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Cell-based High-throughput Screen identifies GALC Enhancers as Potential Small Molecules Therapies for Krabbe Disease

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Abstract

Krabbe disease, also known as globoid-cell leukodystrophy (GLD), is a lysosomal storage disease (LSD) caused by the deficiency of the lysosomal enzyme β -galactocerebrosidase (GALC), resulting in severe neurological manifestations related to demyelination secondary to elevated galactosylsphingosine (psychosine) with its subsequent cytotoxicity. The only available treatment is hematopoietic stem cell transplantation that delays the disease onset but has not prevented further long-term neurological manifestations. Here, we report the identification of small molecules that enhance mutant GALC activity identified by quantitative cell-based high-throughput screen (qHTS). Using a specific neurologically relevant murine cell-line (145M-*Tw1*) modified to express common human hGALC-G270D mutant, we were able to detect GALC activity in 1,536-well microplate format. The qHTS of approximately 46,000 compounds identified 3 small molecules that show significant enhancements of residual mutant GALC activity in primary cell lines from GLD patients. Interestingly, these compounds showed to increase the levels of GALC-G270D mutant in the lysosomal compartment. In kinetic assessments, these small molecules fail to disturb GALC kinetics profile at acidic conditions, which is highly desirable for folding-assisting molecules operating in the endoplasmic reticulum and not affecting GALC catalytic properties once in the lysosomal compartment. In addition, these small molecules rescue the decreased GALC activity at neutral pH and partially stabilize GALC at heat-denaturing conditions. These are drug-like compounds that can be used as the starting point to develop novel small molecule agents to treat the progressive neurodegenerative course of GLD.

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Keywords

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INTRODUCTION

Globoid-cell leukodystrophy (GLD), mostly known as Krabbe disease, is one of the classical neurological lysosomal storage diseases (LSD) with autosomal recessive inheritance caused by a deficiency in β -galactocerebrosidase (GALC) or galactosylceramidase activity (E.C 3.2.1.46). GALC is a soluble lysosomal enzyme that hydrolyzes galactose from several glycosphingolipids including galactosylcerebroside (or galactosylceramide) and galactosylsphingosine, commonly named psychosine. Deficiency of GALC results in intracellular elevation of psychosine in myelin-forming cells triggering apoptotic processes and demyelination, which is manifested radiologically by progressive leukodystrophy (De Gasperi et al. 1996a; Nilsson et al. 1997; Suzuki 1984; Suzuki 1998). The prevalence of GLD is approximately 1/100,000 to 1/250,000 live-births, but in some populations the disease prevalence can be as high as 1/100 to 1/150 live-births (Rafi et al. 1996). Children diagnosed with the infantile form of GLD typically presents prior to 6 months of age with a rapidly progressive neurologic deterioration and death generally before 2 year (Wenger et al. 1997). Late onset GLD clinical forms are characterized by slowly progressive with wider spectrum of neurological manifestations including peripheral neuropathy, motor weakness and psychiatric symptoms (De Gasperi et al. 1996b; Fiumara et al. 2010).

Currently, enzyme replacement therapy (ERT) is the only FDA-approved treatment for LSDs with non-neuronopathic symptoms as Gaucher disease, Pompe disease, Fabry disease, cholesteryl storage disease, mucopolysaccharidoses types I, II, IV-A and VI (Burrow et al. 2007; Hollak and Wijburg 2014). Unfortunately, ERT agents, large-molecular weight molecules, fail to cross the blood-brain barrier (BBB) restricting treatment to non-neurological symptoms in LSDs. Alternatively, hematopoietic cell transplantation (HSCT) is currently the only available treatment for early infantile form of GLD. However, even in presymptomatic infants who underwent HSCT, in the long-term follow-up, GLD-related neurological manifestations are observed (Duffner et al. 2009). More than 110 mutations in the *GALC* gene (Hill et al. 2015) have been associated with severe clinical phenotypes, and a large number of missense mutations in GALC that cause GLD are likely to result in instability and misfolding of GALC, eventually being targeted to endoplasmic reticulum-associated degradation (ERAD) (Deane et al. 2011). Missense mutations in *GALC* gene that result in an enzymatic activity less than 10–20% of wild-type enzymatic activity result in GLD neurological manifestations, which is also observed in several other LSDs (Conzelmann and Sandhoff 1983). Therefore, enhancements of mutant GALC that reduce the cytotoxic elevated psychosine levels, ultimately preventing ultimately arresting the demyelination process, resulting in amelioration of neurological symptoms in GLD patients.

Small molecules as therapeutic agents are more likely to penetrate the BBB and reach diverse neural cells in the CNS. Previously, we developed GLD patient cell-based high-throughput screening (HTS) assay using a specific synthetic fluorescent substrate for GALC

(Ribbens et al. 2013). In the current study, we describe the results of the quantitative HTS (qHTS) screen results against two chemical compound libraries using our cell-based HTS assay. In the primary qHTS of two libraries representing a total of 46,816 molecules, 148 small molecule candidates were active, known as 'hits'. Secondary assays and concentration-response curve (CRC) analysis of compound potency and efficacy (Inglese et al. 2006) allowed for the selection of 10 small molecule candidates. In GLD patient primary fibroblasts, three small molecules show enhancements of mutant GALC enzymatic activity. These small molecule candidates or their analogs are potential therapeutic agents that may be used to treat neurological symptoms of GLD.

MATERIAL AND METHODS

Chemical reagents and equipment

Synthetic fluorescent GALC substrate, 6-hexadecanoylamino-4-methylumbelliferyl- β -D-galactoside (HMUGal, FW 592) used in the cell-based HTS and secondary assays was purchased from CiVentiChem (Cary, NC) and HMU standard (FW 430) was purchased from Moscerdam Substrates (Prinsenlaan, Netherlands). For the GALC kinetics assay, the 4-methylumbelliferyl β -D-galactopyranoside and 2-amino-2-methyl-1-propanol (MAP) were purchased from Sigma-Aldrich (St. Louis, MO) was used. Chemicals such as citric acid, sodium acetate, sodium chloride, sodium hydroxide, sodium bicarbonate (NaHCO_3), sodium carbonate (Na_2CO_3), sodium taurocholate, sodium azide, oleic acid, sodium phosphate dehydrate and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Solvents such as chloroform and methanol were purchased from Fisher Scientific (Waltham, MA). Phosphate buffer saline (PBS) was purchased by Corning Inc. (Manassas, VA). Microplates used in the cellular assay were black 96-well plates with clear flat-bottom (BD; Franklin Lakes, NJ), 384- and 1,536-well plates (Greiner Bio-One, Monroe, NC). All microplates used were tissue culture treated. The immunofluorescence cellular staining was performed using the following reagents: rabbit V5 antibody (Sigma-Aldrich, St. Louis, MO), mouse PDI monoclonal antibody (1D3) (Enzo Life Sciences, Farmingdale, NY), rat LAMP-2 antibody (GLA2A7, Developmental Studies Hybridoma Bank, DSHB), F(ab')₂-goat anti-rabbit IgG (H+L) secondary antibody (Alexa Fluor® 568) (ThermoFisher, Waltham, MA), goat anti-Rat IgG (H+L) secondary antibody, Alexa Fluor® 488 (ThermoFisher, Waltham, MA), goat anti-mouse IgG1 secondary antibody, Alexa Fluor® 488 (ThermoFisher, Waltham, MA). MultiFlo FX Multi-mode Dispenser (Winooski, VT) was used to seed and wash multi-well plates. Cellometer was used for cell counting. Cytation 5 imaging reader (BioTeK, Winooski, VT) was used to measure fluorescence from both 96- and 384-well plates. The GALC assay for the screening performed in 1,536-well plates required the Thermo Scientific Multidrop Combi to seed cells into the plates. The PerkinElmer 1430 ultraHTS Wallac Microplate Imager, ViewLux, was used for reading the fluorescence on the primary screening assay.

Cells, tissue culture conditions and establishment of cell line

Cultured fibroblasts from GLD patients were obtained from the cell core bank at Telethon Network of Genetic Biobanks (Genova, Italy). Primary and cultured fibroblasts cells were grown in Dubelcco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO)

with 10% fetal bovine serum (FBS, Gemini Biologicals, West Sacramento, CA) at 5% CO₂/95% air in a humidified atmosphere at 37°C. Cultured cells underwent washing steps using PBS. Before seeding into 1,536-wells, as well as 384-wells and 96-wells microplates, cells were cultured in 75 cm² flasks until confluent, and cells were trypsinized.

For primary screen (HTS), we generated two stably transfected cell lines using a previous established cell line 145M-*Tw*i (Ribbens et al. 2014). Using lentivirus (pLenti6V5-DEST Gateway Vector), we stably transfected the human *GALC* gene carrying a common late onset mutation, *hGALC-G270D* (*G286D* – alternate numbering)(Deane et al. 2011), establishing the cell line 145M-*Tw*i-*hGALC-G270D*. We also performed lenti-transfections using the wild type human *GALC* gene (*hGALC-WT*), establishing the cell line 145M-*Tw*i-*hGALC-WT* which was used as a control in each 1,536-well microplate assayed. Both these cell lines were used in the primary HTS against the 2 libraries above described. Cells were cultured in DMEM/F12 media with 10%FBS. Using a similar approach, two cell lines were established expressing the *hGALC-WT-V5* and another expression the *hGALC-G270D-V5*. These cell lines 145M-*Tw*i-*hGALC-G270D* expressing for cellular immunostaining studies to examine the intracellular trafficking of *GALC-G270D* mutant.

Primary quantitative cell-based HTS assay

For the HTS campaign, two small molecule libraries were utilized. The NCATS Diversity Collection of 44,000 chemically diverse small molecules, and the NCATS Pharmaceutical Collection (NPC), a library of 2,816 small molecules that have been approved for human use by regulatory agencies. For the NCATS diversity collection, the 145M-*Tw*i-*hGALC-G270D* cells were treated at four different compound concentrations, 0.2, 2.3, 22.7 and 113.7 µM for 48hs. For the NPC library, cells were treated with four different compound concentrations 0.92, 4.6, 22.7 and 113.7 µM. Approximate 1,500 cells were seeded per well of 1,536-well microplate and treated with compounds diluted in DMSO solution and organized in 1,536-wells library plates at different concentrations. Using a Kalypsys pin tool equipped with a 1,536-pin array (V & P Scientific, Palo Alto, CA), 23 nL of compound library plates was dispensed into each well of 1,536-well containing 145M-*Tw*i-*hGALC-G270D* cells (Fig. 1A). After the treatment for 48 h, HMUGal substrate (0.2 mM) was added and incubated for 17-h period at 37 °C (Fig. 1B) as previously described (Ribbens et al. 2013). Adding 60 µl of the same stop buffer to stop the reaction, and fluorescence was measured ViewLux (PerkinElmer, Waltham, MA) at at $\lambda_{\text{excitation}}$ 404 nm and $\lambda_{\text{emission}}$ 450 nm.

Cellular GALC enzymatic activity assays in 384-well and 96-well plates

Cellular GALC enzymatic activity assays were performed in 96- and 384-well plate formats. Briefly, clear-bottom black 384-well plates were seeded 145M-*Tw*i-*hGALC-G270D* (mutant) and 145C-WT-*hGALC-WT* (control) using MultiFlo FX Multimode Dispenser. Each well of 384-well plate was seeded with 10,000 cells and treated with 0.8, 4, 20 and 100 µM concentrations of selected small molecule candidates. After incubation for 5 days, the treated medium was aspirated, and 20 µl of HMUGal substrate was added as previously described (Ribbens et al. 2013). Primary skin fibroblasts from GLD patients were grown in tissue culture treated 75cm² flasks. Once they were fully confluent, the cells were trypsinized and re-suspended in DMEM 10% FBS and seeded to black-clear bottom 96-well

tissue culture treated plates. Each well contained 100 μ l of cell suspension (40,000 to 50,000 cells), and control with wild-type (WT) GALC and two GLD patient fibroblasts were cultured in 96-well plates. After attached, cells were treated with 12.5, 25 and 50 μ M of candidate compounds in 100 μ l of the cultured medium for 5 days. At the end of 5th day, cells were washed with PBS using MultiFlo (Biotek), and the medium containing compounds was replaced with 50 μ l of HMUGal substrate (0.1 mM), as previously described (Ribbens et al. 2013). After incubation for 17-h period at 37 °C, GALC reaction was stopped by adding 100 μ l of 0.5 M NaHCO₃/0.5 Na₂CO₃ buffer (pH 10.7) with 0.25% Trion X-100. The fluorescence was measured by BioTek Cytation 5 at $\lambda_{\text{excitation}}$ 404 nm and $\lambda_{\text{emission}}$ 460 nm (Ribbens et al. 2013).

Establishing V5 expression cell lines for human GALC purification

Bay hamster kidney (BHK-21) cells were grown in 6-well plate until 70% confluent with DMEM containing 10% FBS. BHK-21 cells in the media containing 8 μ g/ml of polybrene (hexadimethrine bromide, Sigma-Aldrich, St. Louis, MO) were then transfected with 500 MOI of lentivirus carrying V5-tagged at N-terminal human GALC plasmid (pCAGGS-GALC-WT). The following day, the media was replaced with the regular media and cultured for another day. Clone selection was performed under 7.5 μ g/ml of Blasticidin (ThermoFisher, Waltham, MA) for the selection with further expansion. For GALC purification, BHK-21 stably transfected with GALC-V5 were grown to confluency in 150 mm tissue culture treated dishes and regular media was replaced by DMEM containing 10 mM of ammonium chloride (Neufeld 1989). After 24 and 48hs, the conditioned media was harvested as well as the cell pellet after 48hs. From conditioned media and cell pellet, human GALC enzyme was purified using the V5-tagged Protein Purification Kit (MBL, Woburn, MA) according to the manufacturer's instructions.

Validation of the selected small molecules

Candidate compounds were selected based on their concentration-response curves (CRCs) of classes in the HTS assay. In 96-well plates, primary cultured skin fibroblasts from controls with GALC WT and two GLD patients were used for validation studies. Primary fibroblasts from GLD patients with carrying G553R/G553R mutations and E130K/N295T missense mutations in *GALC* gene. The cells were treated various concentrations of candidates compounds (12.5, 25 and 50 μ M) for 5 days. After the treatment period, medium was removed, cells were washed with PBS and HMUGal substrate buffer was added. Once the reaction was stopped by adding the same stopping buffer after 17-hr incubation at 37 °C, the fluorescence was immediately measured by the plate reader.

Immunocytochemistry Assays

The 145M-*GALC-G270D-V5* were seeded in 24-well plates containing on polylysine-coated coverslips and grown at 37°C overnight. Cells were treated with 1 μ M of each hits or 0.4% of DMSD for the control for 3 days. After 3 days, cells were fixed with 4% of paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS for 15 minutes. After 3 times wash with PBS, the cells were permeabilized with 0.2% Triton X-100 for 5 min followed by additional 3 times wash with PBS. The cells were blocked by 5% goat-serum (Sigma-Aldrich, St. Louis, MO) was proceeded for 45 min followed by 3 times wash with PBS. The

cells were incubated with primary antibodies that were rabbit V5 monoclonal antibody (Sigma-Aldrich, St. Louis, MO), mouse PDI polyclonal antibody (Enzo Life Sciences, Farmingdale, NY) and rat LAMP-2 monoclonal antibody (Abcam, Cambridge, MA) with 1:250, 1:400 and 1:50 dilutions, respectively, in 1% goat-serum at room temperature for 1 hr. Following 3 times washes in PBS, the cells were incubated for 1 hr at room temperature with appropriate secondary antibodies (1:200) as above described. Following 3 times wash in PBS, DAPI staining was proceeded with 1:20,000 – 50,000 dilution in PBS for 5min. The cells on the coverslips were mounted onto a microscope slide, and fluorescent images were visualized under Cytation 5 imaging reader (BioTeK, Winooski, VT) with appropriate filters.

GALC Enzyme Kinetics

To determine the mode of interaction either inhibition (competitive, mixed, or noncompetitive) or activation of GALC, Michaelis-Menten curves were generated. Human GALC-V5 enzyme and 4-Methylumbelliferyl β -D-galactopyranoside (4MU β Gal) substrate at multiple concentrations ranging from 0.04 to 0.5 mM and NCGC00126350-01, NCGC00138454-01 and NCGC00102820-01 at 12.5×10^{-3} and 25×10^{-3} mM concentrations were used for the kinetics assessment of GALC. Assays were performed in borosilicate glass tubes and aliquots of 200 μ L were transferred to one well of 96-well black-solid bottom plates in triplicate. Reactions were performed under citrate (0.1 M)/sodium phosphate (0.2 M) buffer at pH 4.6. Enzymatic reactions were stopped using 2-amino-2-methyl-1-propanol (MAP; 0.1 M) at pH 10.5. Enzyme reactions were initiated by addition of the diluted specific concentrations of 4MU β Gal substrate solutions to tubes containing the recombinant GALC and specific concentrations of the small molecule or DMSO (solvent only). Tubes were incubated in circulated water bath at 52 °C for 120 minutes before dispensing 800 μ L of the stopping buffer MAP (0.1 mM) and soon after 4-MU fluorescence was measured by BioTek Cytation 5 at λ excitation 365 nm and λ emission 450 nm. The GALC kinetics assays were performed in pH substrate buffers of 4.6 (acidic). This assays was based on the kinetics earlier described from the HMU specific GALC substrate (Martino et al. 2009). Using Prism 6.0, K_m , α and V_{max} values were extracted from the Michaelis-Menten curves (hydrolysis rate in pmol of 4MU β Gal/min/unit of GALC) following nonlinear regression. Similarly, K_i values were obtained by nonlinear regression analysis of these data fitted to either a competitive, mixed, or noncompetitive model of inhibition. The data were found to fit a specific inhibitor model based the alpha scores and other kinetic parameters.

The synthetic fluorogenic 4MU β Gal substrate was also used to examine purified GALC activity at neutral conditions, pH 7.2.

Heat-stability GALC Assay

The effect of small molecule ‘hits’ on the stability of the GALC was examined by heat-stability experiments. Purified human GALC in CP buffer (pH 4.6) was exposed to 52°C and fractions were removed at fixed intervals of 0, 5, 10, 20, 30, 45 and 75 min at 52 °C and put on ice. Aliquots from each fractions (n=3) were then assayed as above described for GALC kinetics assessments.

Data Analysis

Where applicable, data are expressed as the mean $\pm$ standard deviation (S.D.). Comparisons of parametric data were analyzed in the use of conventional parametric statistical methods as two-tailed Student's *t* test. The statistical test *Z'* factor was used to measure the quality of the assay and its applicability to high throughput screening. Assays with *Z'* factor of 0.5 indicate that the assay is robust enough to identify enhancement of HMU fluorescent signal indicating the GALC enzymatic activity. Coefficient of variance (CV) and signal-to-noise ratios were also calculated when comparing signals from GALC activity from wild type (WT) and mutant (MUT) cell lines. Data from the qHTS was analyzed using Curve Response Class (CRC) classification from dose response HTS, in which normalized data is fitted to a 4-parameter dose response curves using a custom grid-based algorithm to generate curve response class (CRC) score for each compound dose response (Inglese et al. 2006). CRC values of 1.1, 1.2, 2.1, 2.2 are considered highest quality hits; CRC values of 1.3, 1.4, 2.3, 2.4 and 3 are inconclusive hits; and a CRC value of 4 are inactive compounds. Concentration curve analysis was performed utilizing non-linear regression curve fit using Prism version 6.07. Two-way analysis of variance (ANOVA) was applied for *P* calculation where appropriate, and *P*<0.05 was considered statistically significant.

RESULTS

Selective primary screening of NCATS diversity collection and NPC collection of approved drugs for GALC enhancers

Using the 145M-*Twi-hGALC*-G270D cultured in 1,536-well plates, cell-based HTS assay was performed against the NCATS diversity collection (44,000 small molecules) and NPC (2,816 small molecules) libraries – Fig. 1A. The NPC library was screened as this collection comprises of compounds that have been approved for use by the Food and Drug Administration (FDA) or related agencies in other countries. The NCATS Diversity library was chosen for the study because it consists in a medium-size library with chemically diverse small molecules mostly design for probe discovery. The method utilized is a cell-based HTS assay previously developed by our group (Ribbens et al. 2013). In this study, however, a neurologically relevant 145M-*Twi-hGALC*-G270D cell line was utilized in the primary screen. To generate concentration-response curves (CRCs), during the treatment period of 48hs different treatment concentrations of the two small molecule collection libraries were utilized (Fig. 1B). At the primary screen stage, concentrations of 0.23, 2.3, 22.7 and 113.7 μ M of the NCATS diversity collection and 0.92, 4.6, 22.7 and 113.7 μ M of NPC collection were used to treat live 145M-*Twi-hGALC*-G270D cells seeded in 1,536-well plates. The statistical parameters from each 1,536-well plates showed signal windows (SW) 5, coefficient of variance (CV) ranging from 10–15% and *Z'* factor 0.4 from the majority of ultra-dense 1,536-well microplates (Fig. 2 and Table S1). In total, 148 small molecules were identified as qHTS small molecule 'hits' by the CRC classification analysis (Inglese et al. 2006) (Fig. 3 and Table 1). The pre-plate reading of HMU fluorescence before dispensing the HMU substrate into 1,536-well plates identified several auto-fluorescent compounds (Fig. 1B and Fig. 2). A significant number of false-positives due to compound auto-fluorescence (n=171) were filtered by performing the pre-plate HMU fluorescence

reading at the primary qHTS level (Fig. 2 and Table 1). The 148 small molecules were processed for follow-up validation and characterization.

CRC-based prioritization of the small molecule candidates from the primary screen

Before further mechanistic validation, the 148 small molecule 'hits' were assessed using the similar cell-based GALC activity assay in 384-wells plates as detailed in *Materials and Methods* section. To control the cell-based GALC 384-well plate assay, extra 28 small molecules were included in the validation ('cherry-pick') sub-collection along with 148 small molecules. In 384-well plates, 145M- *Tw1-hGALC*-G270D cells were treated over 5 days at 0.8, 4, 20 and 100 μ M concentrations of each selected small molecule 'hits' (Fig. 4). Based on their CRC profile, 10 small molecules were prioritized for follow-up validation in primary skin fibroblasts cell lines obtained from GLD patients (Fig. 5). Patient skin fibroblasts carrying misfolding GALC mutants GALC-G553R, GALC-E130K and GALC-N295T (Deane et al. 2011) were used to validate the candidate small molecules as described in *Materials and Methods* section. Among top 10 candidates, two molecules were filtered out because they failed to enhance GALC enzymatic activity (Fig. 5). Out of 8 selected small molecules, three molecules, NCGC00126350-01 (CID 16023334), NCGC00102820-01 (CID 5297522) and NCGC00138454-01 (CID 3139704) generated significant increases mutant GALC enzymatic activity in one GLD patient fibroblast cell line carrying the mutants GALC-E130K and GALC-N295T (Figs. 5A and 5B). In the GLD patient fibroblast carrying the GALC-G553R mutant in homozygosity, only NCGC00102820-01 showed enhancements of its residual enzyme activity but without statistical significance (figs. 5C and 5D).

GALC small molecule enhancers showed no disturbances of kinetics profile at acidic conditions

In order to examine the GALC enzyme kinetics in the presence of the small molecule candidates, human GALC was purified from BHK-21 as detailed in *Material and Methods* section. Since the HMU substrate demonstrated considerable variability due to a prolonged hydrolysis of the HMU synthetic substrate (Wiederschain et al. 1992a; Wiederschain et al. 1992b), the 4MU β Gal, another fluorogenic synthetic substrate, was utilized for the kinetics examinations of GALC (Fig. S8). Previous studies assessing GALC kinetics with 4MU β Gal have been reported (Martino et al. 2009). Therefore, using the 4MU β Gal substrate, we determined the steady-state kinetics and initial kinetic parameters of the purified human GALC for this specific studies (Fig. S8). Under traditional acidic conditions of a lysosomal enzyme as GALC (pH 4.6), small molecules NCGC00126350-01, NCGC00138454-01 and NCGC00102820-01 showed no alterations in the hGALC kinetics profile (Fig. 6). Under, neutral conditions (pH 7.2), as expected for most lysosomal enzyme, the hGALC catalytic activity is significantly reduced (Fig. 7), though still measurable. At pH 7.2, the low level of GALC activity did not allow proper and reliable kinetic assessments, especially at lower substrate concentrations. Interestingly, when GALC activity was examined with 1 μ M of each of these three small molecule candidates, significant recovery of enzymatic activity was observed (Fig. 7). Interestingly, NCGC00126350-01 and NCGC00102820-01 showed substantial increase that was similar to the GALC catalytic performance at pH 4.6 (Fig. 7).

Small molecule hits increase the stability of GALC

In addition to cellular and kinetics assays, GALC enzymatic stability was examined in the presence of the three small molecules candidates in heat-denaturation conditions (Fig. 8). The purified GALC was exposed to three small molecules candidates at 0.75 and 1.5 μM concentrations in heat-denaturation conditions. The purified GALC underwent a series of incubation periods of 0, 5, 10, 30, 45, 60 and 75 minutes at 37°C, the three small molecules showed to significantly maintain the GALC enzymatic activity (Fig. 8). The NCGC00126350-01, NCGC00102820-01 and NCGC00138454-01 interacts with GALC significantly increasing its stability as shown by maintaining GALC enzymatic activity longer than GALC incubated only with solvent ($p < 0.001$) (Fig. 7). The GALC increased stability predicts the potential folding-assisting role of these small molecules and ultimately evading the ERAD and degradation.

Small molecule candidates decrease the levels of GALC-V5 into the ER

Certain missense mutations in *GALC* gene results in misfolded mutant GALC proteins resulting in increased retention in the endoplasmic reticulum (ER). After failing several cycles of folding in the ER, misfolded GALC may be ultimately directed to the ER-associated degradation (ERAD) pathway. To examine how the identified small molecule candidates may increase the folding and outside trafficking of mutant misfolded GALC, the 145M-*TwihGALC*-G270D-V5 cells were treated over 3 days under 0.5 and 1 μM concentrations of NCGC00126350-01, NCGC00102820-01 and NCGC00138454-01 molecules (Fig. 9). In comparison to solvent (DMSO)-treated cells, the NCGC00126350-01, NCGC00102820-01 and NCGC00138454-01 molecules rescue mutant GALC-V5 enzyme from ERAD and assisting their enzyme trafficking to lysosomal compartment.

The small molecule properties of the three small molecules are shown in table 2. Two of them, NCGC00102820-01 (CID 5297522) and NCGC00138454-01 (CID 3139704) showed a molecular weight <500 g/mol with a reasonable XlogP_3 indicating a potential blood-brain barrier penetration.

DISCUSSION

The present study is focused on the identification and characterization of small molecules capable of enhancing the residual GALC enzymatic activity found in patients affected with GLD, known as Krabbe disease. In earlier studies, a robust cell-based HTS assay in 1,536-well microplates was developed by our group to screen small molecule libraries for mutant GALC enhancers (Ribbens et al. 2013). Here we report the utilization of the quantitative cell-based HTS assay against two small molecule libraries: an NCATS diversity collection (44,000) and the NPC (2,816) collection of approved drugs. The HTS assay statistical parameters revealed significant robustness and reproducibility across the over 120 microplates with wide signal-assay window, CVs ranging on 5–15% and consistent Z' factors for each of the over 120 ultra-dense plates tested (Fig. 2 and Table S1).

In GLD, the majority of the misfolded mutant GALC are early degraded through ERAD (Deane et al. 2011). Therefore, the low levels of mutant GALC, that eventually fold

properly, reach the lysosomal compartment resulting in residual GALC enzymatic activity, though still below a 10–20% critical threshold (Berardi et al. 2014; Tropak and Mahuran 2007). Below the GALC enzymatic critical threshold GLD neurological manifestations are observed (Conzelmann and Sandhoff 1983; Ribbens et al. 2013). In this context, enhancements of the mutant GALC residual activity close to the critical threshold can prevent the psychosine elevation, progressive oligodendrocytes and myelin loss, and subsequent leukodystrophy and astrocytic gliosis and infiltration of the unique “globoid cells” - pathologic hallmarks labeling the name of “globoid-cell leukodystrophy” for this LSD (Suzuki and Suzuki 1970).

Based on the previous cell-based qHTS assay (Ribbens et al. 2014), one modification was performed by using a different cell line. The 145M-*Tw*i-GALC-G270D cell line was generated by transfecting with human *hGALC-G270D* gene using a lentivirus. In the HTS setting, the generated 145M-*Tw*i-*hGALC-G270D* line was more stable and reliable than the previous patient-derived SV-40 transformed G270D/G270D line, which was reflected in the plate statistics assessments (Fig. 1 and Table S1) (Ribbens et al. 2013). In addition, the 145M-*Tw*i-*hGALC-G270D* is a more neurologically relevant cell line as it was derived from brain cortices of neonatal *Tw*i-*hGALC-G270D* mice (Ribbens et al. 2014), which may allow the identification of small molecules that are more suitable to tackle the neurodegenerative molecular processes involved in the pathogenesis of GLD.

Performing a quantitative HTS allowed the selection and prioritization of small molecule candidates based on their concentration-response curves (CRC). The adoption of a fluorescent pre-reading step of each 1,536-well microplate before adding the HMU GALC substrate (Fig. 1B and Fig. 2) excluded a substantial number false positives (n=171; Table 1). Large numbers of false positives are commonly observed in cell-based HTS assay (Inglese et al. 2007). One of the fundamental reasons for the high false-positive rate is auto-fluorescence originated from several and diverse small molecules, especially in the setting of a highly sensitive fluorescence-based HTS assay (Thorne et al. 2010). Through this process, the ‘hit’ rate of 0.32% (n=148) at the primary screen was observed, which is a considered a low rate given the large collection of compounds screened (n=46,816) (Inglese et al. 2007). Apart from performing a fluorescence reading pre-dispensing the HMU substrate (Fig. 2), the cell-based nature of the assay may further reduce the number of false positives. Despite being a biochemical assay, the HTS assay is preceded by the treatment of live-cells with small molecules from two selected libraries at multiple concentrations (Fig. 1). Therefore, since the qHTS for GALC is focus on positive CRCs that indicate increases in the residual *hGALC-G270D* activity, small molecules producing negative CRCs are very likely cytotoxic and will be filtered out already in the primary stage. In addition, those other molecules with poor cell permeability, and eventually unable to achieve adequate levels in the ER compartment – where the GALC folding process occurs - will be also filtered out. All these factors further increase the stringency of the qHTS reducing substantially the number of small molecule ‘hits’ identified in the primary screen.

Eventually, some small molecules from the two screened libraries may be missed. These are small molecules that eventually have a biological effect in GALC-G270D folding outside the range of concentrations tested (0.94 – 113.7 μ M). In addition, some small molecules may

require further time to assist the GALC-G270D folding. The 48hs treatment period may not be sufficient to observe the resultant enhancement of the residual GALC-G270D activity. However, in early experiments developing this cell-based GALC assay in high-dense multi-well plates as the 1,536-wells (Ribbens et al. 2013), after 72hs of culturing the cells with 2 μ L of media/well, substantial variability of the assay was observed very likely due to evaporation and subsequent cell death.

Primary fibroblast from GLD patients with late onset forms were used for the validation of the small molecule as these cells are clinically relevant to disease-associated mutations in GLD (Fig. 5). Two GLD patient fibroblast lines were selected as they carry *GALC* mutations that encode mutants predicted to affect substantially the folding and structure stability (Deane et al. 2011). One of the GLD patient fibroblast cell lines carries homozygous *GALC*G553R mutation showing very low but measurable residual enzymatic activity (Tappino et al. 2010). The mutant GALC-G553R is predicted to present severe misfolding by interactions of large and basic side chains (Deane et al. 2011). Whereas, another culture primary fibroblast cell line from a GLD patient carries compound heterozygous mutation in *GALC* gene E130K/N295T. In the heterozygosity state, it is difficult to assume whether mutant GALC-E130K and/or GALC-N295T are responsible for the residual activity detected in this cells line. The mutant GALC-E130K and GALC-N295T showed significant degree of misfolding on predictions based on the murine GALC crystal structure (Deane et al. 2011). Out of 10 selected small molecules, three showed that to increase significantly the residual GALC enzymatic activity in the GLD patient fibroblasts with mutant GALC-E130K and GALC-N295T (Fig. 4). The GALC-E130K (also known as E-114K) was identified in GLD patients with juvenile form in the Sicilian region of south Italy along with a common founder mutation (GALC-G41S) (Lissens et al. 2007). Whereas, the GALC-N295T (also known as N279T) was identified in the Irish GLD patients (Deane et al. 2011). Further studies will be required to determine whether the enhancement of the residual GALC activity is due to both or one of these two different mutants in this GLD patient fibroblasts. Whereas, the GLD patient fibroblast line carrying the mutant GALC-G553R fail to show statistically significant responses when treated with the 10 small molecule candidates at same concentrations (Fig. 4). These results indicate that some compounds may function as folding-assisting molecules only to specific subset or even a specific mutant GALC enzyme. Nevertheless, in general, the *GALC* missense mutations that affect protein folding result in a residual GALC activity, which is encountered in the majority of patients presenting the late onset forms of GLD. The hGALC-G270D, a common pathogenic allele in the late onset forms, was chosen as the surrogate mutant misfolded GALC mutant for primary screen to identify small molecules that can assist GALC protein folding.

Pharmacological chaperones (PC) bind to target proteins to stabilize the conformation of a mutant protein, thereby allowing it to escape degradation targeted by the ER-associated degradation (ERAD) pathway (Leidenheimer and Ryder 2014). Once folded properly with the assistance of specific PC, the mutant lysosomal enzyme evades the ERAD and interacts with ER-export pathways elements achieving the lysosomal compartment (Ong and Kelly 2011). The majority of PCs identified bind to the catalytic sites of their target enzymes as competitive inhibitors. Due to the requirement of long-reaction time, the HMU substrate, a

very sensitive and specific GALC substrate used in the biochemical diagnosis and newborn screen for GLD, failed several attempts to examine the kinetics of the purified human GALC. For these reasons, the 4MUBGal substrate, a sensitive fluorogenic synthetic substrate, was utilized allowing a short assessment and reliable and consistent results. First, the steady-state and linearity of the GALC kinetics using the 4MUBGal substrate was characterized (Fig. 8S). Later, through enzyme kinetics assessments using the human purified GALC, we examined the GALC kinetics profiles in the presence of each of the three small molecules (Fig. 6). At optimal acidic GALC assay conditions (pH 4.6), NCGC00126350-01, NCGC00102820-01 and NCGC00138454-01 fail to affect the GALC kinetics (Fig. 6). Whereas, in neutral conditions (pH 7.2), the GALC catalytic activity was dramatically reduced as expected for a lysosomal hydrolase (Fig. 7). Given that the neutral pH conditions are not the physiological environment of GALC, no reliable kinetics can be generated with considerable variation of enzymatic activity (data not shown). However, at the maximum substrate concentration (0.5 mM of 4MUBGal) under neutral conditions, GALC activity was measurable (Fig. 7). Interestingly, at pH 7.2, the three small molecule hits showed to rescue the reduced GALC enzymatic activity (Fig. 7), indicating potential interactions resulting in increased stability of GALC at neutral conditions. Therefore, these three small molecules have a highly desirable profile as specific folding-assisting molecules at the neutral pH (7.2) conditions as those encountered in the ER compartment. In acidic condition, typically noted in the lysosomal compartment, these molecules fail to perturb the kinetic profile of human purified GALC (Fig. 6). For these reasons, the small molecules ‘hits’ may very likely interact at allosteric sites located away from the GALC active-site, which consequently remains available for interactions with the natural substrates including galactosylceramide, and especially psychosine.

Under heat-stability assays with purified human GALC, the three small molecule candidates showed to significantly stabilize the GALC enzyme (Fig. 8). The structure of GALC suggests that nearly 70% of the missense mutations are involved in the substitutions of residues that lead to instability or misfolding of GALC (Deane et al. 2011). Therefore, the three small molecule ‘hits’ here identified may interact with misfolding mutant GALC enzymes, increasing their stability, assisting folding and preventing their degradation. In addition, in 145M-*Tw1-hGALC*-G270D-V5 cells, under treatment with small molecule ‘hits’, increased localization of mutant GALC-G270D in the lysosomal compartment and reduction in the ER (Fig. 9). However, it is important to point out that the mutant GALC-G270D was tagged with V5 to its N-terminal, which may also affect the interactions of small molecules to GALC-G270D.

Physical properties such as molecular weight, lipophilicity (XLogP3) and the number of hydrogen bond donors and acceptors in any compound are considered, when predicting the bioavailability of the compound. The three small molecule candidates show physical properties predicting good absorption or permeation given the molecular weight 500, calculated XLogP3 5, hydrogen bond donors 5 and hydrogen bond acceptors 10 (Lipinski et al. 2001). The structure of small molecules is depicted in figure S8.

Further studies, testing different mutant GALCs along with examination of small molecule analogs will be needed to optimize the small molecules here identified. In addition, whether

the enhancements of mutant GALC enzymatic activity generated by the identified small molecules will reduce the elevated and consequently cytotoxic levels of psychosine is unknown. The establishment of neural-stem cells (NSCs) lines from primary GLD patient fibroblasts carrying the specific mutant GALCs, as the GALC-E130K and GALC-N295T are ongoing and will allow studies on the effects of these and other molecules in GLD patient specific neural cells. At this point, the misfolding GALC-E130K and GALC-N295T showed enhancements on the residual GALC activity. Further GALC mutants will need to be tested to the three small molecules here identified. Eventually, the generation of analogs will be able to expand the repertoire of GALC misfolded mutants rescued by the molecules.

In summary, we report a cell-based HTS assay against approximately 46,000 compounds resulting in the identification and further characterization of small molecules capable of enhancing the residual GALC enzymatic activity by increasing mutant GALC stability, ultimately assisting the folding and consequently escaping the ERAD. These three small molecules showed physical properties that may allow BBB penetration. Eventually these molecules or their future analogs are potential therapeutic agents for the treatment of neurological manifestations of GLD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

Globoid-cell leukodystrophy (GLD), or Krabbe disease, is a neurodegenerative, lysosomal storage disease caused by the deficiency of β -galactocerebrosidase (GALC) activity. This study describes the identification and characterization of small molecule enhancers of residual enzymatic activity for mutant GALC. Using a quantitative cell-based high-throughput screening (qHTS) assay, we screened two small molecule libraries comprising a total collection of 46,816 drug-like compounds and identified three small molecules capable of enhancing the residual activity of GALC in primary fibroblasts from GLD patients. These molecules showed to rescue GALC activity at neutral pH and thermo-denaturing conditions without disturbing its kinetic performance at acidic conditions. In addition, the small molecule candidates also increase trafficking of mutant GALC to lysosomal compartment. These small molecules or their analogs are potential therapeutic agents for GLD.

Figure 1. The 1,536-well plate design and timeline of the primary screen events
(A) The primary screen was performed in 1,536-well plates as described above. The plate location of different cell-lines is depicted in the figure of one 1,536-well plate. **(B)** The timeline of events of the live cell-based HTS assay. Since the HTS assay was quantitative, four concentrations of the NCATS Diversity collection and NPC collection of approved drugs, comprising total of 46,816 small molecules were used to treat cells.

Figure 2. Scatter plot of the HMU fluorescent readings of 1,536-well plates from primary screen Selected scatter plot in plate-assay format are shown here. Each scatter plot corresponds to one 1,536-well plate from the primary screen which contains the 145M-*Twi-hGALC-G270D* (higher HMU fluorescence signals) and 145M-*Twi-hGALC-WT* (medium-low HMU fluorescence signal) cells and wells containing only media, labeled as background. Each plate underwent two HMU fluorescent readings: pre-substrate dispensing (after 48hs treatment) and post-stopping buffer dispensing (final reading). The two readings allowed to identify and filter out the auto-fluorescent compounds. Selected plates are shown here, in which the pre-substrate dispensing reading allowed the identification of auto-fluorescent compounds. The values of the subtraction of the post-stopping buffer dispensing signals from pre-substrate dispensing signals are shown in a scatter assay-plate format – third column. The Z' score of the HMU reading performed post-substrate (final). Further statistical parameters are shown in Table S1. The NCATS Diversity Library screening plates are here represented. The remaining scatter plot plates from primary screen are shown in Supplemental Data (Figs S1–7).

Figure 3. Concentration-response curves (CRCs) of small molecule candidates from the primary HTS against NCATS Diversity Compound Collection

In total, 148 hits out of 46,816 compounds were selected by CRC analysis. The quantitative cell-based qHTS was performed at four concentrations: 0.2, 2.3, 22.7 and 113.7 μM (NCATS diversity collection). Each small molecule 'hit' is listed and labeled as NCATS Chemical Genomics Center (NCGC)'s compound ID under is labeled in each panel (A–H) and subjected to the confirmation process. Black arrows are indicating ten selected small molecule 'hits' which were ultimately selected for further validation assays in primary GLD patient cell lines.

Figure 4. Confirmation and identification of the selected small molecules in the HTS assay
 The small molecule candidates from the primary screening (n=148) were re-examined by the qHTS assay in 384-well plate format at 0.8, 4, 20 and 100 μ M. Concentration-response curves (CRCs) of 176 small molecules, being 148 from the primary screening of 46,816 small molecules and 28 (de-identified controls) are plotted in different panels (A–H). In each panel, mean value of 145M-*Twi-hGALC-G270D* treated with DMSO and its $\pm 2 \times$ Standard Deviation (2SD) were shown as red-dotted lines. Black arrows are indicating ten of the selected small molecule candidates with the highest ranked positive CRC.

Figure 5. Validation of the selected hits in GLD primary skin fibroblasts

The selected 10 of the small molecule ‘hits’ with the highest ranked positive CRC classes were examined in two GLD primary skin fibroblasts: one compound heterozygous carrying the mutants GALC-E130K/GALC-N295T and another homozygous carrying the mutant GALC-G553R/GALC-G553R. Each small molecule was tested at 12.5, 25 and 50 μ M concentrations (triplicate/concentration) in GALC-E130K/GALC-N295T (A, B) and GALC-G553R (C, D) cell lines. Red-dotted lines show mean and $\pm$ 2SD of each primary GLD patient cell line treated only with the compound solvent (DMSO). Two selected hits were not shown in the panels due to their inhibitory effects on GALC. At the highest concentration (50 μ M), histograms were depicted with comparison with the respective DMSO treated fibroblasts panel (B) and (D). Black-dotted lines indicate fluorescent level of DMSO only treated fibroblasts. Three potential hits; NCGC001126350-01, NCGC00102820-01 and NCGC00138454-01 were selected through this validation process. Data represent mean $\pm$ standard deviations, $n=3$. * $p<0.01$, ** $p<0.001$.

Figure 6. Kinetics evaluation of three small molecule candidates with human GALC
Using purified human GALC and two different concentrations of each small molecule (0.5×10^{-3} and 1×10^{-3} mM), Michaelis-Menten curves were generated in the presence of NCGC00126350-01 (A), NCGC00138454-01 (B) and NCGC00102820-01 (C) in acidic conditions (pH 4.6). Kinetic parameters including α value, K_s and V_{max} are depicted in each panel.

Figure 7. Effects of small molecule candidates on GALC at neutral conditions

At pH 7.2, the GALC catalytic activity (blue bars) is significantly decreased in comparison to its activity at physiological acidic conditions (pH 4.6; red bars). However, in the presence of the three small molecule hits (at 1 μ M), NCGC00126350, NCGC00138454-01 and NCGC0010282 (magenta bars) at pH 7.2, significant recovery of GALC enzymatic activity was observed. Interestingly, no dramatic effect was noted when GALC was exposed to these small molecules at acidic conditions (pH 4.6; green bars).

Figure 8. Heat-stability assay to assess stability of GALC in the presence of small molecule candidates

The purified GALC was exposed to denature conditions in the presence of NCGC00126350-01 (A), NCGC00138454-01 (B) and NCGC00102820-01 (C) was assessed by thermal denaturation. *P* values were calculated by two-way ANOVA analysis. Data represent mean $\pm$ standard deviations, $n=4$.

Figure 9. Cell-based trafficking assay using 145M-Twi-GALC-G270D-V5

One of the small molecule candidates, NCGC00126350-01, was used at 1 μ M concentration to treat 145-Twi-GALC-V5 cell lines. (A and C) After 48hs of treatment, the increased localization of GALC-V5 was observed in lysosomal compartment – increased signal apposition from LAMP-2 (lysosomal-associated membrane protein-2 (green) and GALC-G270D (red). (B and D) In addition, decrease GALC-G270D levels were noted in ER compartment labeled with phosphodisulfate isomerase (PDI) – indicated by decreased apposition of signals of PDI (green) and GALC-G270D-V5 (red). A small panel with a higher resolution is focus on the colocalization signal associated with the dislocation of the GALC-G270D-V5 and either PDI or LAMP-2.

Table 1
Selection and prioritization of small molecule candidates from primary screen based on CRC classes.

CRC Class	Asymptotes	r ²	Efficacy	Number of Small Molecules	Candidates NCATS Diversity (44,000) and NPC (2,812) Collections
				Activity	Activity (excluding auto-fluorescent molecules)
1.1		0.9	>80%	0	0
1.2	2		Min - 80%	1	0
1.3		0.9	>80%	2	0
1.4			Min - 80%	14	13
2.1		0.9	>80%	13	0
2.2	1		Min - 80%	0	0
2.3		0.9	>80%	50	6
2.4			Min - 80%	36	9
3	1		> Min.	195	116
5	None		Min	7	5
Total				319	148

CRC, Classification of Concentration-Response Curves. *Original classification in Inglesse *J et al.* 2005. Min, minimal.

Table 2

Small molecule candidates that showed significant enhancement of residual GALC.

Compound NCGC ID	NCGC00126350-01	NCGC00138454-01	NCGC00102820-01
Compound ID	16023334	3139704	5297522
Molecular Weight	531.97 g/mol	347.43 g/mol	422.5 g/mol
Molecular Formula	C ₂₈ H ₂₃ ClFN ₅ O ₃	C ₂₁ H ₁₇ NO ₂ S	C ₂₂ H ₂₂ N ₄ O ₃ S
XLogP3	4.4	5.5	3.9
Hydrogen Bond Donor Count	3	0	2
Hydrogen Bond Acceptor Count	6	4	7
Rotatable Bond Count	6	3	5
Exact Mass	531.15 g/mol	347.1 g/mol	422.14 g/mol
Monoisotopic Mass	531.15 g/mol	347.1 g/mol	422.14 g/mol
Topological Polar Surface Area	95.1 Å ²	67.4 Å ²	126 Å ²
Heavy Atom Count	38	25	30
Formal Charge	0	0	0
Complexity	1000	526	663
Isotope Atom Count	0	0	0
Defined Atom Stereocenter Count	0	0	0
Undefined Atom Stereocenter Count	1	0	0
Defined Bond Stereocenter Count	0	0	0
Undefined Bond Stereocenter Count	0	0	0
Covalently-Bonded Unit Count	1	1	1

* Source, PubChem. <https://pubchem.ncbi.nlm.nih.gov>