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Synthesis and characterization of a new fluorogenic substrate for alpha-galactosidase

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Abstract

Alpha-galactosidase A hydrolyzes the terminal alpha-galactosyl moieties from glycolipids and glycoproteins in lysosomes. Mutations in α -galactosidase cause lysosomal accumulation of the glycosphingolipid, globotriaosylceramide, which leads to Fabry disease. Small-molecule chaperones that bind to mutant enzyme proteins and correct their misfolding and mistrafficking have emerged as a potential therapy for Fabry disease. We have synthesized a red fluorogenic substrate, resorufinyl

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α -D-galactopyranoside, for a new α -galactosidase enzyme assay. This assay can be measured continuously at lower pH values, without the addition of a stop solution, due to the relatively low pK_a of resorufin (~6). In addition, the assay emits red fluorescence, which can significantly reduce interferences due to compound fluorescence and dust/lint as compared to blue fluorescence. Therefore, this new red fluorogenic substrate and the resulting enzyme assay can be used in high-throughput screening to identify small-molecule chaperones for Fabry disease.

Keywords

Alpha-galactosidase; Enzyme assay; Assay optimization; Assay miniaturization; Fabry disease

Introduction

Fabry disease (OMIM301500) is a hereditary X-linked lysosomal storage disorder with an estimated incidence of one in 40,000 males. The disorder is caused by a deficiency of the lysosomal enzyme α -galactosidase A (GLA), which results in the accumulation of the glycosphingolipid, globotriaosylceramide (Gb3), in different cells and organs, most notably the endothelial and smooth muscle cells of blood vessels [1]. Manifestations of this disease are not only encountered in affected hemizygous males, but are also seen in many heterozygous females. Patients with Fabry disease present clinically with chronic neuropathic pain, gastrointestinal disturbances, angiokeratomata, progressive renal impairment, cardiomyopathy, premature myocardial infarctions, and stroke. Both life expectancy and quality of life are severely compromised.

To date, 430 mutations have been identified in the GLA gene (Human Gene Mutation Database, www.hgmd.cf.ac.uk). Of these, more than 57% are missense mutations. Studies of residual GLA activity of mutant forms of the enzyme revealed that many of these mutant enzymes had kinetic properties similar to the wild-type enzyme, but were significantly less stable [2–4]. These results suggest that the enzyme deficiency of these mutants is caused primarily by misfolding, and/or the inability to traffic to lysosomes. It is possible that the mutant enzyme is retained in the endoplasmic reticulum after synthesis and degraded prematurely.

Enzyme replacement therapy (ERT) has recently become available for the treatment of patients with Fabry disease [5,6]. However, the cost of ERT is very high, and patients require lifelong treatment. ERT does not appear to prevent premature strokes in patients with Fabry disease [7,8]. In addition, some patients receiving ERT develop immune responses to the infused enzyme [7]. Chemical chaperone therapy has recently emerged as a potential therapeutic alternative for Fabry disease. Chemical chaperones bind the misfolded proteins and assist in their folding, maturation and/or trafficking to lysosomes. It has been reported that some enzyme inhibitors and receptor agonists/antagonists can serve as chemical chaperones for mutant enzymes and receptors [9,10]. Recently, a GLA inhibitor, 1-deoxygalactonojirimycin (DGJ, marketed as AmigalTM by Amicus Therapeutics, Inc.), has been studied under a phase II clinical trial as a therapeutic chaperone agent for Fabry disease [11]. However, the effects of DGJ appear to be mutation-specific. Many types of mutant GLA are not responsive, including mutants such as R356W, which have poor protein stability with nearly normal catalytic activity [2,12]. Novel chaperone compounds with broader activity towards the mutant enzyme are needed to serve as starting points for new drug development for Fabry disease.

The current enzyme assays available for GLA were initially developed for the diagnosis of Fabry disease. The most common GLA assay uses 4-methylumbelliferyl- α -D-galactopyranoside as the fluorogenic substrate, where the enzyme reaction product emits blue fluorescence at a peak of 440 nm [13,14]. This assay is not ideal for a compound library screen

because it is prone to interference from naturally fluorescent compounds in the collections. Chromogenic assays are also available that use *p*-nitrophenol- α -D-galactopyranoside [15,16] or naphthyl- α -D-galactopyranoside [17] as substrates. But absorbance assays are usually less sensitive than fluorescence assays, and cannot easily be miniaturized into the 1,536-well plate format for high-throughput screening (HTS). We report the synthesis of a new fluorogenic substrate, resorufinyl α -D-galactopyranoside (res- α -galc), and the development of a red fluorogenic GLA enzyme assay. This new GLA assay has been miniaturized into the 1,536-well plate format and is ready for HTS of large compound collections.

Materials and methods

Reagents and chemicals

4-Methylumbelliferyl- α -D-galactocopyranoside, 1-deoxygalactonojirimycin, and the buffer components were purchased from Sigma-Aldrich (St. Louis, MO). The recombinant (–) galactosidase A (GLA) enzyme was obtained from residual solution after clinical infusions of Fabrazyme® (Genzyme, Cambridge, MA). 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. 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.

Enzyme assay

Development and optimization of the assay was performed in black 384-well plates. Typically, 20 μl /well of enzyme solution was added followed by 10 μl /well of substrate solution. After a 10-min incubation at room temperature, the reaction was terminated by the addition of 30 μl /well of stop solution. The plate was read with a ViewLux CCD-based imaging 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 2.2 nM and 20 μM , respectively, unless otherwise indicated.

The enzyme assay was miniaturized to the 1,536-well plate format for HTS. Using black 1,536-well plates, 2 μl /well of enzyme solution was added followed by 23 nl/well of compound in DMSO solution. After a 5-min incubation at room temperature, the enzyme reaction was initiated by the addition of 1 μl /well of substrate (res- α -galc). After a 10-min incubation at room temperature, the reaction was terminated by the addition of 3 μl /well of stop solution and the assay plate was measured in the ViewLux plate reader.

Instruments for the 1,536-well assay—Reagents were dispensed into 1,536-well plates at volumes of 1–3 μl using a BioRAPTR FRD™ Microfluidic Workstation (Beckman Coulter, Inc., Fullerton, CA). Each compound was 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 1,536-well plates at 7 μl /well. An automated pin-tool station (Kalypsys, San Diego, CA) was used to transfer nanoliter volumes of these compounds to 1,536-well assay plates, and fluorescence detection at the speed of 30 s per plate was performed with the ViewLux. The fluorescence excitation and emission spectra of the product, resorufin, was determined in a Safire2™ 4-monochromator scanning fluorescence plate reader (Tecan, Männedorf, Switzerland).

Enzyme kinetics assay

The enzyme kinetics assay was performed in the 384-well plate format with 10 μl /well of varying substrate concentrations in each well. A 2.2 nM enzyme solution (20 μl /well) was added

at 2-, 4-, 6-, 8-, 10-, and 12-min intervals to initiate the reaction. The res- α -galc stock solution was serially diluted 1:2 to give eight concentrations. The final concentrations of substrate used in the assay were 125, 62.50, 31.25, 15.63, 7.81, 3.91, 1.95, and 0.98 μ M. At pH 5.5, the assay was measured continuously at 2-min intervals for up to 12 min in the ViewLux. At pH 5.0, 30 μ l/well of stop solution was added to all the wells after the final room temperature incubation time (12 min). 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 data were calculated as the mean $\pm$ standard error, unless specifically indicated. The results for the assay optimization and enzyme kinetics experiments were analyzed with Prism® (Graphpad, San Diego, CA).

Results and discussion

Chemical synthesis of resorufinyl α -D-galactopyranoside

The synthesis of the α -configuration of resorufinyl galactopyranoside includes two steps: an SN2 glycosidation and subsequent deprotection (Fig. 1). Starting from the known compound tetraacetyl- β -chloro-galactose (**1**), glycosidation was performed in the presence of a two-equivalent excess of resorufin sodium salt in the aprotic solvent hexamethylphosphoric triamide (HMPA). This provided a mixture of the desired major α -configuration product (**2**), as well as the unwanted minor β -configuration product in a ratio of 2:1, in medium yields. The α,β -configuration products could be separated by repeated flash chromatography, although deprotection of tetraacetyl groups proved troublesome. We tried multiple basic or acidic conditions including sodium methoxide, potassium carbonate, triethylamine, and hydrochloric acid. All of these reagents gave complex mixtures of products which could not be purified either by flash chromatography or by preparative HPLC. Finally, neutral conditions using samarium and iodine were employed for the successful cleavage of the four acetyl-protecting groups. This deprotection reaction mixture was purified by preparative HPLC to provide the final product **3** with good and sufficient quality (over 98% purity) for use in fluorogenic enzyme assays.

General—Thin-layer chromatography analyses were performed with Analtech (Newark, DE) silica gel GHLF 0.25 mm plates using UV and iodine detection. Flash chromatography was performed on an Analogix Intelliflash Workstation with a SuperFlash Septra Si 50 SF column. APCI mass spectrometry (APCI-MS) was performed on an LC/MSD TrapXCI Agilent Technologies instrument. Analytical and preparative HPLC analysis was performed on an Agilent 1200 series instrument equipped with a multi-wavelength detector using an Agilent Eclipse XDB C18, 5.0 μ m, 4.6 $\times$ 150-mm column and 21.2 $\times$ 50-mm column, respectively. Solvent A was 0.1% TFA in water, Solvent B was 0.1% TFA in acetonitrile, and a linear gradient of 5% B to 95% B over 12 min was used. $^1\text{H-NMR}$ spectra was recorded with a Varian spectrometer at 400 MHz. Chemical shifts are reported in parts per million (δ) and are referenced to tetramethylsilane (TMS). All chemicals are commercially available from Aldrich.

Resorufinyl 2,3,4,6-tetra-O-acetyl- α -D-galactopyranoside (2**)**—To a solution of 2,3,4,6-tetra-O-acetyl- β -D-galactopyranosyl 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 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** (610 mg, 37% yield) as an orange oil. $^1\text{H-NMR}$ (CDCl_3 , 400 MHz)

δ 7.67 (d, 1H, $J=8.6$ Hz), 7.35 (d, 1H, $J=9.8$ Hz), 7.02 (dd, 1H, $J=2.6$ Hz and 8.5 Hz), 7.00 (d, 1H, $J=2.3$ Hz), 6.78 (dd, 1H, $J=2.0$ Hz and 9.8 Hz), 6.29 (d, 1H, $J=2.0$ Hz), 5.81 (d, 1H, $J=3.7$ Hz), 5.49 (dd, 1H, $J=3.3$ Hz and 10.6 Hz), 5.47 (m, 1H), 5.24 (dd, 1H, $J=3.5$ Hz and 10.6 Hz), 4.22 (t, 1H, $J=6.5$ Hz), 4.04–4.02 (m, 2H), 2.11 (s, 3H), 2.02 (s, 3H), 1.97 (s, 3H), 1.88 (s, 3H). MS (m/z): 544 ($M+H$)⁺.

Resorufinyl α -D-galactopyranoside (3)—To a suspension of compound **2** (110 mg, 0.20 mmol) in a mixture of methanol–tetrahydrofuran (7 ml, 1:1 v/v) was added samarium (123 mg, 0.82 mmol) and iodine (208 mg, 0.82 mmol). The mixture was refluxed for 5 h. The solvents were removed *in vacuo*, and the residue was purified by preparative HPLC to provide **3** (12 mg, 16% yield) as an orange powder. ¹H NMR (DMSO- d_6 , 400 MHz) δ 7.79 (d, 1H, $J=8.8$ Hz), 7.54 (d, 1H, $J=9.8$ Hz), 7.21 (d, 1H, $J=2.5$ Hz), 7.14 (dd, 1H, $J=2.6$ Hz and 8.8 Hz), 6.80 (dd, 1H, $J=2.0$ Hz and 9.8 Hz), 6.30 (d, 1H, $J=2.2$ Hz), 5.68 (d, 1H, $J=3.3$ Hz), 5.06 (d, 1H, $J=6.3$ Hz), 4.84 (d, 1H, $J=5.5$ Hz), 4.66 (d, 1H, $J=4.1$ Hz), 4.56 (t, 1H, $J=5.7$ Hz), 3.86–3.76 (m, 3H), 3.65 (t, 1H, $J=6.4$ Hz), 3.53 (m, 1H), 3.39 (m, 1H). MS (m/z): 376 ($M+H$)⁺.

Assay principle

The fluorogenic substrate, res- α -galc, is hydrolyzed by GLA to form two products, galactose and resorufin (Fig. 2). Resorufin has a pK_a of ~ 6.0 and emits at a peak of 590 nm. In contrast, the product of the existing 4-methylumbelliferyl- α -D-galactopyranoside (4MU- α -galc) substrate emits at a peak of 440 nm and is prone to interference from fluorescent compounds. It has been reported that 4.9% of compounds in a compound library were fluorescent at the emission of 440 nm [18], which can cause false positives in library screens. In addition, lint and dust emit blue fluorescence, which can also result in false positives. However, the product of this new red fluorogenic substrate emits red fluorescence that is less prone to interference by both fluorescent compounds and lint/dust. In addition, the lower pK_a of resorufin (~ 6.0) enables continuous measurement for kinetic assays in a lower pH buffer than 4-MU ($pK_a \sim 8.0$). The assay using 4MU- α -galc requires the addition of a stop solution to raise the buffer pH in order to obtain adequate fluorescence signal.

Assay development and optimization

Buffer pH—GLA is a lysosomal enzyme whose activity is dependent on the local acidic environment in lysosomes. To determine the optimal pH of enzyme activity with this new substrate, the enzyme activity was measured in a series of assay buffers with pH values ranging from 4.0 to 7.5 (Fig. 3a). The optimal assay pH was found to be 5.0, similar to the existing blue fluorogenic substrate (data not shown), and was used in the following experiments.

Enzyme concentration—To increase the assay sensitivity for compound screens, minimal amounts of enzyme that produce enough signal should be used, and the enzyme response should be linear. Reduction in enzyme concentration can also lower the cost for large scale compound screens. Thus, the enzyme concentration was optimized by varying the concentrations from 0.04 to 200 nM. A nearly linear enzyme response was observed at enzyme concentrations up to 25 nM, after which the response became increasingly non-linear (Fig. 3b). Based on this result, an enzyme concentration of 2.2 nM was selected as the optimal assay condition, as it yielded sufficient fluorescence intensity with less than 10% substrate consumption.

Time course of enzyme reaction—The time course of the enzyme reaction was studied by varying incubation times using 2.2 nM GLA. The enzyme activity showed a nearly linear increase for up to 180 min incubation of GLA with the substrate (Fig. 3c). An incubation time of 10 min was selected as the optimal assay condition for the later experiments as it produced sufficient signal.

DMSO tolerance—Because DMSO is used to dissolve the compounds for HTS, the DMSO tolerance of this assay was examined by adding various concentrations of DMSO into the assay mixture. The enzyme activity was not significantly changed at DMSO concentrations of up to 2% (Fig. 4a), indicating that this assay is well tolerated by the addition of DMSO. The final DMSO concentration was 0.76% in the later compound-screening experiments.

Activity of a known GLA inhibitor—The inhibitory activity of 1-deoxygalactonojirimycin (DGJ), a known GLA inhibitor, was tested in this enzyme assay. The inhibition was concentration dependent with an IC_{50} value of 45.2 ± 0.5 nM, similar to the IC_{50} value of 59.5 ± 0.8 nM determined in the blue fluorogenic GLA assay (Fig. 4b). This result indicates that the assay sensitivity of the red fluorogenic GLA assay is similar to that of the existing enzyme assay that uses the blue fluorogenic substrate.

Enzyme kinetics—The enzyme kinetic parameters were measured by varying the substrate concentrations and incubation times. Stop solution was added at the end of the incubation times to raise the pH to 8.0 for optimal fluorescence measurement. The K_m and V_{max} values were determined as $20.9 \pm 2.0 \mu M$ and 21.5 ± 0.7 pmol/min, respectively, in this red fluorogenic GLA assay at pH 5.0 (Fig. 5a). For the compound-screening assays, the substrate concentration is usually selected around or less than the K_m value to maintain the sensitivity of the enzyme assay for compound identification. Thus, $20 \mu M$ of res- α -galc was chosen as the optimal condition for compound screens.

Since the pK_a of the enzyme reaction product, resorufin, in this assay is ~ 6.0 , we have determined the K_m and V_{max} values of GLA at pH 5.5 without addition of the stop solution. The K_m and V_{max} values were found to be $24.4 \pm 1.3 \mu M$ and 41.1 ± 0.8 pmol/min, respectively, which are comparable to those obtained above (Fig. 5b). The continuous assay format, without the addition of stop solution, simplifies the enzyme kinetic experiment, and can thus make the K_i measurements of GLA enzyme inhibitors more efficient.

Assay miniaturization and DMSO plate test

Assay miniaturization into the 1,536-well plate format enables HTS for the screening of large compound libraries and reduces the screening cost by saving enzyme and substrate. The assay was miniaturized by the proportional reduction of the volumes of reagents. The final assay volume was $3 \mu l$ /well, a tenfold reduction compared to the $30 \mu l$ /well in the 384-well plate format. To validate this enzyme assay, a DMSO plate was used to assess the statistical parameters of the assay in the 1,536-well plate format. The signal-to-basal ratio was 13.2-fold, CV was 4.6% and Z' factor was 0.88 (Fig. 6a), indicating a robust assay for HTS. These statistics were comparable to those of the existing blue substrate assay, which had a signal-to-basal ratio of 34.3, a CV of 10% and a Z' factor of 0.66 (Fig. 6b). These data indicate that the robust performance of the GLA assay using this new red substrate is similar to the assay using the blue substrate.

In summary, we have synthesized a new red fluorogenic substrate, res- α -galc, for the GLA enzyme assay. We have also optimized and miniaturized an enzyme assay using this red fluorogenic substrate in a 1,536-well plate format that is appropriate for the screening of large compound collections. This GLA enzyme assay is less prone to interference from fluorescent compounds and lint/dust due to its red fluorescence. Thus, it provides a robust new screening tool for the identification of compounds for potential chaperone treatment of Fabry disease.

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References

1. Desnick, R.; Ioannou, Y.; Eng, C. Alpha-galactosidase A deficiency: Fabry disease. In: Scriver, CR.; Baudet, AL.; Sly, WS.; Valle, D., editors. The metabolic and molecular bases of inherited disease. Vol. 8. Vol. 1. McGraw-Hill; New York: 2001. p. 3733-3774.
2. Ishii S, et al. Mutant alpha-galactosidase A enzymes identified in Fabry disease patients with residual enzyme activity: biochemical characterization and restoration of normal intracellular processing by 1-deoxygalactonojirimycin. *Biochem J* 2007;406 (2):285–295. [PubMed: 17555407]
3. Ishii S, Suzuki Y, Fan JQ. Role of Ser-65 in the activity of alpha-galactosidase A: characterization of a point mutation (S65T) detected in a patient with Fabry disease. *Arch Biochem Biophys* 2000;377 (2):228–233. [PubMed: 10845698]
4. Ishii S, et al. Characterization of a mutant alpha-galactosidase gene product for the late-onset cardiac form of Fabry disease. *Biochem Biophys Res Commun* 1993;197(3):1585–1589. [PubMed: 7904161]
5. Brady RO. Enzyme replacement for lysosomal diseases. *Annu Rev Med* 2006;57:283–296. [PubMed: 16409150]
6. Lidove O, et al. Clinical results of enzyme replacement therapy in Fabry disease: a comprehensive review of literature. *Int J Clin Pract* 2007;61(2):293–302. [PubMed: 17263716]
7. Wilcox WR, et al. Long-term safety and efficacy of enzyme replacement therapy for Fabry disease. *Am J Hum Genet* 2004;75 (1):65–74. [PubMed: 15154115]
8. Moore DF, et al. Regional cerebral hyperperfusion and nitric oxide pathway dysregulation in Fabry disease: reversal by enzyme replacement therapy. *Circulation* 2001;104(13):1506–1512. [PubMed: 11571244]
9. Bernier V, Bichet DG, Bouvier M. Pharmacological chaperone action on G-protein-coupled receptors. *Curr Opin Pharmacol* 2004;4(5):528–533. [PubMed: 15351360]
10. Ulloa-Aguirre A, et al. Pharmacologic rescue of conformationally-defective proteins: implications for the treatment of human disease. *Traffic* 2004;5(11):821–837. [PubMed: 15479448]
11. Fan JQ, Ishii S. Active-site-specific chaperone therapy for Fabry disease. Yin and yang of enzyme inhibitors. *Febs J* 2007;274 (19):4962–4971. [PubMed: 17894781]
12. Shin SH, et al. Screening for pharmacological chaperones in Fabry disease. *Biochem Biophys Res Commun* 2007;359(1):168–173. [PubMed: 17532296]
13. Hultberg B, Sjoblad S, Ockerman PA. Glycosidases in human skin fibroblast cultures. Alpha-fucosidase, alpha-galactosidase, alpha-glucosidase, beta-mannosidase, and N-acetyl-alpha-glucosaminidase. *Acta Paediatr Scand* 1975;64(1):123–131. [PubMed: 234663]
14. Mapes CA, Sweeley CC. Galactosyl (alpha 1–4) galactosylceramide: galactosyl hydrolase activity in normal and Fabry plasma. *Biochem Biophys Res Commun* 1973;53(4):1317–1324. [PubMed: 4201302]
15. de Groot PG, et al. A new immunochemical method for the quantitative measurement of specific gene products in man-rodent somatic cell hybrids. *Hum Genet* 1978;44(3):295–304. [PubMed: 83282]
16. Yagi F, Eckhardt AE, Goldstein IJ. Glycosidases of Ehrlich ascites tumor cells and ascitic fluid—purification and substrate specificity of alpha-N-acetylgalactosaminidase and alpha-galactosidase: comparison with coffee bean alpha-galactosidase. *Arch Biochem Biophys* 1990;280(1):61–67. [PubMed: 2162155]
17. Tsou KC, Su HC. A study of yeast alpha-galactosidase with naphthyl alpha-D-galactopyranosides as chromogenic substrates. *Anal Biochem* 1964;8:415–423. [PubMed: 14218908]
18. Simeonov A, et al. Fluorescence spectroscopic profiling of compound libraries. *J Med Chem* 2008;51 (8):2363–2371. [PubMed: 18363325]

Fig. 1.
Scheme of the synthesis of resorufinyl-labeled α -D-galactopyranoside

Fig. 2.

Schematic representation of the GLA enzyme assay. The fluorogenic substrate, res- α -galc, is hydrolyzed by GLA to yield the two products, galactose and resorufin. Resorufin has an excitation peak at 573 nm and an emission peak at 590 nm. An excitation filter of 573 (10)nm and an emission filter of 610 (10)nm were used in the experiment as it yielded a better signal-to-noise ratio

Fig. 3.

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 nearly linearly up to 25 nM GLA concentration. **c** Time course of the enzyme reaction at room temperature. The enzyme activity increased nearly linearly from 5 to 180 min incubation times

Fig. 4.

a DMSO tolerance in the GLA enzyme assay. The enzyme activity was not affected by up to a 2% DMSO concentration. **b** Concentration responses of a known GLA inhibitor, DGJ, in the GLA enzyme assays using the red substrate (*solid line*) and blue substrate (*dashed line*). The IC₅₀ values were 45.2±0.5 nM in the red substrate assay and 59.5±0.8 in the blue substrate assay, respectively

Fig. 5.

Enzyme kinetics. **a** The enzyme reaction obeyed the Michaelis–Menten equation with a K_m of $20.9 \pm 2.0 \mu\text{M}$ and a V_{max} of $21.5 \pm 0.7 \text{ pmol/min}$ as determined in a buffer at pH 5.0 with addition of stop solution. **b** The enzyme kinetic parameters were obtained in a continuous experiment without addition of stop solution in a buffer at pH 5.5. The K_m and V_{max} values were $24.4 \pm 1.3 \mu\text{M}$ and $41.1 \pm 0.8 \text{ pmol/min}$, respectively

Fig. 6. Scatter plot of the screen results from a DMSO plate. **a** In the red substrate assay, the signal-to-basal ratio was 13.2 fold with a CV of 4.6% and Z' factor of 0.88. **b** In the blue substrate assay, the signal-to-basal ratio was 34.3 fold with a CV of 10.0% and a Z' factor of 0.66