*, 2014). However, the β -glucuronidase variant 101C7 displayed high catalytic activity against MUI but was relatively ineffective at correcting the phenotype of MPS I cells. This might result from differences between the substrate used for screening (MUI) and natural GAG substrates in MPS I cells. Screening systems developed against the natural substrates may be able to select enzyme variants which exert better therapeutic effects for MPS I and other LSDs.

The ECSTASY mammalian expression and screening system (Fig. 1B) (Chen *et al.*, 2012) is well suited for evolving enzymes with posttranslational modifications such as glycosylation and disulfide bridge formation. FACS was utilized to select for stable β -glucuronidase variants by immunofluorescence staining of β -glucuronidase present on the surface of the cells. β -Glucuronidase is normally located intracellularly inside lysosomes, but addition of a GPI-anchor peptide sequence directs the variant enzymes to the cell surface. Misfolded, unstable and truncated enzymes that do not pass the quality control processes in the endoplasmic reticulum are not displayed on the cells and can be eliminated from subsequent screening steps. Indeed, we discarded \sim 93% of the library cells, which displayed no or low levels of β -glucuronidase on their surface, which can increase the 'hits' in the screening process. Besides, lysosomes usually maintains an acidic environment about pH 4.5 to provide the lysosomal enzyme catalytic activity for substrates degradation (De Duve and Wattiaux, 1966). The catalytic activity of human β -glucuronidase is optimal at pH 4 (Chen *et al.*, 2008) while α -iduronidase has optimal pH around 3.5 depending on different substrates and buffers used (Freeman and Hopwood, 1992). To better mimic the catalytic mechanism of α -iduronidase, we performed the screening assay at pH 3.5 (Kakkis *et al.*, 1996), in which the library cells cannot survive. Thus, GPI-anchored β -glucuronidase variants were released in soluble form by treatment of the cells with PI-PLC to allow measurement of soluble enzyme concentrations and activity. Up to 0.5 μ g β -glucuronidase could be released from the surface of the cells in a single confluent colony in the well of a 96-well plate, which is sufficient for enzyme quantitation by ELISA and activity assays.

Analysis of the amino acid sequences of the nine selected variants (Supplementary Fig. S1C), in which the activity was confirmed in purified soluble enzymes, revealed that the wild-type amino acids were highly preferred at S447 (100%, 9 of 9 clones), G542 (78%, 7/9), L565 (78%, 7/9), W587 (78%, 7/9), T594 (67%, 6/9) and R600 (78%, 7/9). When compared with sequencing results of the initial library (Supplementary Fig. S1A) and the surface expression negative variants after FACS (Supplementary Fig. S1B), preference of S447, L565 and T594 may result

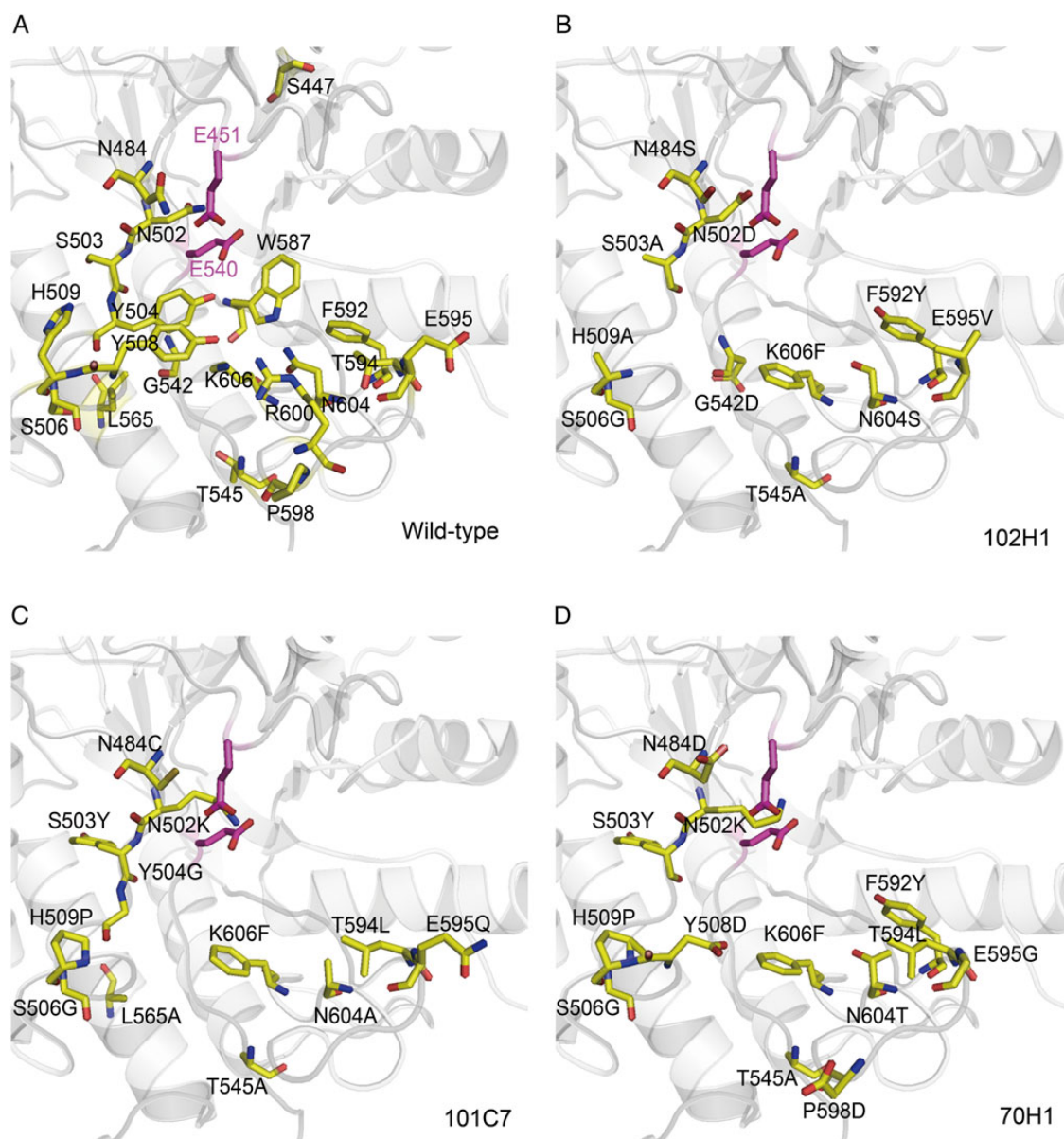


Fig. 4 Illustration of mutated residues mapped onto the 3D structure of human β -glucuronidase. (A) The active pocket of human β -glucuronidase (PDB ID: 3HN3, Klei *et al.*) is shown and the residues are represented as stick models with carbon atoms for designed mutated residues and catalytic residues E451 and E540. Putative 3D structures of β -glucuronidase variants 102H1 (B), 101C7 (C) and 70H1 (D) are shown with the mutated residues labeled. This graph is generated using PyMol.

from the deficient surface expression of S447E, L565A and T594L mutation which corresponded to 78, 75 and 63% in surface negative variants, respectively. Some residues were about equally abundant between wild-type and screened library (Y504, T545 and P598), suggesting that these residues are not involved in the enzyme specificity change. On the other hand, five predicted 'hot spot' amino acids (N484, S503, H509, E595 and N604) were almost all changed from the wild-type residue in variants that displayed high α -iduronidase activity (Supplementary Fig. S1C). These hot spots may be interesting sites for site-saturation mutagenesis. Besides, N502K (67%, 6/9), S506G (78%, 7/9), F592Y (78%, 7/9) and K606F (78%, 7/9) were observed at high frequency in β -glucuronidase variants that displayed high α -iduronidase activity, which may indicate their importance for enzyme specificity change. The putative 3D structures of wild-type β -glucuronidase and variants were generated by PyMol (Schrodinger, 2010) to reveal the relative

sites of mutations in the active pocket (Fig. 4). N502 was very close to the active residues E451 and E540 and mutation to charged amino acids such as lysine (67%, 6/9) may directly affect the catalytic mechanism leading to increased α -iduronidase activity. S506G (78%, 7/9) along with H509P (55%, 5/9) was located in the turn and loop region and thus mutation with glycine and proline may allow local structure alterations. F592Y (78%, 7/9) and N604S/T (78%, 7/9) were relatively close and the hydroxyl groups of the side chains may possibly interact with each other. In conclusion, the variants identified in our study provide some hints for rational design of new β -glucuronidase variants with higher activity. However, more valuable information might be deduced from the real crystal structures of the variants with substrates.

Previous research suggests a correlation between immune responses and ERT therapeutic efficacy (Linthorst *et al.*, 2004; Dickson *et al.*, 2008; Ohashi *et al.*, 2008; Kishnani *et al.*, 2009; Patel *et al.*, 2012;

Banugaria *et al.*, 2013; Langereis *et al.*, 2014). However, LSDs are usually heterogeneous in individual patients and thus a universal deimmunization method such as removing immunogenic epitopes of the therapeutic protein is difficult to achieve (Parker *et al.*, 2010; Cantor *et al.*, 2011; Osipovitch *et al.*, 2012; Choi *et al.*, 2013; Salvat *et al.*, 2014). Here, we investigated an alternative strategy in which an endogenous enzyme is altered to compensate for the defective enzyme. The β -glucuronidase variants are expected to display reduced immunogenicity when compared with α -iduronidase in MPS I patients because only a few amino acids are changed from the wild-type β -glucuronidase sequence. For example, the selected β -glucuronidase variants 102H1, 101C7 and 70H1 possess 11, 13 and 13 amino acid changes, which correspond to 1.7, 2 and 2% of the total amino acids. On the other hand, injection of endogenous proteins possessing only a few mutations may induce autoantibody responses in the host (Tsujihata *et al.*, 2001). However, in LSD patients who express inactive enzymes due to deleterious mutations, administration of recombinant wild-type enzyme during ERT can induce antibody responses but no significant autoimmune responses to the endogenous mutant enzyme have been reported. Along the same lines, in a recent clinical study of a dendritic cell tumor vaccine, patients who received peptides with amino acid substitutions that differed from their endogenous proteins did not develop adverse autoimmune events (Carreno *et al.*, 2015). These studies suggest that β -glucuronidase variants may not induce autoimmune responses. Moreover, most mutations in the β -glucuronidase variants are buried in the interior active pocket and may be inaccessible to antibodies (Fig. 4). However, whether the β -glucuronidase variants are less immunogenic than α -iduronidase in MPS I patients must ultimately be experimentally determined.

In future studies, several aspects should be investigated to further estimate the feasibility of this strategy toward reducing immunogenicity of ERT. First, the β -glucuronidase variants should be tested for their immunogenicity in appropriate animal models, such as transgenic mice that express human β -glucuronidase but not human α -iduronidase. Human β -glucuronidase transgenic animal models may also be useful for estimating whether human β -glucuronidase variants possess neoantigens that induce host autoimmune responses to endogenous human β -glucuronidase. Secondly, β -glucuronidase variants with higher α -iduronidase activity might be promising to exhibit good therapeutic efficacy in MPS I patients. A new generation library can be rationally designed by site-saturation mutagenesis to select the best amino residues in putative hot spots residues N484, S503, H509, E595 and N604 while maintaining the residues favored in the variants that display high α -iduronidase activity such as N502K, S506G, F592Y and K606F. DNA shuffling of the selected variants displaying high α -iduronidase activity can also be employed to select the best combination of mutant residues. Both approaches may provide valuable information for improving the iduronidase activity of β -glucuronidase variants.

In conclusion, this study provides a proof of principle that the specificity of a normally expressed endogenous human enzyme can be shifted to compensate for a separate defective enzyme. We successfully isolated β -glucuronidase variants that displayed significant α -iduronidase activity and exhibited phenotypic effects on MPS I cells. Our results suggest the feasibility of this strategy, which may be worthy of further investigation for therapeutic applications.

Supplementary data

Supplementary data are available at *PEDS* online.

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References

- Amalfitano, A., Bengur, A.R., Morse, R.P., *et al.* (2001) *Genet. Med.*, **3**, 132–138.
- Banugaria, S.G., Patel, T.T., Mackey, J., Das, S., Amalfitano, A., Rosenberg, A.S., Charrow, J., Chen, Y.T. and Kishnani, P.S. (2012) *Mol. Genet. Metab.*, **105**, 677–680.
- Banugaria, S.G., Prater, S.N., Patel, T.T., *et al.* (2013) *PLoS ONE*, **8**, e67052.
- Cantor, J.R., Yoo, T.H., Dixit, A., Iverson, B.L., Forsthuber, T.G. and Georgiou, G. (2011) *Proc. Natl Acad. Sci. U.S.A.*, **108**, 1272–1277.
- Caras, I.W., Weddell, G.N., Davitz, M.A., Nussenzweig, V. and Martin, D.W., Jr. (1987) *Science*, **238**, 1280–1283.
- Carreno, B.M., Magrini, V., Becker-Hapak, M., *et al.* (2015) *Science*, **348**, 803–808.
- Chen, K.C., Cheng, T.L., Leu, Y.L., Prijovich, Z.M., Chuang, C.H., Chen, B.M. and Roffler, S.R. (2007) *Cancer Gene Ther.*, **14**, 187–200.
- Chen, K.C., Wu, C.H., Chang, C.Y., *et al.* (2008) *Chem. Biol.*, **15**, 1277–1286.
- Chen, C.P., Hsieh, Y.T., Prijovich, Z.M., *et al.* (2012) *Protein Eng. Des. Sel.*, **25**, 367–375.
- Choi, Y., Griswold, K.E. and Bailey-Kellogg, C. (2013) *J. Computat. Chem.*, **34**, 879–891.
- De Duve, C. and Wattiaux, R. (1966) *Annu. Rev. Physiol.*, **28**, 435–492.
- Dickson, P., Peinovich, M., McEntee, M., *et al.* (2008) *J. Clin. Invest.*, **118**, 2868–2876.
- Di Domenico, C., Villani, G.R., Di Napoli, D., Reyero, E.G., Lombardo, A., Naldini, L. and Di Natale, P. (2005) *Hum. Gene Ther.*, **16**, 81–90.
- Di Domenico, C., Di Napoli, D., Gonzalez, Y.R.E., Lombardo, A., Naldini, L. and Di Natale, P. (2006) *Hum. Gene Ther.*, **17**, 1112–1121.
- Durand, P., Fabrega, S., Henrissat, B., Mornon, J.P. and Lehn, P. (2000) *Hum. Mol. Genet.*, **9**, 967–977.
- El-Amouri, S.S., Dai, M., Han, J.F., Brady, R.O. and Pan, D. (2014) *Mol. Ther.*, **22**, 2028–2037.
- Ensa, L.F., Aranda, C.S., de Lacerda, A.E., Camelo-Nunes, I., Sole, D., Martins, A.M. and Castells, M. (2014) *Pediatr. Allergy Immunol.*, **25**, 498–499.
- Fletcher, J.M. (2006) *J. Inherit. Metab. Dis.*, **29**, 405–408.
- Freeman, C. and Hopwood, J.J. (1992) *Biochem. J.*, **282**(Pt 3), 899–908.
- Geddie, M.L. and Matsumura, I. (2004) *J. Biol. Chem.*, **279**, 26462–26468.
- Goding, J.W. (1976) *J. Immunol. Methods*, **13**, 215–226.
- Harmatz, P., Giugliani, R., Schwartz, J., *et al.* (2006) *J. Pediatr.*, **148**, 533–539.
- Herati, R.S., Ma, X., Tittiger, M., Ohlemiller, K.K., Kovacs, A. and Ponder, K.P. (2008) *J. Gene Med.*, **10**, 972–982.
- Islam, M.R., Tomatsu, S., Shah, G.N., Grubb, J.H., Jain, S. and Sly, W.S. (1999) *J. Biol. Chem.*, **274**, 23451–23455.
- Jain, S., Drendel, W.B., Chen, Z.W., Mathews, F.S., Sly, W.S. and Grubb, J.H. (1996) *Nat. Struct. Biol.*, **3**, 375–381.
- Kakkis, E.D., McEntee, M.F., Schmidtchen, A., *et al.* (1996) *Biochem. Mol. Med.*, **58**, 156–167.
- Kishnani, P.S., Nicolino, M., Voit, T., *et al.* (2006) *J. Pediatr.*, **149**, 89–97.
- Kishnani, P.S., Goldenberg, P.C., Dearmeyer, S.L., *et al.* (2009) *Mol. Genet. Metab.* doi: S1096-7192(09)00244-3.
- Langereis, E.J., van Vlies, N., Church, H.J., *et al.* (2014) *Mol. Genet. Metab.* doi: 10.1016/j.ymgme.2014.10.012.

- Lin,H.Y., Lin,S.P., Chuang,C.K., Chen,M.R., Chen,B.F. and Wraith,J.E. (2005) *J. Inherit. Metab. Dis.*, **28**, 1146–1148.
- Linthorst,G.E., Hollak,C.E., Donker-Koopman,W.E., Strijland,A. and Aerts,J.M. (2004) *Kidney Int.*, **66**, 1589–1595.
- Maita,N., Tsukimura,T., Taniguchi,T., Saito,S., Ohno,K., Taniguchi,H. and Sakuraba,H. (2013) *Proc. Natl Acad. Sci. U.S.A.*, **110**, 14628–14633.
- Matsumura,I. and Ellington,A.D. (2001) *J. Mol. Biol.*, **305**, 331–339.
- Natowicz,M.R., Chi,M.M., Lowry,O.H. and Sly,W.S. (1979) *Proc. Natl Acad. Sci. U.S.A.*, **76**, 4322–4326.
- Nieman,C.E., Wong,A.W., He,S., Clarke,L., Hopwood,J.J. and Withers,S.G. (2003) *Biochemistry*, **42**, 8054–8065.
- Ohashi,T., Iizuka,S., Ida,H. and Eto,Y. (2008) *Mol. Genet. Metab.*, **94**, 313–318.
- Ohashi,T., Iizuka,S., Shimada,Y., Higuchi,T., Eto,Y., Ida,H. and Kobayashi,H. (2012) *Mol. Ther.*, **20**, 1924–1931.
- Osipovitch,D.C., Parker,A.S., Makokha,C.D., Desrosiers,J., Kett,W.C., Moise,L., Bailey-Kellogg,C. and Griswold,K.E. (2012) *Protein Eng. Des. Sel.*, **25**, 613–623.
- Parker,A.S., Zheng,W., Griswold,K.E. and Bailey-Kellogg,C. (2010) *BMC Bioinform.*, **11**, 180.
- Pastores,G.M., Sibille,A.R. and Grabowski,G.A. (1993) *Blood*, **82**, 408–416.
- Patel,T.T., Banugaria,S.G., Case,L.E., Wenninger,S., Schoser,B. and Kishnani,P.S. (2012) *Mol. Genet. Metab.*, **106**, 301–309.
- Peroni,D.G., Pescollderung,L., Piacentini,G.L., Cassar,W. and Boner,A.L. (2009) *J. Pediatr.*, **155**, 940–941.
- Rempel,B.P., Clarke,L.A. and Withers,S.G. (2005) *Mol. Genet. Metab.*, **85**, 28–37.
- Salvat,R.S., Parker,A.S., Williams,A., Choi,Y., Bailey-Kellogg,C. and Griswold,K.E. (2014) *Cell. Mol. Life Sci.*, **71**, 4869–4880.
- Schrodinger,LLC (2010) The PyMOL Molecular Graphics System, Version 1.7.4.
- Staretz-Chacham,O., Lang,T.C., LaMarca,M.E., Krasnewich,D. and Sidransky,E. (2009) *Pediatrics*, **123**, 1191–1207.
- Sun,B., Bird,A., Young,S.P., Kishnani,P.S., Chen,Y.T. and Koeberl,D.D. (2007) *Am. J. Hum. Genet.*, **81**, 1042–1049.
- Tsujihata,Y., So,T., Hashimoto,Y., Ueda,T. and Imoto,T. (2001) *Mol. Immunol.*, **38**, 375–381.
- Tsukimura,T., Tajima,Y., Kawashima,I., *et al.* (2008) *Biol. Pharm. Bull.*, **31**, 1691–1695.
- Unger,E.G., Durrant,J., Anson,D.S. and Hopwood,J.J. (1994) *Biochem. J.*, **304** (Pt 1), 43–49.
- Wang,J., Lozier,J., Johnson,G., Kirshner,S., Verthelyi,D., Pariser,A., Shores,E. and Rosenberg,A. (2008) *Nat. Biotechnol.*, **26**, 901–908.
- Wang,D., El-Amouri,S.S., Dai,M., Kuan,C.Y., Hui,D.Y., Brady,R.O. and Pan,D. (2013) *Proc. Natl Acad. Sci. U.S.A.*, **110**, 2999–3004.
- Wu,C.H., Balasubramanian,W.R., Ko,Y.P., Hsu,G., Chang,S.E., Prijovich,Z.M., Chen,K.C. and Roffler,S.R. (2004) *Biotechnol. Appl. Biochem.*, **40**, 167–172.
- Xu,M., Liu,K., Swaroop,M., Sun,W., Dehdashti,S.J., McKew,J.C. and Zheng,W. (2014) *J. Biomol. Screen.*, **19**, 168–175.
- Zarate,Y.A. and Hopkin,R.J. (2008) *Lancet*, **372**, 1427–1435.