Published in final edited form as:

Anal Bioanal Chem. 2012 January ; 402(2): 731–739. doi:10.1007/s00216-011-5496-z.

A high throughput glucocerebrosidase assay using the natural substrate glucosylceramide

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Abstract

Glucocerebrosidase is a lysosomal enzyme that catalyzes the hydrolysis of glucosylceramide to form ceramide and glucose. A deficiency of lysosomal glucocerebrosidase due to genetic mutations results in Gaucher disease, in which glucosylceramide accumulates in the lysosomes of certain cell types. Although enzyme replacement therapy is currently available for the treatment of type 1 Gaucher disease, the neuronopathic forms of Gaucher disease are still not treatable. Small molecule drugs that can penetrate the blood-brain barrier, such as pharmacological chaperones and

enzyme activators, are new therapeutic approaches for Gaucher disease. Enzyme assays for glucocerebrosidase are used to screen compound libraries to identify new lead compounds for drug development for the treatment of Gaucher disease. But the current assays use artificial substrates that are not physiologically relevant. We developed a glucocerebrosidase assay using the natural substrate glucosylceramide coupled to an Amplex-red enzyme reporting system. This assay is in a homogenous assay format and has been miniaturized in a 1,536-well plate format for high throughput screening. The assay sensitivity and robustness is similar to those seen with other glucocerebrosidase fluorescence assays. Therefore, this new glucocerebrosidase assay is an alternative approach for high throughput screening.

Keywords

Glucocerebrosidase; Natural substrate; Gaucher disease; Enzyme substrate; Inhibitor

Introduction

Gaucher disease is a hereditary lysosomal storage disorder with an estimated incidence of 1 in 40,000–60,000 people in the general population [1]. This disease is caused by the deficiency of the lysosomal enzyme glucocerebrosidase (EC 3.2.1.45), which results in the accumulation of glucosylceramide in macrophages of the reticuloendothelial system. To date, 271 mutations have been identified in the glucocerebrosidase gene (GBA; Human Gene Mutation Database, www.hgmd.cf.ac.uk), including 212 missense/nonsense alterations that may result in misfolding, decreased stability, and/or mistrafficking of this lysosomal protein. Patients with Gaucher disease can present at any age and frequently have organomegaly, anemia, thrombocytopenia, and bone crises. Some patients have neuronopathic forms of the disorder. Enzyme replacement therapy (ERT) has been successfully used to treat type 1 or non-neuronopathic Gaucher disease, but it is extremely expensive and not effective in treating CNS manifestations [2].

In the past several years, efforts have focused on the development of small molecule drugs that can penetrate the blood-brain barrier and treat all types of Gaucher disease, including the neuronopathic forms of the disease. Compared to protein based drugs such as ERT, small molecule drugs are advantageous, as they can be administered orally, do not cause autoimmune responses, and are relatively easy to manufacture. Several approaches have emerged for new drug development to treat Gaucher disease, including pharmacological chaperone therapy, activity enhancement of mutant enzyme, and substrate reduction therapy [3]. Pharmacological chaperones are small molecules that can bind to mutant proteins and assist in their correct folding, maturation, and trafficking to the functional site, such as lysosomes. Isfagomine, an iminosugar analog developed by Amicus Therapeutics, was in clinical trials for the treatment of Gaucher disease [4]. Two closely related pharmacological chaperones are also in clinical trials for the treatment of Fabry disease and Pompe disease, other lysosomal storage disorders [5–8]. Substrate reduction therapy for Gaucher disease employs a small molecule enzyme inhibitor of ceramide glucosyltransferase to block the synthesis of glucosylceramide, which accumulates in the lysosomes of patients with Gaucher disease. The long-term clinical efficacy and drug safety of substrate reduction therapy are still under investigation [9]. In addition, small molecule activators that can enhance the residual mutant enzyme activity in lysosomes could be therapeutically useful for the treatment of Gaucher disease, especially those with chaperone capability.

Enzyme assays are commonly used for lead compound discovery in drug development. In order to simplify enzyme assay procedures and detection methods, artificial chromogenic and fluorogenic substrates are commonly used, enabling a homogeneous assay format to

increase the compound screening throughput. Enzyme assays using natural substrates are more physiologically relevant, but are often not feasible for the high throughput screening (HTS) of large compound libraries because of complicated assay protocols and limited screening throughput. Several fluorogenic enzyme substrates are available for the measurement of glucocerebrosidase activity. The most commonly used ones are the bluefluorogenic substrate, 4-methylumbelliferyl- β -D-glucopyranoside (4MU- β -glc) [10, 11], and the chromogenic substrate, *p*-nitrophenyl- β -D-glucopyranoside [10]. Both substrates are used in assays for the clinical diagnosis of Gaucher disease and in compound screens. The red-fluorogenic substrate, resorufin- β -D-glucopyranoside (res- β -glc) [12, 13], is advantageous for compound screens because it is less prone to compound autofluorescence, and thus produces less false positives. In addition, a green fluorogenic substrate, fluorescein di- β -D-glucopyranoside (flu- β -glc), is occasionally used [14, 15]. However, these substrates are artificially designed, consisting of a glucose linked to a fluorophore, which is not naturally present in cells. Although these fluorogenic substrates are sensitive in the assay and easy to use, they may not reflect physiological conditions in cells. In addition, the specificity of fluorogenic substrates could be a problem in assays using cell or tissue preparations instead of purified recombinant enzymes, as they may be cleaved by other cellular enzymes present in these crude preparations [16–18]. Alternatively, the natural substrate, glucosylceramide, can be used in the glucocerebrosidase assay to more accurately measure glucocerebrosidase activity in enzyme preparations from cells and tissues. It has been reported that glucocerebrosidase activity can be measured with a fluorophore tagged natural substrate, bodipyglucosylceramide, but an additional step using high-performance liquid chromatography (HPLC) is needed [19]. With this fluorophore tagged glucosylceramide as a substrate, the specific glucocerebrosidase activity can be measured in crude cell and tissue preparations by separating the bodipy-labeled product from the substrate by a HPLC step. This assay enables the measurement of glucocerebrosidase activity with the real natural substrate, glucosylceramide, which is more relevant to physiological conditions in cells and tissues. We describe here the development of a glucocerebrosidase assay with natural substrate, using an Amplex-red glucose oxidase reporting system in a homogenous assay format. We also compare the advantages and disadvantages of this assay in compound screens to those performed with three other available glucocerebrosidase enzyme assays using different substrates, including red- and blue-fluorogenic substrates, and bodipy-glucosylceramide.

Materials and methods

Reagents and chemicals

4-Methylumbelliferyl β -D-glucopyranoside (4MU- β -glc), a blue-fluorogenic substrate, was purchased from Sigma-Aldrich (St. Louis, MO), as were citric acid, potassium phosphate trihydrate, Tween-20, glycine, and sodium hydroxide. Taurocholic acid sodium salt was from Calbio-Chem (a subsidiary of EMD Bioscience, San Diego, CA). Resorufin β -D-glucopyranoside (res- β -glc), a red-fluorogenic substrate, was purchased from Marker Gene Technologies, Inc. (Eugene, OR). Natural glucosylceramide and bodipy-glucosylceramide, *N*-[11-(dipyrrometheneboron difluoride) undecanoyl]-D-glucosyl- β 1-1'-D-erythrosphingosine, catalog # 810267P) were purchased from Avanti Polar Lipids, Inc. (Alabaster, Alabama). The Amplex[®] Red Glucose/ Glucose Oxidase Assay Kit was purchased from Invitrogen (Eugene, OR) to measure the amount of glucose produced when glucosylceramide is cleaved by glucocerebrosidase.

The glucocerebrosidase inhibitors, *N*-nonyl-deoxynojirimycin (NN-DNJ) and isofagomine D-tartrate, were purchased from Sigma-Aldrich and Toronto Research Chemicals (Toronto, Canada), respectively. The non-iminosugar inhibitor, NCGC00166331, was synthesized internally as a glucocerebrosidase inhibitor.

The recombinant glucocerebrosidase enzyme was obtained from residual solution after clinical infusions of Cerezyme® (Genzyme, Cambridge, MA). The enzyme solution was mixed with 30% glycerol and small aliquots were stored at –80 °C for up to 3 years.

The assay buffer was composed of 50 mM citric acid, 176 mM K₂HPO₄, 10 mM sodium taurocholate, and 0.01% Tween-20 at pH 5.9. It was stored at 4 °C for use up to 6 months. A solution of 1 M sodium hydroxide, 1 M glycine at pH 10 was used as the stop solution.

Enzyme kinetics assay with glucosylceramide

The kinetics assay with the natural substrate glucosylceramide was carried out in a 384-well plate. Ten microliters per well of substrate solution was added to the plate. The substrate solutions were diluted 1:3 to give final concentrations of 0.5, 1.6, 4.9, 14.8, 44.4, 133, and 400 μM. As described above, reactions were initiated by the addition of 20 μl/well of enzyme solution at a 5-nM final concentration. The assay plate was incubated at 37 °C for 5, 10, 15, 20, and 25 min. This was followed by the addition of 30 μl/well of the detection mixture, the Amplex-red glucose oxidase assay solution (Invitrogen). This detection mixture contained glucose oxidase, horseradish peroxidase and its fluorogenic substrate, the Amplex-red® reagent, which changed the pH of 5.9 in the original assay buffer to neutral. The negative control had all the reagents except glucocerebrosidase. The assay plate was then incubated at room temperature (RT) for 20 min and measured in a ViewLux plate reader with an excitation at 573 nm and an emission at 610 nm. A standard curve for glucose (one of the two products of the reaction), run in the same volume of detection mixture, was generated to normalize the enzyme reaction products.

Enzyme kinetics assay with bodipy-glucosylceramide

This kinetics assay for the glucocerebrosidase enzyme reaction was initially performed in a 96-well plate with the substrate, bodipy-glucosylceramide. The substrate was diluted in a 1:2 ratio to final concentrations of 0.24, 0.49, 0.98, 2, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, and 500 μM. The enzyme reaction was initiated by the addition of 40 μl/well of enzyme solution (5 nM final concentration) to 20 μl/well of substrate solution. The reaction was incubated at 37 °C for 20 h in order to acquire sufficient signal. Sixty microliters per well of stop solution were added to terminate the enzyme reaction. An Agilent 1100 Series HPLC equipped with a Model G1321A fluorescence detector was used to separate the product from the substrate in the resulting reaction mixture. A 10 μl aliquot was injected onto a Zorbax Eclipse XDB-C18 column (4.6×250 mm, 5 μm) at room temperature. A 10-min gradient (85/15 methanol/ (0.1% formic acid in water) to 100% Methanol) at 2 ml/min was used. Fluorescent peaks (Ex=505 nm, Em=540 nm) were identified by comparing their retention times with those of the standards. The apparent K_m was calculated by using the velocity of product formation calculated from the ratio of the amount of product over the reaction time.

Enzyme kinetics assays with blue- and red-fluorogenic substrates

Both the blue-fluorogenic (4MU-β-glc) and red-fluorogenic (res-β-glc) assays were carried out in 384-well plates using 5 nM final concentration of recombinant glucocerebrosidase. In the blue-fluorogenic enzyme assay, substrate solutions were serially diluted 1:2 to give final substrate concentrations of 23, 47, 94, 188, 375, 750, 1,500, and 3,000 μM. In the red-fluorogenic enzyme assay, substrate solutions were serially diluted 1:1.5 to give final substrate concentrations of 7.8, 11.7, 17.6, 26.3, 39.5, 59.3, 88.9, 133, and 200 μM. The enzyme reaction was initiated by the addition of 20 μl/well of enzyme solution to 10 μl/well of substrate solution in the assay plate. For the blue-fluorogenic assay, 30 μl/well of stop solution was added to terminate the reaction after incubations of 2, 4, 6, 8, and 10 min at room temperature. The assay plate was measured in a ViewLux plate reader (Perkin Elmer, Wellesly, MA) at an excitation wavelength of 365 nm and an emission wavelength of 440

nm. For the red-fluorogenic assay, stop solution was not required, and the fluorescence intensity was measured at 2 min intervals in the ViewLux plate reader at an excitation wavelength of 573 nm and an emission wavelength of 610 nm to obtain five readings (2, 4, 6, 8, and 10 min). Standard curves of the free fluorophores, 4-methylumbelliferone and resorufin, in the same volume of assay buffer and stop solution were generated for normalization of the enzyme reaction products.

Compound screens

The enzyme assays with the blue-fluorogenic substrate, red-fluorogenic substrate, and glucosylceramide were miniaturized in the 1,536-well plate format. A Bio-RAPTR™ FRD Workstation (Beckman Coulter, Brea, CA) was used to dispense 2 μ l/well of enzyme solution (1 nM final concentration) to black assay plates. An automated pintool station (Kalypsys, San Diego, CA) was used to transfer 23 nl/well of the compound DMSO solutions to the enzyme solution in the assay plates. NN-DNJ and NCGC00166331 were serially diluted from 10 mM stock solution in DMSO at a 1:3 ratio for 12 concentrations, while isofagomine was serially diluted at a 1:2 ratio for 24 concentrations. The enzyme reaction was initiated by the addition of 1 μ l/well of substrate solution. The assay plates with the blue- and red-fluorogenic substrates were incubated for 20 min at room temperature, while the plates with the natural substrate were incubated for 30 min at 37 °C. Only the blue-fluorogenic assay was terminated using 3 μ l/well of stop solution. The plates were then measured in the ViewLux plate reader at the same excitation and emission wavelengths specified above. For the glucocerebrosidase assay with natural substrate, 3 μ l/well of Amplex-red reaction mixture was added to the reaction mixture in the assay plates, which were then incubated at room temperature for 20 min before being measured in the ViewLux plate reader. The final enzyme concentration for all assays was 5 nM, unless otherwise specified. The final substrate concentrations of the blue-fluorogenic, red-fluorogenic, and natural substrates were 800, 30, and 50 μ M, respectively.

The assay with bodipy-glucosylceramide was performed manually in three steps, an initial glucocerebrosidase assay in the presence of compounds in 96-well plates, an HPLC separation of product from substrate in the reaction mixture, and detection of the separated products. Three known glucocerebrosidase inhibitors, NN-DNJ, isofagomine, and NCGC00166331, were diluted at a 1:3 ratio for 12 concentrations. The enzyme reaction was initiated by the addition of 20 μ l/well substrate solution to 40 μ l/well enzyme-compound solution in 96-well plates. The enzyme and substrate final concentrations were 1 nM and 25 μ M, respectively. The assay plate was incubated at 37 °C for 20 h, and then the reaction was terminated with 60 μ l/well stop solution. The product was separated from the substrate (both labeled with a bodipy dye) by the HPLC method as described above. The fluorescence intensity of the resulting product was measured using a fluorescence plate reader as described above.

Data analysis

The data were calculated as the mean $\pm$ standard error. The results for the enzyme assays, including the enzyme kinetics and IC₅₀ calculations, were analyzed with Prism® (Graphpad, San Diego, CA).

Results and discussion

Assay principle and protocol

This glucocerebrosidase assay uses the natural substrate of glucocerebrosidase, glucosylceramide, which is hydrolyzed to form ceramide and glucose (Fig. 1a). To detect glucocerebrosidase activity using glucosylceramide without fluorescence labeling, an

enzyme reporting system consisting of a glucose oxidase, a horseradish peroxidase (HRP), and the fluorogenic Amplex-red dye, is used. This assay format has been used in alpha-glucosidase (GAA) and cytokinin-O-glucoside-specific β -glucosidase assays, which have the natural substrates glycogen and cytokinin-O-glucoside, respectively [20, 21]. The Amplex-red enzyme reporting system enables quantitation of the amount of glucose generated by the glucocerebrosidase reaction with high sensitivity. Glucose, one of two products of the glucocerebrosidase reaction, is first oxidized by glucose oxidase to produce H_2O_2 . The resulting H_2O_2 serves as a substrate for HRP to convert the pro-fluorescent Amplex-red dye to the red fluorescent product, resorufin. This assay is homogenous and has been miniaturized into the 1,536-well plate format, similar to the fluorogenic enzyme assays (Fig. 2). Since glucose oxidase and HRP are used in this assay, the compounds interacting with these reporting enzymes may become false positives in compound screens. However, these false positive compounds can be eliminated using a counter-screen assay with the same reporting system, where glucocerebrosidase is replaced with glucose. In addition, the purified enzyme is required for this assay because in impure enzyme preparations, such as whole cells and tissue homogenates, endogenous glucose can significantly reduce the assay window or produce inaccurate results. Despite these shortcomings, this assay using the natural substrate of glucocerebrosidase is useful as an alternative method for compound screens because it may yield different lead compounds for drug development.

We also optimized three other glucocerebrosidase fluorescence assays and compared them with this new assay using the natural substrate. The glucocerebrosidase enzyme assays using the two fluorogenic substrates, 4MU- β -glc and res- β -glc, are in homogenous assay formats previously adapted to the 1,536-well plate format for HTS [12]. Once the substrates are hydrolyzed by glucocerebrosidase, one of the products, either 4MU or resorufin, becomes fluorescent and can be detected with a fluorescence plate reader (Fig. 1b, c). 4MU- β -glc is the substrate most commonly used for the glucocerebrosidase enzyme assay, as well as for assaying many other lysosomal hydrolases. The compound screen results generated from this assay can be directly compared to those in the screening database and literature [22, 23]. Because the pK_a of the product (4MU) is ~ 8 , a stop solution is required to raise the pH of the reaction mixture to 10 for optimal fluorescence detection. The glucocerebrosidase assay using the red-fluorogenic substrate, res- β -glc (Fig. 1c), does not require the addition of stop solution because the product, resorufin, has a pK_a of ~ 6 , which is close to the pH in the assay buffer. The assay protocols using these two substrates are identical, except for the addition of stop solution. Because the fluorescence emissions of the two assays (440 vs. 610 nm) are considerably different, one assay can be used in compound screens as a counter-screen for the other to eliminate autofluorescent compounds.

The fourth glucocerebrosidase assay uses the bodipy-glucosylceramide substrate, which has a complicated assay protocol requiring an additional HPLC step to separate the enzyme reaction products from the remaining substrate because both the substrate and product have the bodipy label (Fig. 1d). Thus, this enzyme assay has two stages, an initial enzyme reaction in 96-well plates followed by a HPLC separation method. The mixtures of the enzyme reaction in 96-well plates are collected and then undergo HPLC to separate the products from unhydrolyzed substrates. The fluorescence intensity of the resulting products is then measured in a fluorescence detector. Because of the additional HPLC step, this assay has very limited throughput for compound screens (Fig. 2).

Enzyme kinetics

The enzyme kinetics of the glucocerebrosidase assay using glucosylceramide and recombinant human enzyme was compared in parallel with the assays using the three other substrates, 4MU- β -glc, res- β -glc, and bodipyglucosylceramide. The K_m values determined from these assays using the natural substrates, glucosylceramide and bodipy-

glucosylceramide, were 108 and 145 μM , respectively (Fig. 3a, b). Similar values were expected because the structures of these two substrates are almost identical, except for a bodipy label attached to the end of one of the ceramide chains on bodipy-glucosylceramide. Therefore, attaching a bodipy tag to the natural substrate does not have a significant effect on glucocerebrosidase activity. The reporter enzymes, glucose oxidase and HRP, in the glucocerebrosidase assay with natural substrate did not react with glucosylceramide which was used as a negative control. This assay is useful for the high throughput screening of large compound collections to identify new lead compounds, but only with the purified enzyme. On the other hand, the assay using bodipy-glucosylceramide can be performed with crude enzyme preparations, including permeabilized whole cells and crude tissue homogenates, but its screen throughput is significantly lower, and can only be used as a confirmation screen with a small number of compounds.

The K_m values of the assays using the fluorogenic substrates, 4MU- β -glc and res- β -glc, were 862 and 31 μM , and the V_{max} values were 1.7 and 42 pmol/min, respectively (Fig. 3c, d). K_m is an important parameter for compound screening assays, as the substrate concentration is usually set equal to or less than the K_m value, in order to maintain compound sensitivity. Thus, 800 μM 4MU- β -glc and 30 μM res- β -glc were chosen as the optimal substrate concentrations for subsequent assays. V_{max} is the maximal rate at which products are generated, and a greater value means a more efficient enzyme reaction. These values, as well as the V_{max}/K_m ratio (representing the catalytic efficiency of enzyme), are summarized in Table 1. The V_{max}/K_m ratios of 4MU- β -glc and res- β -glc were 0.002 and 1.4 pmol/min/ μM , respectively. Therefore, the catalytic efficiency of the res- β -glc assay was about 700 times greater than that of the 4MU- β -glc assay. These substrates are both suitable for compound screens, although this large difference in catalytic efficiency values makes the assay using res- β -glc more sensitive. A drawback with fluorogenic substrates is that they may be hydrolyzed by other enzymes in crude enzyme preparations, whereas the natural substrate is more specific for lysosomal glucocerebrosidase [24]. In addition, the binding affinity of fluorogenic substrates to the enzyme's active site may differ from that of the natural substrate, which may affect compound activities in the late cell-based follow-up assays.

Inhibitor activities determined in the four screening assays

We used three known inhibitors of glucocerebrosidase to compare the compound sensitivity in this new assay to the three other fluorescence assays (data summarized in Table 1). Two of the three compounds were the iminosugar analogs, isofagomine, and NN-DNJ. The third compound, NCGC00166331, a non-iminosugar glucocerebrosidase inhibitor, was identified and synthesized at the NIH Chemical Genomics Center (unpublished data). The potency of isofagomine in this assay (IC_{50} =39 nM) was similar to those obtained from the blue- and red-fluorogenic assays, but it was ~46-fold more potent than that determined in the assay using bodipy-glucosylceramide as the substrate (Table 1). The IC_{50} value of NN-DNJ in this assay was 4.8 μM , which was ~20-fold less potent than the values obtained in the assays using the blue- and red-fluorogenic substrates. However, it was ~5-fold more potent than the IC_{50} measured in the assay using bodipy-glucosylceramide. The IC_{50} value for NCGC00166331 in this assay was 1.5 μM , which was ~10-fold less potent than that in the fluorogenic substrate assays, but ~13-fold more potent in the bodipy-glucosylceramide assay (Table 1). Similar IC_{50} values were obtained in the blue- and red-fluorogenic substrate assays (0.16 and 0.12 μM , respectively). We noticed that the concentration–response curves for isofagomine, NN-DNJ, and NCGC00166331 were significantly shifted to the right in the assay using bodipy-glucosylceramide (Fig. 4). The activities of all three inhibitors were ~100-fold weaker in the bodipy-glucosylceramide assay as compared with those in the assays using fluorogenic substrates. Thus, in addition to its lower throughput, the enzyme

assay using bodipy-glucosylceramide is less sensitive to inhibitors, further limiting its application in compound screens.

Taken together, all four types of glucocerebrosidase assays described here have pros and cons based upon the substrate properties and experimental methods. Fluorogenic substrates, such as 4MU- β -glc and res- β -glc, are useful for compound screening involving large compound collections, where 300,000 to 2–3 million compounds are typically screened. Both enzyme assays in the 1,536-well plate format are robust. The glucocerebrosidase assay using glucosylceramide as a substrate is also homogenous and conducive to a 1,536-well plate format. The signal-to-basal ratio of this assay was 5.6-fold (s.d.=0.59) and the Z' factor was 0.75 (s.d.=0.01) in the 1,536-well plates. Thus, this assay can be used in HTS applications, which may result in new lead compounds due to the use of the natural substrate. The glucocerebrosidase assay using bodipy-glucosylceramide as a substrate is useful for determining compound activities in crude enzyme preparations, but is not suitable for screening large numbers of compounds. Therefore, these four glucocerebrosidase assays provide multiple assay choices for the primary screen, counter-screen of primary screen hits, and follow-up assays for hit confirmation.

In summary, we have developed a new homogenous glucocerebrosidase assay using the natural substrate, glucosylceramide, and recombinant enzyme. Compared to three other assays using fluorogenic substrates or bodipy-labeled glucosylceramide, this new assay is sensitive, robust, and more physiologically relevant for the high throughput screening of large compound collections.

Acknowledgments

This research was supported by the Molecular Libraries Initiative of the NIH Roadmap for Medical Research and the Intramural Research Programs of the National Human Genome Research Institute and the National Institutes of Health.

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Fig. 1.

Schematic presentations of assay principles for four glucocerebrosidase assays. **(a)** The glucocerebrosidase assay using natural substrate glucosylceramide, which is coupled to an Amplex-red enzyme reporting system. **(b)** The glucocerebrosidase assay using the blue-fluorogenic substrate, 4MU-β-glc. **(c)** The glucocerebrosidase assay using the red-fluorogenic substrate, res-β-glc. **(d)** The glucocerebrosidase assay using bodipy-glucosylceramide as a substrate, which includes a 96-well plate enzyme assay and a HPLC step for the separation of bodipy-labeled product and substrate

Fig. 2. Comparison of assay steps and compound screen throughput of four glucocerebrosidase assays using the different substrates

Fig. 3.

Results of enzyme kinetics determined for four glucocerebrosidase assays using different substrates, including (a) natural substrate glucosylceramide, (b) fluorescent substrate bodipy-glucosylceramide, (c) blue fluorogenic substrate 4MU-β-glc, and (d) red fluorogenic substrate res-β-glc. The values of K_m and V_{max} are listed in Table 1

Fig. 4.

Concentration–response curves of three known inhibitors, (a) isofagomine, (b) NN-DNJ, and (c) NCGC00166331, determined in four glucocerebrosidase assays using different substrates, including glucosylceramide (*filled circle*), bodipy-glucosylceramide (*diamond*), 4MU-β-glc (*square*), and res-β-glc (*triangle*). The IC_{50} values of these three compounds are listed in Table 1

The K_m , V_{max} , V_{max}/K_m , and IC_{50} values of three known inhibitors determined in four glucocerebrosidase assays using different substrates

Table 1

Substrate	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m	NN-DNJ IC_{50} (μM)	Isfagomine IC_{50} (μM)	NCGC0016631 IC_{50} (μM)
Glucosylceramide	108	305	2.80	4.80	0.039	1.50
Bodipy-glucosylceramide	145 ^a	N/D	–	22.7	1.80	19.0
4MU- β -glc	874	1.7	0.002	0.25	0.018	0.16
Res- β -glc	31	42	1.4	0.24	0.012	0.12

^a Apparent K_m