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A New Resorufin-Based Alpha-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.

Keywords

alpha-glucosidase; enzyme assay; assay optimization; assay miniaturization; high throughput screening; HTS; Pompe disease

Introduction

Alpha-glucosidase (GAA) (EC3.2.1.20/3) 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, which predominantly disturbs the intracellular architecture of skeletal muscle fibers and cardiomyocytes. Pompe disease, also called glycogen

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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 α -glucosidase (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, 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, about half of which are missense mutations [3, Human Gene Mutation Database, 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, and its derivatives showed a chaperone effect, which 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 for 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 as the fluorogenic substrate [10–12]. The product of this substrate, 4MU, emits at a peak of 440 nm in the fluorescence spectra, which is prone to interference from fluorescent compounds in the library collection [13]. Chromogenic assays are also available for GAA that use paranitrophenyl- α -D-glucopyranoside [14] or 2-naphthyl- α -D-glucopyranoside [15] as substrates. In addition, p-nitrophenyl- α -D-maltoheptaoside has been used for 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 assays. Resorufin, which emits red fluorescence, is a viable choice for labeling the substrate, as 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 high throughput screening (HTS).

Materials and methods

Reagents and chemicals

4-methylumbelliferyl- α -D-glucopyranoside (4MU- α -glc), N-butyldeoxynojirimycin (NB-DNJ), a known inhibitor of GAA, and the buffer components were purchased from Sigma-Aldrich (St. Louis, MO). 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 two 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. 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 resorufin α -D-glucopyranoside (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 HPLC. The experimental details are described below.

In addition, the synthesis of res- α -glc was reproducible as 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 $\times$ 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. ^1H NMR (CDCl₃, 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 Hz and 8.8 Hz), 7.09 (d, 1H, J = 2.5 Hz), 6.85 (dd, 1H, J = 2.0 Hz 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 Hz and 10.4 Hz), 4.26 (dd, 1H, J = 5.2 Hz and 12.8 Hz), 4.09–4.04 (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 21.2 $\times$ 50 mm column. A linear gradient of 5% to 95% acetonitrile with 0.1% TFA over 12 min was used. ^1H NMR (DMSO- d_6 /D₂O, 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 Hz and 8.8 Hz), 6.78 (dd, 1H, J = 2.2 Hz 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 Hz and 9.5 Hz), 3.50 (dd, 1H, J = 2.0 Hz 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 Hz 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 μ l/well enzyme solution was added followed by 10 μ l/well substrate solution. After a 10 minute incubation at room temperature, 30 μ l/well stop solution was added to terminate the reaction, and the fluorescence was measured using the Viewlux, a CCD-based plate reader (PerkinElmer, Boston, MA), 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 μ M, 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 μ l/well enzyme solution was added, followed by 23 nl/well compound in DMSO solution. After a 5 minute incubation at room temperature, the enzyme reaction was initiated by the addition of 1 μ l/well substrate. The reaction was terminated by the addition of 3 μ l/well

stop solution followed by a 10 minute 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 μ M, respectively.

Instruments for the 1536-well assay

A BioRAPTR FRDTM Microfluidic Workstation (Beckman Coulter, Inc. Fullerton, CA) was used to dispense reagents into 1536-well plates at volumes of 1–3 μ l. Initially, the compounds were serially diluted in DMSO in 384-well plates using a CyBi[®]-Well dispensing station with a 384-well head (Cybio Inc., Woburn, MA), and then reformatted into 1536-well plates at 7 μ l/well. Nanoliter volumes of these compounds were transferred to 1536-well assay plates using an automated pin-tool station (Kalypsys, San Diego, CA). A ViewLux CCD-based imaging plate reader (PerkinElmer, Boston, MA) was used for fluorescence detection at the speed of 30 seconds per plate. A Safire2TM 4-monochromator scanning fluorescence plate reader (Tecan, Männedorf, Switzerland) was used for the determination of fluorescence excitation and emission spectra.

Enzyme kinetics assay

The enzyme kinetics assay was carried out in a 384-well plate format using 7.4 nM enzyme with varying concentrations of substrate. Initially, 10 μ l/well of varying concentrations of substrate were added to a 384-well plate. The reaction was initiated by the addition of 20 μ l/well of enzyme solution. The res- α -glc stock solution was serially diluted 1:2 to give eight concentrations. The final concentrations of substrate used in the assay were 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 μ M. 30 μ l/well stop solution was added after 2, 4, 6, 8, and 10 minute room temperature incubation times. A standard curve of the free fluorophore, resorufin, in the same volume of assay buffer and stop solution was generated for the calculation of the enzyme product.

Data analysis

The quantitative data were calculated as mean $\pm$ standard error unless they were specifically indicated in figures. The results for the assay optimization and enzyme kinetics experiment were analyzed with Prism[®] (Graphpad, San Diego, CA).

Results and discussion

Upon hydrolysis by GAA, fluorogenic res- α -glc forms two products, glucose and resorufin. Resorufin has a pK_a of $\sim$ 6.0 and emits red fluorescence at a peak of 590 nm (Fig. 1). This substrate has advantages over the existing substrate, 4MU- α -glc, that emits blue fluorescence at a peak of 440 nm, as explained below. The fluorescence enzyme assay is usually more sensitive than the chromogenic enzyme assay, and is a better choice for assay miniaturization for high throughput screening (HTS). However, a number of compounds in the library collections are fluorescent at the blue and green wavelengths, which can cause interference during compound screening. A previous report indicated that approximately 4.9% of compounds in a compound library were fluorescent at the emission peak of 440 nm [13]. These fluorescent compounds could be identified as “positive” hits in the enzyme assay when using 4MU- α -glc as the substrate. In addition, lint and dust that emit at or near the blue spectral region are other common sources of fluorescence interference. Most of these interferences, as well as false positive compounds identified from the HTS, can be avoided or significantly reduced when using red fluorogenic substrates. In addition, the lower pK_a value of the enzyme reaction product resorufin ($pK_a = \sim$ 6), enables continuous kinetic assay both at the lower pH of 5.0 and higher (data not shown). It is known that the fluorescence intensity is dependent on the pH in the assay buffer and is decreased progressively at pH values that are less than the pK_a [22, 23]. The fluorophore 4MU has a pK_a of $\sim$ 8 and shows almost no fluorescence at the lower pH

of the cell (4.5 to 6.8). Thus, the enzyme assays with 4MU labeled substrates require an addition of a stop solution to raise the pH for optimal fluorescence detection.

Assay development and optimization

Assay buffer pH—GAA is a lysosomal enzyme and its activity is dependent on the local acidic environment in the lysosomal compartment. To determine the optimal pH for enzyme activity with this substrate, a series of assay buffers ranging from pH 4.0 to 7.5 were tested. The optimal pH for GAA activity using this new substrate was 5.0 (Fig. 2A), similar to the existing blue fluorogenic substrate, 4MU- α -glc. Subsequent assays were performed at this optimal pH.

Enzyme concentration—The relationship between enzyme concentration and enzyme activity was then determined using enzyme concentrations ranging from 0.2 to 60 nM. The results showed a nearly linear response up to 30 nM (Fig. 2B). Based on this result, 7.4 nM enzyme was selected. This concentration yielded sufficient fluorescence intensity for detection while keeping the substrate consumption under 10%.

Incubation time for enzyme reaction—The time course study of enzyme activity showed a linear increase in enzyme activity up to an incubation time of 40 minutes at room temperature (Fig. 2C). An incubation time of 10 minutes was selected for further experiments, as it produced sufficient signal with a good signal-to-basal ratio.

DMSO tolerance—Since DMSO is a commonly used solvent for compounds in HTS, the DMSO tolerance of this assay was also examined. It was found that enzyme activity was slightly affected with increasing DMSO concentrations. The enzyme response was reduced 8.2, 16.2 and 16.3 % at 0.5, 1.0 and 2.0% DMSO, respectively (Fig. 2D). The DMSO effect on the compound screens was minimized by using the proper DMSO controls in the compound screen. The final concentration of DMSO selected was 0.76% and the same amount of DMSO was used in both positive and negative control wells.

Enzyme kinetics—Enzyme kinetics were also assessed to determine the GAA activity with this new substrate. We found that the K_m and V_{max} were $166 \pm 20.6 \mu M$ and 16.5 ± 0.708 pmol/min, respectively (Fig. 3A), as compared with the K_m of $76 \pm 2.9 \mu M$ and V_{max} of 5.6 ± 0.075 pmol/min for the 4MU- α -glc substrate. The substrate concentration selected should be around or less than the K_m value in order to maintain the sensitivity of the enzyme assay. Thus, a concentration of 80 μM res- α -glc was chosen for the compound screen experiments.

Activity of a known inhibitor—The activity of a known GAA inhibitor, N-butyldoxynojirimycin (NB-DNJ), was tested in this GAA assay using the new substrate. Using 7.4 nM GAA and 80 μM res- α -glc (Fig. 3B), the IC_{50} value of NB-DNJ was $6.8 \pm 0.46 \mu M$. This value was similar to that determined in the GAA assay using the blue fluorogenic substrate, 4MU- α -glc ($IC_{50} = 9.2 \pm 0.63 \mu M$).

Assay miniaturization and LOPAC library screen

The 384-well plate assay conditions described above were successfully miniaturized to the 1536-well plate format by proportionally reducing the assay volume from 30 μl /well to 3 μl /well without significantly affecting the assay window. To further validate this enzyme assay, we carried out a test screen with a DMSO plate. The signal-to-basal ratio was 14.2 fold, CV was 3.5% and Z factor was 0.87 determined from the DMSO plate test in the 1536-well plate format (Fig. 4A). These results demonstrated that the new GAA assay is robust and suitable for compound screens in the 1536-well plate format.

We then performed a test screen with this 1536-well plate assay using the LOPAC library of 1,280 pharmacologically active compounds. The LOPAC library is commonly used to test the screening assays before HTS to ensure the quality of HTS and to estimate the potential hit rate from the screening of large compound collections. A quantitative HTS screening format was used in which all the compounds were screened in a 7-concentration titration as previously described [24]. Two positive compounds were identified from the screen as GAA inhibitors and the hit rate for this screen was 0.16%. We found that these two active compounds were both iminosugar analogs, and confirmed their activities in a follow-up assay (Fig. 4B). Thus, the results indicate that the GAA assay with the new red fluorogenic substrate is a useful tool for HTS that can be used to identify new enzyme inhibitors and activators without interference from blue fluorescent compounds.

In a previous screen with the blue fluorogenic substrate, additional candidate GAA activators were identified from the LOPAC library screen (pubchem.ncbi.nlm.nih.gov) that were not identified in this screen. We retested these three “active compounds” with both the red and blue fluorogenic substrates that were coupled with a pre-read before the enzyme reactions. It was found that all three of these compounds were fluorescent in the blue wavelength (440 nm), but not in the red wavelength (610 nm) (Fig. 5), demonstrating that the increase in enzyme activity using the blue fluorogenic substrate was due to compound fluorescence at that wavelength. These results indicate that in compound library screens, the enzyme assay with a red fluorogenic substrate can avoid or minimize false positives due to the significant amount of fluorescent compounds seen in the blue wavelength [13]. Thus, res- α -glc is a better assay choice for the identification of new activators and inhibitors using HTS. Although the pre-read with the assay plate prior to the enzyme reaction may help eliminate the fluorescent compounds, it would reduce the throughput of HTS, and the results may not be distinguishable due to the normal variations between the plate reads.

In conclusion, we have developed a new GAA enzyme assay that uses a red fluorogenic substrate. This assay has been miniaturized into the 1536-well plate format for HTS. The advantages of this new GAA assay include high assay sensitivity, robust signal for compound screens, and less interference due to fluorescent compounds and lint/dust. Thus, this red fluorogenic GAA enzyme assay should be a useful tool for compound screens to develop new treatments for Pompe disease.

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References

1. Hirschhorn, R.; Reuser, A.J.J. Glycogen storage disease type II: acid α -glucosidase (acid maltase) deficiency. In: Scriver, C.R.; Beaudet, A.L.; Sly, W.S.; Valle, D., editors. *The Metabolic and Molecular Bases of Inherited Disease*. New York: McGraw-Hill Professional; 2001. p. 3389-3420.
2. Martiniuk F, Chen A, Mack A, Arvanitopoulos E, Chen Y, Rom WN, Codd WJ, Hanna B, Alcibes P, Raben N, Plotz P. Carrier frequency for glycogen storage disease type II in New York and estimates of affected individuals born with the disease. *Am J Med Genet* 1998;79:69–72. [PubMed: 9738873]
3. Raben N, Plotz P, Byrne BJ. Acid alpha-glucosidase deficiency (glycogenosis type II, Pompe disease). *Curr Mol Med* 2002;2:145–166. [PubMed: 11949932]
4. Montalvo AL, Cariati R, Deganuto M, Guerici V, Garcia R, Ciana G, Bembi B, Pittis MG. Glycogenosis type II: identification and expression of three novel mutations in the acid alpha-glucosidase gene causing the infantile form of the disease. *Mol Genet Metab* 2004;81:203–208. [PubMed: 14972326]

5. Hermans MM, van Leenen D, Kroos MA, Beesley CE, Van Der Ploeg AT, Sakuraba H, Wevers R, Kleijer W, Michelakakis H, Kirk EP, Fletcher J, Bosshard N, Basel-Vanagaite L, Besley G, Reuser AJ. Twenty-two novel mutations in the lysosomal alpha-glucosidase gene (GAA) underscore the genotype-phenotype correlation in glycogen storage disease type II. *Hum Mutat* 2004;23:47–56. [PubMed: 14695532]
6. Reuser AJ, Kroos M, Willemsen R, Swallow D, Tager JM, Galjaard H. Clinical diversity in glycogenesis type II. Biosynthesis and in situ localization of acid alpha-glucosidase in mutant fibroblasts. *J Clin Invest* 1987;79:1689–1699. [PubMed: 3108320]
7. Reuser AJ, Kroos M, Oude Elferink RP, Tager JM. synthesis Defects in phosphorylation and maturation of acid alpha-glucosidase in glycogenesis type II. *J Biol Chem* 1985;260:8336–8341. [PubMed: 3159730]
8. Parenti G, Zuppaldi A, Gabriela Pittis M, Rosaria Tuzzi M, Annunziata I, Meroni G, Porto C, Donaudy F, Rossi B, Rossi M, Filocamo M, Donati A, Bembi B, Ballabio A, Andria G. Pharmacological Enhancement of Mutated alpha-Glucosidase Activity in Fibroblasts from Patients with Pompe Disease. *Mol Ther* 2007;15:508–514. [PubMed: 17213836]
9. Okumiya T, Kroos MA, Vliet LV, Takeuchi H, Van der Ploeg AT, Reuser AJ. Chemical chaperones improve transport and enhance stability of mutant alpha-glucosidases in glycogen storage disease type II. *Mol Genet Metab* 2007;90:49–57. [PubMed: 17095274]
10. Hultberg B, Sjoblad S, Ockerman PA. Glycosidases in human skin Alpha-fucosidase fibroblast cultures. alpha-galactosidase, alpha-glucosidase, beta-mannosidase and N-acetyl-alpha-glucosaminidase. *Acta Paediatr Scand* 1975;64:123–131. [PubMed: 234663]
11. Okumiya T, Keulemans JL, Kroos MA, Van der Beek NM, Boer MA, Takeuchi H, Van Diggelen OP, Reuser AJ. A new diagnostic assay for glycogen storage disease type II in mixed leukocytes. *Mol Genet Metab* 2006;88:22–28. [PubMed: 16359900]
12. Porter WH, Jennings CD Jr, Wilson HD. Measurement of alpha-glucosidase activity in serum from patients with cystic fibrosis or pancreatitis. *Clin Chem* 1986;32:652–656. [PubMed: 3513992]
13. Simeonov A, Jadhav A, Thomas CJ, Wang Y, Huang R, Southall NT, Shinn P, Smith J, Austin CP, Auld DS, Inglese J. Fluorescence spectroscopic profiling of compound libraries. *J Med Chem* 2008;51:2363–2371. [PubMed: 18363325]
14. Mahmoud AM, Geslevich J, Kint J, Depuydt C, Huyse L, Zalata A, Comhaire FH. Seminal plasma alpha-glucosidase activity and male infertility. *Hum Reprod* 1998;13:591–595. [PubMed: 9572418]
15. Guerin JF, Ali HB, Rollet J, Souchier C, Czyba JC. Alpha-glucosidase as a specific epididymal enzyme marker. Its validity for the etiologic diagnosis of azoospermia. *J Androl* 1986;7:156–162. [PubMed: 3522516]
16. Walker JM, Winder JS, Kellam SJ. High-throughput microtiter plate-based chromogenic assays for glycosidase inhibitors. *Appl Biochem Biotechnol* 1993;38:141–146. [PubMed: 8346902]
17. Mazura P, Fohlerova R, Brzobohaty B, Kiran NS, Janda L. A new, sensitive method for enzyme kinetic studies of scarce glucosides. *J Biochem Biophys Methods* 2006;68:55–63. [PubMed: 16730803]
18. Hays WS, Wheeler DE, Eghtesad B, Glew RH, Johnston DE. Expression of cytosolic beta-glucosidase in guinea pig liver cells. *Hepatology* 1998;28:156–163. [PubMed: 9657108]
19. Parzefall W, Berger W, Kainzbauer E, Teufelhofer O, Schulte-Hermann R, Thurman RG. Peroxisome proliferators do not increase DNA synthesis in purified rat hepatocytes. *Carcinogenesis* 2001;22:519–523. [PubMed: 11238195]
20. Courtin-Duchateau M, Veyrières A. Synthesis of 4-methylumbelliferyl 1,2-cis-glycosides. *Carbohydr. Res* 1978;65:23–33.
21. Yamada R, Negoro N, Bessho K, Yamada K. Metallic samarium and iodine in alcohol. Deacylation and dealkyloxycarbonylation of protected alcohols and lactams. *Synlett* 1995:1261.
22. Bueno C, Villegas ML, Bertolotti SG, Previtali CM, Neumann MG, Encinas MV. The excited-state interaction of resazurin and resorufin with amines in aqueous solutions. Photophysics and photochemical reactions. *Photochem Photobiol* 2002;76:385–390. [PubMed: 12405144]
23. Coleman DJ, Studler MJ, Naleway JJ. A long-wavelength fluorescent substrate for continuous fluorometric determination of cellulase activity: resorufin-beta-D-cellobioside. *Anal Biochem* 2007;371:146–153. [PubMed: 17927946]

24. Inglese J, Auld DS, Jadhav A, Johnson RL, Simeonov A, Yasgar A, Zheng W, Austin CP. Quantitative high-throughput screening: a titration-based approach that efficiently identifies biological activities in large chemical libraries. *Proc Natl Acad Sci U S A* 2006;103:11473–11478. [PubMed: 16864780]

Fig. 1.

Schematic representation of the α -glucosidase enzyme assay. (A) The pro-fluorescence substrate, res- α -glc, is hydrolyzed by GAA to produce two products, glucose and resorufin, which emits red fluorescence. (B) The fluorescence spectra of res- α -glc has an excitation peak at 570 nm and an emission peak at 590 nm, (determined at 200 μ M). Its fluorescence intensity is only 0.4% of resorufin's, the enzyme reaction product (data not shown). (C) The fluorescence spectra of resorufin has an excitation peak at 573 nm and an emission peak at 590 nm (determined at 2 μ M). For the optimal signal-to-basal ratio in the enzyme assay, a 573 (10) nm excitation filter and a 610 (10) nm emission filter was used. Under the assay conditions, the substrate fluorescence is insignificant compared to the product fluorescence.

Fig. 2.

Enzyme assay optimization. (A) Effect of pH on the enzyme reaction. The optimal pH for the reaction was 5.0. (B) Enzyme concentration response. The enzyme activity increased linearly up to 30 nM GAA. (C) Time course of the enzyme reaction at room temperature. The enzyme activity increased linearly with longer incubation times ranging from 5 to 40 minutes. (D) DMSO tolerance test. The enzyme activity decreased slightly as DMSO concentrations increased from 0% to 2% of the final concentration.

Fig. 3.

(A) Enzyme kinetics. The enzyme reaction obeyed the Michaelis-Menten equation with a K_m of $166 \pm 20.6 \mu\text{M}$ and a V_{max} of $16.5 \pm 0.708 \text{ pmol/min}$ at an enzyme concentration of 7.4 nM . (B) Activity of the known GAA inhibitor NB-DNJ. The IC_{50} of this compound was $6.8 \pm 0.46 \mu\text{M}$ at 7.4 nM GAA with $80 \mu\text{M}$ substrate.

Fig. 4.

(A) Scatter plot of the screen results from a DMSO plate. The signal-to-basal ratio was 14.2 fold with a CV of 3.5% and a Z' factor of 0.89. (B) Concentration-responses of two positive compounds identified from the LOPAC library screen in the confirmation assay. NCGC00093860 is 1-Deoxynojirimycin HCl obtained from Sigma-Aldrich (catalog number: D9305), and NCGC00162133 is 1,4-Dideoxy-1,4-imino-D-Arabinitol (D1542). Their activities were similar in both the blue and red fluorogenic GAA assays.

Fig. 5.

Three “activator” compounds identified from the LOPAC library screen using a blue fluorogenic GAA assay that were false positives due to compound fluorescence in the blue spectral region. (A) Triamterene (T4143), (B) Camptothecin (C9911), and (C) (6R)-5,6,7,8-Tetrahydro-L-biopterin HCl (T4425). These compounds were identified as activators in the blue fluorogenic GAA assay, but were not confirmed with the red fluorogenic GAA assay. However, in the pre-read before the enzyme reaction, they were fluorescent in the blue wavelength (440 nm) and not fluorescent in the red wavelength (610 nm).

Scheme 1.

Synthesis of resorufinyl-labeled α-D-glucopyranoside