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A new resorufin-based α-glucosidase assay for high-throughput screening

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ABSTRACT

Mutations in α-glucosidase cause accumulation of glycogen in lysosomes, resulting in Pompe disease, a lysosomal storage disorder. Small molecule chaperones that bind to enzyme proteins and correct the misfolding and mistrafficking of mutant proteins have emerged as a new therapeutic approach for the lysosomal storage disorders. In addition, α-glucosidase is a therapeutic target for type II diabetes, and α-glucosidase inhibitors have been used in the clinic as alternative treatments for this disease. We have developed a new fluorogenic substrate for the α-glucosidase enzyme assay, resorufin α-D-glucopyranoside. The enzyme reaction product of this new substrate emits at a peak of 590 nm, reducing the interference from fluorescent compounds seen with the existing fluorogenic substrate, 4-methylumbelliferyl-α-D-glucopyranoside. Also, the enzyme kinetic assay can be carried out continuously without the addition of stop solution due to the lower pK_a of the product of this substrate. Therefore, this new fluorogenic substrate is a useful tool for the α-glucosidase enzyme assay and will facilitate compound screening for the development of new therapies for Pompe disease.

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α-Glucosidase (GAA,[1] EC 3.2.1.20/3) is a lysosomal enzyme that catalyzes the hydrolysis of terminal α-1,4- and α-1,6-glucosidic linkages of glycogen. The deficiency of this enzyme results in lysosomal accumulation of glycogen that predominantly disturbs the intracellular architecture of skeletal muscle fibers and cardiomyocytes. Pompe disease, also called glycogen storage disease type II or acid maltase deficiency (OMIM 232300), is an autosomal recessive disorder with an estimated incidence of 1:40,000 live births [1,2] that is caused by mutations in the gene encoding GAA. The clinical manifestations of Pompe disease include a range of phenotypes and a spectrum of disease severity. All patients suffer from progressive muscle weakness, affecting their mobility and respiratory function. The most severely affected patients present clinically with prominent cardiomegaly, hypotonia, and hepatomegaly and have a devastating clinical course, with death usually occurring before 2 years of age due to cardiorespiratory failure [1].

More than 100 mutations have been identified in the GAA gene, and approximately half of these are missense mutations (see Ref. [3] and Human Gene Mutation Database [http://www.hgmd.cf.ac.uk]). Missense mutations can cause misfolding or mistrafficking of the enzyme proteins and subsequently result in degradation of mutant proteins before they reach lysosomes [4–7]. Recently, there has been interest in the potential use of small molecule chemical chaperones to rescue misfolded or unstable proteins from degradation and to transport them to lysosomes, thereby restoring their activity. Some reports have demonstrated that an iminosugar analog, *N*-butyldeoxynojirimycin (NB–DNJ), and its derivatives showed a chaperone effect that increased the activity of mutant GAA [8,9]. However, the iminosugar analogs have shortcomings such as their limited pharmacokinetic profiles and nonspecific interactions with other lysosomal enzymes. Therefore, the screening of compound libraries for the identification of additional chaperone molecules, especially enzyme activators, is a new approach to drug development for the treatment of Pompe disease.

There are a few enzyme assays available for GAA that were initially developed for the diagnosis of Pompe disease. One fluorescence assay uses 4-methylumbelliferyl-α-D-glucopyranoside (4MU-α-glc) as the fluorogenic substrate [10–12]. The product of this substrate, 4MU, emits at a peak of 440 nm in the fluorescence spectra, and this is prone to interference from fluorescent compounds in the library collection [13]. Chromogenic assays that use *p*-nitrophenyl-α-D-glucopyranoside [14] and 2-naphthyl-α-D-glucopyranoside [15] as substrates are also available for GAA. In addition, *p*-nitrophenyl-α-D-maltoheptaoside has been used for

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[1] *Abbreviations used:* GAA, α-glucosidase; NB–DNJ, *N*-butyldeoxynojirimycin; 4MU-α-glc, 4-methylumbelliferyl-α-D-glucopyranoside; HTS, high-throughput screening; res-α-glc, resorufin-α-D-glucopyranoside; HMPA, hexamethylphosphoric triamide; HPLC, high-performance liquid chromatography; EtOAc, ethyl acetate; TFA, trifluoroacetic acid; DMSO, dimethyl sulfoxide; CCD, charge-coupled device; CV, coefficient of variation.

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compound screening [16], but the screen throughput was relatively low. A fluorescence assay that is not prone to interferences from fluorescent compounds and dust/lint is ideal for high-throughput screening (HTS) assays. Resorufin, which emits red fluorescence, is a viable choice for labeling the substrate because it has been used in substrates of other enzymes [17–19]. We report the development of a robust screening assay for GAA using a new fluorogenic substrate, resorufin-α-D-glucopyranoside (res-α-glc). The GAA enzyme assay using this new substrate has been miniaturized into a 1536-well plate format for HTS.

Materials and methods

Reagents and chemicals

4MU-α-glc, NB–DNJ, a known inhibitor of GAA, and the buffer components were purchased from Sigma–Aldrich (St. Louis, MO, USA). GAA was obtained from residual solution after clinical infusions of Myozyme (Genzyme). The enzyme solution was mixed with 30% glycerol, and small aliquots were stored at −80 °C for up to 2 years.

The assay buffer was composed of 50 mM citric acid, 115 mM K_2PO_4, 110 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, and 0.01% Tween 20 at pH 5.0. It was stored at 4 °C for up to 6 months. A solution of 1 M Tris–HCl at pH 8.0 was used as the stop solution to increase the fluorescence signal.

Synthesis of res-α-glc

The synthesis of the α-configuration of resorufin glucopyranoside is outlined in Scheme 1 and includes two steps: an SN2 glycosidation and subsequent deprotection. Glycosidation was performed in the presence of excess resorufin sodium salt and hexamethylphosphoric triamide (HMPA), which provided the separable α-configuration products **2** in medium yields [20]. The deprotection reaction was carried out in neutral conditions using samarium and iodine [21] and was purified by preparative high-performance liquid chromatography (HPLC). The experimental details are described below.

In addition, the synthesis of res-α-glc was reproducible given that the enzyme assays were performed with three different batches and produced similar experimental results.

*Resorufinyl 2,3,4,6-tetra-O-acetyl-α-D-glucopyranoside (**2**)*

To a solution of 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl chloride (1.21 g, 3.0 mmol) in dry HMPA (12 ml) was added resorufin sodium salt (1.41 g, 6.0 mmol). The reaction mixture was stirred for 48 h at room temperature. The mixture was diluted in ethyl acetate (EtOAc, 300 ml), washed with water (2 × 150 ml) and brine, and dried over sodium sulfate. After evaporation of the solvent, the residue was purified by column chromatography using a gradient of toluene–EtOAc (4:1 to 2:1) on silica gel to give **2** (670 mg, 41% yield) as an orange oil. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.75 (d, 1H, *J* = 8.6 Hz), 7.42 (d, 1H, *J* = 10.0 Hz), 7.12 (dd, 1H, *J* = 2.5 and 8.8 Hz), 7.09 (d, 1H, *J* = 2.5 Hz), 6.85 (dd, 1H, *J* = 2.0 and 9.8 Hz), 6.33 (d, 1H, *J* = 2.2 Hz), 5.84 (d, 1H, *J* = 3.5 Hz), 5.70 (t, 1H, *J* = 10.0 Hz), 5.18 (t, 1H, *J* = 9.7 Hz), 5.08 (dd, 1H, *J* = 3.7 and 10.4 Hz), 4.26 (dd, 1H, *J* = 5.2 and 12.8 Hz), 4.09 to 4.04 (m, 2H), 2.09 (s, 3H), 2.07 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H). MS (*m*/*z*): 544 $(M + H)^+$.

*Resorufinyl α-D-glucopyranoside (**3**)*

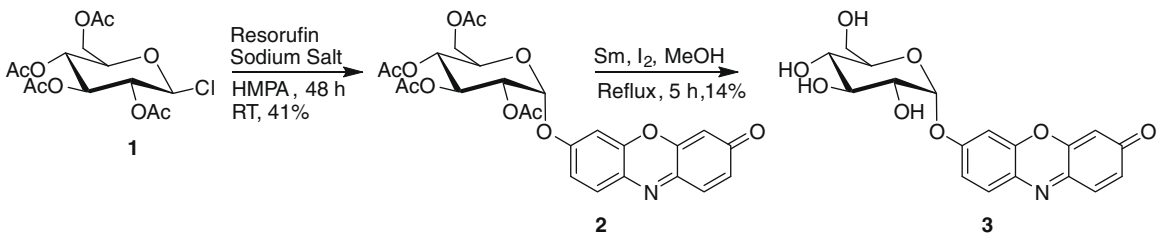
To a suspension of compound **2** (203 mg, 0.37 mmol) in a mixture of methanol–tetrahydrofuran (12 ml, 1:1, v/v) was added samarium (225 mg, 1.5 mmol) and iodine (380 mg, 1.5 mmol). The mixture was refluxed for 5 h. The solvents were removed in vacuo, and the residue was purified by preparative HPLC to give **3** (19 mg, 14% yield) as an orange powder. Preparative HPLC was performed on an Agilent 1200 series instrument using an Agilent Eclipse XDB C18 column (21.2 × 50 mm). A linear gradient of 5 to 95% acetonitrile with 0.1% trifluoroacetic acid (TFA) over 12 min was used. ^{1}H NMR (dimethyl sulfoxide [DMSO]–d_6/D_2O, 400 MHz) δ 7.76 (d, 1H, *J* = 8.6 Hz), 7.51 (d, 1H, *J* = 10.0 Hz), 7.19 (d, 1H, *J* = 2.5 Hz), 7.15 (dd, 1H, *J* = 2.5 and 8.8 Hz), 6.78 (dd, 1H, *J* = 2.2 and 9.8 Hz), 6.27 (d, 1H, *J* = 2.0 Hz), 5.59 (d, 1H, *J* = 3.7 Hz), 3.61 (dd, 1H, *J* = 8.9 and 9.5 Hz), 3.50 (dd, 1H, *J* = 2.0 and 12.1 Hz), 3.43 (t, 1H, *J* = 5.8 Hz), 3.41 (m, 1H), 3.35 (m, 1H), 3.18 (dd, 1H, *J* = 8.9 and 9.9 Hz). MS (*m*/*z*): 376 $(M+H)^+$.

Enzyme assay in 384-well plates

The assay development and optimization was performed in black 384-well plates. Typically, 20 μl/well enzyme solution was added, followed by 10 μl/well substrate solution. After a 10-min incubation at room temperature, 30 μl/well stop solution was added to terminate the reaction and the fluorescence was measured using the ViewLux, a charge-coupled device (CCD)-based plate reader (PerkinElmer, Boston, MA, USA), with an excitation at 573 nm and an emission at 610 nm. The final concentrations of enzyme and substrate were 7.4 nM and 80 μM, respectively, unless otherwise indicated.

Enzyme assay in 1536-well plate format

The enzyme assay was miniaturized to the 1536-well plate format for HTS. In a black 1536-well plate, 2 μl/well enzyme solution was added, followed by 23 nl/well compound in DMSO solution. After a 5-min incubation at room temperature, the enzyme reaction was initiated by the addition of 1 μl/well substrate. The reaction was terminated by the addition of 3 μl/well stop solution, followed by a 10-min incubation. The assay plate was then measured in the ViewLux with an excitation at 573 nm and an emission at 610 nm. The final concentrations of GAA and substrate were 7.4 nM and 80 μM, respectively.



Scheme 1. Synthesis of resorufinyl-labeled α-D-glucopyranoside.