haematologica
Journal of The Ferrata Storti Foundation

Functional assessment of glucocerebrosidase modulator efficacy in primary patient-derived macrophages is essential for drug development and patient stratification

by Natalie J. Welsh, Christina A. Gewinner, Kavita Mistry, Mumta Koglin, Juniebel Cooke, Matthew Butler, Ben Powney, Malcolm Roberts, James M. Staddon, and Anthony H.V. Schapira

Haematologica 2019 [Epub ahead of print]

Citation: Natalie J. Welsh, Christina A. Gewinner, Kavita Mistry, Mumta Koglin, Juniebel Cooke, Matthew Butler, Ben Powney, Malcolm Roberts, James M. Staddon, and Anthony H.V. Schapira. Functional assessment of glucocerebrosidase modulator efficacy in primary patient-derived macrophages is essential for drug development and patient stratification.
Haematologica. 2019; 104:xxx
doi:10.3324/haematol.2019.224717

Functional assessment of glucocerebrosidase modulator efficacy in primary patient-derived macrophages is essential for drug development and patient stratification.

Natalie J. Welsh[#,1,], Christina A. Gewinner[#,1,], Kavita Mistry[2], Mumta Koglin[2], Juniebel Cooke[3], Matthew Butler[1], Ben Powney[2], Malcolm Roberts[2], James M. Staddon[2] and Anthony H. V. Schapira[4,*].

[#]Authors contributed equally to this work. [1.] Eisai-UCL Drug Discovery and Development Collaboration, UCL Queen Square Institute of Neurology, School of Life Sciences and Medicine, University College London, UK. [2.] Neurology Innovation Centre, Hatfield Research Laboratories, Eisai Ltd, Hatfield, Hertfordshire, UK. [3.] Lysosomal Storage Disorders Unit, Royal Free Hospital, Pond Street, London, UK. [4.] Dept. of Clinical and Movement Neurosciences, UCL Queen Square Institute of Neurology, University College London, UK. * Corresponding author.

Running title: Glucocerebrosidase modulation in macrophages

Author contributions:

Natalie Welsh: produced figures 1 and 2, designed and prepared the manuscript, experimental design, discussed data
Christina Gewinner: performed experiments in figures 1 and 2 and experimental design
Kavita Mistry: performed experiments in figure 1A-C, discussed data
Mumta Koglin: performed experiment in figure 1C
Juniebel Cooke: clinician responsible for patient sample collection
Matt Butler: performed experiment figure 1D
Ben Powney: experimental design, discussed data
Malcom Roberts: experimental design, discussed data
James Staddon: experimental design, discussed data
Anthony Schapira: drafting and critical review of manuscript

Disclosures:

N. J. W., C. A. G., and M. B., were funded by Eisai Ltd.
K. M., M. K., B. P., M. R. And J. M. S. are employees of Eisai Ltd.
This work was possible due to funding from the industrial funder Eisai Ltd.

Letter to the Editor:

Gaucher disease (GD) is a lysosomal storage disorder caused by mutations in the glucocerebrosidase 1 (*GBA1)* gene encoding the lysosomal enzyme glucocerebrosidase (GCase). Type 1 patients present with accumulation of glucosylceramide in macrophages leading to a range of systemic manifestations, whilst type 2 and type 3 patients also exhibit

central nervous system (CNS) involvement (1, 2). Importantly, mutations in *GBA1* are also the main risk factor for the neurodegenerative disorders Parkinson disease (PD) and dementia with Lewy bodies (DLB) (3). Mechanisms linking *GBA1* mutations to neurodegeneration are not elucidated but hypothesized to involve the CNS resident macrophage population of microglia (4). Although enzyme replacement therapy has been successful in treating systemic features of lysosomal storage, due to its lack of brain penetrance there are currently no treatments available for the neuronopathic features of type 2 and type 3 GD, although substrate reduction therapy is under evaluation (5, 6). The difficulty in developing neuroprotective therapeutics for the treatment of these diseases is compounded by genetic diversity amongst patients, with over 300 disease-causing mutations in *GBA1 (7).* Moreover, the distribution of disease alleles varies between ethnic groups (8). Therapeutic promise may be found in brain-penetrant, small molecule chemical chaperone compounds that stabilize mutant, misfolded GCase in the endoplasmic reticulum, allowing efficient trafficking to lysosomes where GCase can function (9, 10). To date, many candidate compounds fall into the inhibitory class, where they bind and stabilize GCase to facilitate trafficking to the lysosome but they may concomitantly inhibit GCase enzymatic activity even at low lysosomal pH (11). How to evaluate the downstream functional consequences of these therapeutic compounds, what this means for patient treatments and how to stratify a genetically diverse population for therapeutic intervention are three key issues that remain to be solved.

Using an *in vitro* patient blood monocytic cell (PBMC)-derived macrophage model described by Aflaki et al. 2014 (12), we explored the effectiveness of inhibitory chaperones on lipid metabolism and compared the response to such compounds in a panel of genetically heterogeneous GD patient-derived material. We found that inhibitory chaperone compounds can have positive effects on lipid metabolism despite their mode of action but, crucially, this depends on how the treatment is applied. Moreover, we found that despite genetic diversity amongst patients tested, response to compounds can be similar across patients, suggesting that it may be possible to base inhibitory compound clinical trial stratification on *in vitro* phenotype rather than *GBA1* genotype. This would allow for clinical trials with greater power. The development of a pre-clinical *in vitro* biomarker to identify treatment responders is critical for advancing current pipeline therapeutics (6).

Inhibitory chaperone compound treatment in GD patient-derived fibroblasts has been shown to increase GCase activity via the stabilization of the mutant protein (13, 14). We show here that in total cell extracts from a patient-derived fibroblast line, even at high concentrations of the

inhibitory chaperone compounds ambroxol (15) and isofagomine (11),GCase protein levels were increased (Figure 1A-B) and GCase enzymatic activity was concurrently elevated (Figure 1C). However, analysis of total cell extract does not provide information about the activity of GCase in the lysosomal compartment *in situ*. It is thus not clear to what extent lysosomal localized GCase is affected by inhibitory compounds: whether large increases in GCase protein correctly targeted to the lysosome are enough to overcome any residual inhibitory effect of compound binding at low pH.

To address this issue, we used an *in vitro* functional model of GD that was developed to evaluate the effects of this class of compound on the downstream functional consequence of GCase enzyme activity modulation, namely substrate degradation (12). Loss of GCase enzyme function leads to intracellular accumulation of its lipid substrate glucosylceramide (GlcCer); cells of the monocyte-macrophage lineage are severely affected by the functional impairment of GCase leading to visible accumulation of glycolipids in the cell (1, 16). We used human PBMC-derived macrophages from GD patient blood (Figure 1D and E), pre-fed with unlabeled patient derived erythroblast ghosts (Figure E) to measure degradation of fluorescently-conjugated glucosylceramide (TopFluor-GlcCer)-labelled erythroblast ghosts, 24hrs after feeding (Figure F and G) (12). Aflaki and colleagues showed that in this model a reduction in TopFluor signal denotes increased degradation of TopFluor-GlcCer-labelled erythroblast ghosts, thus reflecting increased GCase activity in lysosomes (12). By adding 10μM isofagomine to PBMC-derived macrophages in a continuous manner throughout the total erythroblast ghost incubation period (Figure 1G) we found a robust two-fold increase in total cellular GCase activity compared to vehicle-treated control, but no concurrent downstream increase in erythroblast ghost degradation (Figure 1H) and thus no positive functional consequence of the observed increase in GCase activity. We surmised that this might be due to an overriding effect of lysosomal GCase inhibition, masking the effect of increased total levels of stabilized GCase protein to degrade its substrate. We therefore used the model to understand whether inhibitory chaperones can be delivered in such a way that the compound-driven lysosomal inhibition of GCase function can be overcome.

To address this question we compared the dose-response profiles of PBMC-derived macrophages cultured in the continued presence of compound, with PBMC-derived macrophages that were transferred to compound-free media for the final 24hrs in a discontinuous treatment protocol (Figure 1G). Our findings showed that reduction of TopFluor signal was far greater when compound was removed for the last 24hrs compared with continued

application of compound (Figure 2). This demonstrated that the capacity to degrade erythroblast ghosts was enhanced by chaperone compound treatment compared to vehicle control, but that removal of the compound was necessary to observe this experimentally. These results showed that by allowing a 24hr compound-free period, the functional impact of increased GCase protein was unmasked in the absence of lysosomal inhibition and would indeed indicate a therapeutic value of such compounds under the right treatment regimen. We therefore conclude that assessment of inhibitory compound efficacy on total GCase activity alone is insufficient to determine the therapeutic potential of such compounds. Additional testing of the downstream functional impact of such treatment is essential to understand the effect on lysosomal GCase function.

It is thought that inhibitory chaperones such as isofagomine require a washout period to be effective (11), which would lead to a complicated dosing regimen for patient treatment. We show that even for a mixed-type GCase inhibitor such as ambroxol, previously shown to have no inhibitory activity at low pH *in vitro* (15), when assessed in live cells there is a masking effect on lysosomal functional improvement under continued compound exposure at high concentrations. Molecular mechanisms that modulate the action of ambroxol in a cellular context may contribute to this finding. Importantly, we show that, although under washout conditions (24hr compound-free protocol), the response was greater as concentration increased, it should be noted that the lowest concentration of compound tested (5μM) gave an equivalent outcome independent of the treatment protocol used. However, it is also important to highlight that in some patients only a two-fold increase in compound concentration was needed before the inhibitory mechanism was demonstrated, indicating a small therapeutic window. This emphasizes the importance of assessing both treatment paradigms when evaluating inhibitory compounds *in vitro* to understand likely responses in individual patients. Together our data demonstrate an optimization point where continuous application at sub-inhibitory concentrations could still be therapeutically effective, avoiding the need to employ a washout dosing strategy. It also underscores the need to perform such *in vitro* biomarker testing to first understand how individual patients may respond to treatment.

Finally, current thinking cites the inappropriate patient stratification as a contributing factor to the failure of clinical trials for disease-modifying compounds used for the treatment of nervous system disorders (17-19). Having repeated the study in a number of GD patient cells harboring a panel of different *GBA1* mutation allele combinations, we saw that individual patients did demonstrate subtly different responses to the two treatment protocols. However, of key

significance is the fact that there was a general trend for the lower concentrations of compound to be effective in all patients under both treatment strategies, regardless of *GBA1* allele combination. This provides promising evidence (i) for the ability to identify groups of patients that are likely to respond well to treatment and (ii) that grouping patients together for clinical trials based on their *in vitro* phenotypic response to candidate compounds could be a valid method for effectively stratifying cohorts. Additional studies are required to confirm that this *in vitro* assay is representative of an *in vivo* response. However, we would like to highlight the implication of our findings to improving the design and outcome of clinical trials.

In sum, we describe a potential *in vitro* biomarker assay for the stratification of inhibitory chaperone compound clinical trials, highlight the importance of using a dual approach treatment regimen to gain mechanistic insight into the therapeutic effectiveness of inhibitory chaperones to identify likely responders and, importantly, show that phenotype-based patient stratification might be a plausible method to determine an inclusion or a stratification criterion to ensure that the right population of patients will benefit from well-designed clinical trials.

Bibliography:

1. Beutler E, Grabowski GA. Glucosylceramide lipidosis–Gaucher disease. The metabolic and molecular basis of inherited diseases. 2001;8th Edition(Scriver C. R., Beaudet A. L., Sly W. S and Valle D., eds):3635-3668.
2. Grabowski GA. Phenotype, diagnosis, and treatment of Gaucher's disease. Lancet. 2008;372(9645):1263-1271.
3. Migdalska-Richards A, Schapira AH. The relationship between glucocerebrosidase mutations and Parkinson disease. J Neurochem. 2016;139 Suppl 1:77-90.
4. Gegg ME, Schapira AHV. The role of glucocerebrosidase in Parkinson disease pathogenesis. FEBS J. 2018;285(19):3591-3603.
5. Bennett LL, Mohan D. Gaucher disease and its treatment options. Ann Pharmacother. 2013;47(9):1182-1193.
6. Shihabuddin LS, Brundin P, Greenamyre JT, Stephenson D, Sardi SP. New Frontiers in Parkinson's Disease: From Genetics to the Clinic. J Neurosci. 2018;38(44):9375-9382.
7. Hruska KS, LaMarca ME, Scott CR, Sidransky E. Gaucher disease: mutation and polymorphism spectrum in the glucocerebrosidase gene (GBA). Hum Mutat. 2008;29(5):567-583.
8. Sawkar AR, D'Haeze W, Kelly JW. Therapeutic strategies to ameliorate lysosomal storage disorders--a focus on Gaucher disease. Cell Mol Life Sci. 2006;63(10):1179-1192.
9. Schapira AH, Chiasserini D, Beccari T, Parnetti L. Glucocerebrosidase in Parkinson's disease: Insights into pathogenesis and prospects for treatment. Mov Disord. 2016;31(6):830-835.
10. McMahon B, Aflaki E, Sidransky E. Chaperoning glucocerebrosidase: a therapeutic strategy for both Gaucher disease and Parkinsonism. Neural Regen Res. 2016;11(11):1760-1761.
11. Steet RA, Chung S, Wustman B, et al. The iminosugar isofagomine increases the activity of N370S mutant acid beta-glucosidase in Gaucher fibroblasts by several mechanisms. Proc Natl Acad Sci U S A. 2006;103(37):13813-13818.
12. Aflaki E, Stubblefield BK, Maniwang E, et al. Macrophage models of Gaucher disease for evaluating disease pathogenesis and candidate drugs. Sci Transl Med. 2014;6(240):240ra273.
13. Sawkar AR, Cheng WC, Beutler E, et al. Chemical chaperones increase the cellular activity of N370S beta -glucosidase: a therapeutic strategy for Gaucher disease. Proc Natl Acad Sci U S A. 2002;99(24):15428-15433.
14. Bendikov-Bar I, Maor G, Filocamo M, Horowitz M. Ambroxol as a pharmacological chaperone for mutant glucocerebrosidase. Blood Cells Mol Dis. 2013;50(2):141-145.
15. Maegawa GH, Tropak MB, Buttner JD, et al. Identification and characterization of ambroxol as an enzyme enhancement agent for Gaucher disease. J Biol Chem. 2009;284(35):23502-23516.
16. Beutler E. Gaucher's disease. N Engl J Med. 1991;325(19):1354-1360.
17. Vellas B, Carrillo MC, Sampaio C, et al. Designing drug trials for Alzheimer's disease: what we have learned from the release of the phase III antibody trials: a report from the EU/US/CTAD Task Force. Alzheimers Dement. 2013;9(4):438-444.
18. Cook D, Brown D, Alexander R, et al. Lessons learned from the fate of AstraZeneca's drug pipeline: a five-dimensional framework. Nat Rev Drug Discov. 2014;13(6):419-431.
19. Pankevich DE, Altevogt BM, Dunlop J, Gage FH, Hyman SE. Improving and accelerating drug development for nervous system disorders. Neuron. 2014;84(3):546-553.
20. Stone DL, Tayebi N, Orvisky E, et al. Glucocerebrosidase gene mutations in patients with type 2 Gaucher disease. Hum Mutat. 2000;15(2):181-188.
21. von der Heul C, Kroos MJ, de Jeu-Jaspars CM, von Eijk HG. The uptake of iron by reticulocytes. The influence of purification of the ghosts on iron-containing components in the ghost suspension. Biochim Biophys Acta. 1980;601(3):572-583.

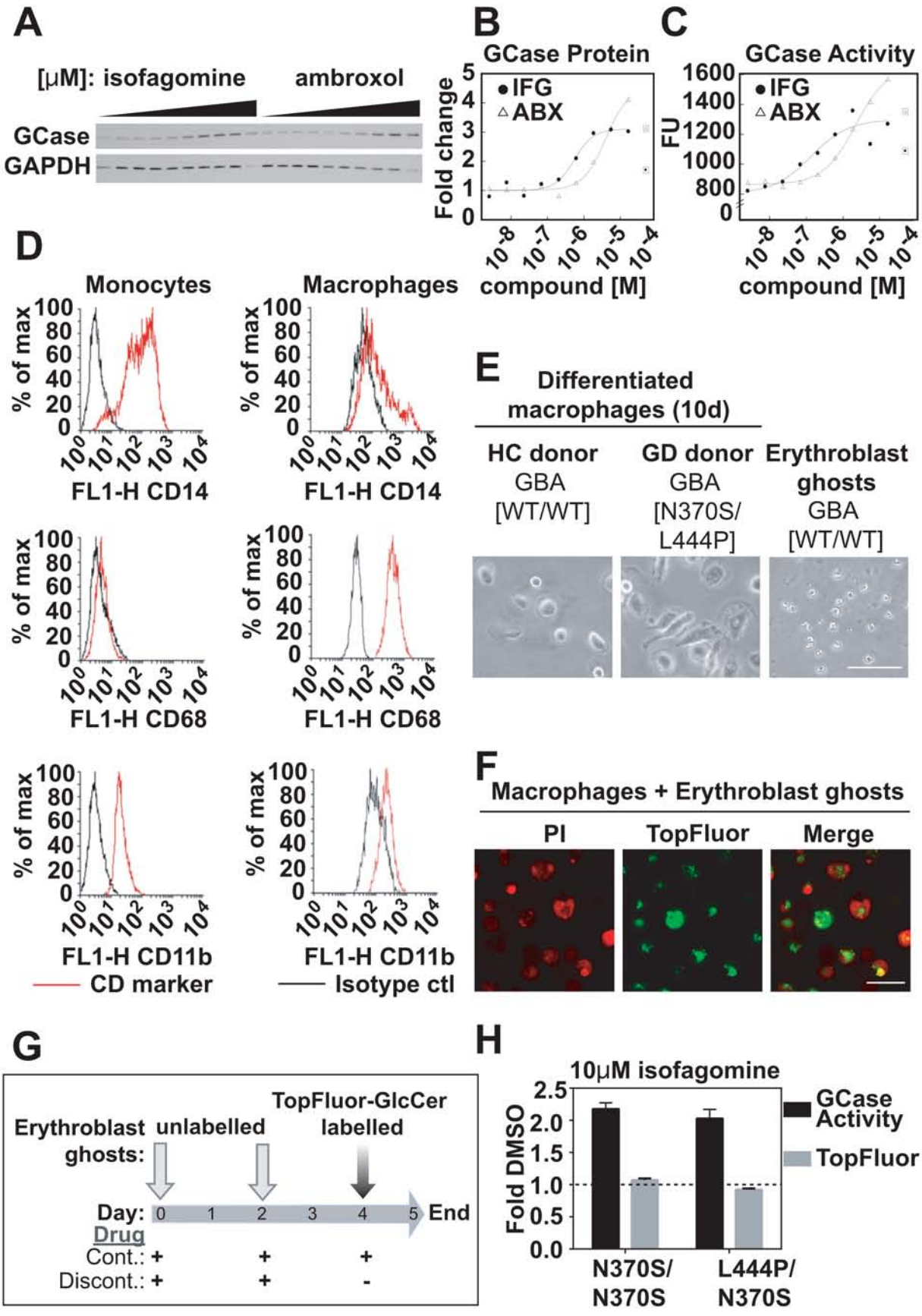
22. Gegg ME, Burke D, Heales SJ, et al. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. Ann Neurol. 2012;72(3):455-463.

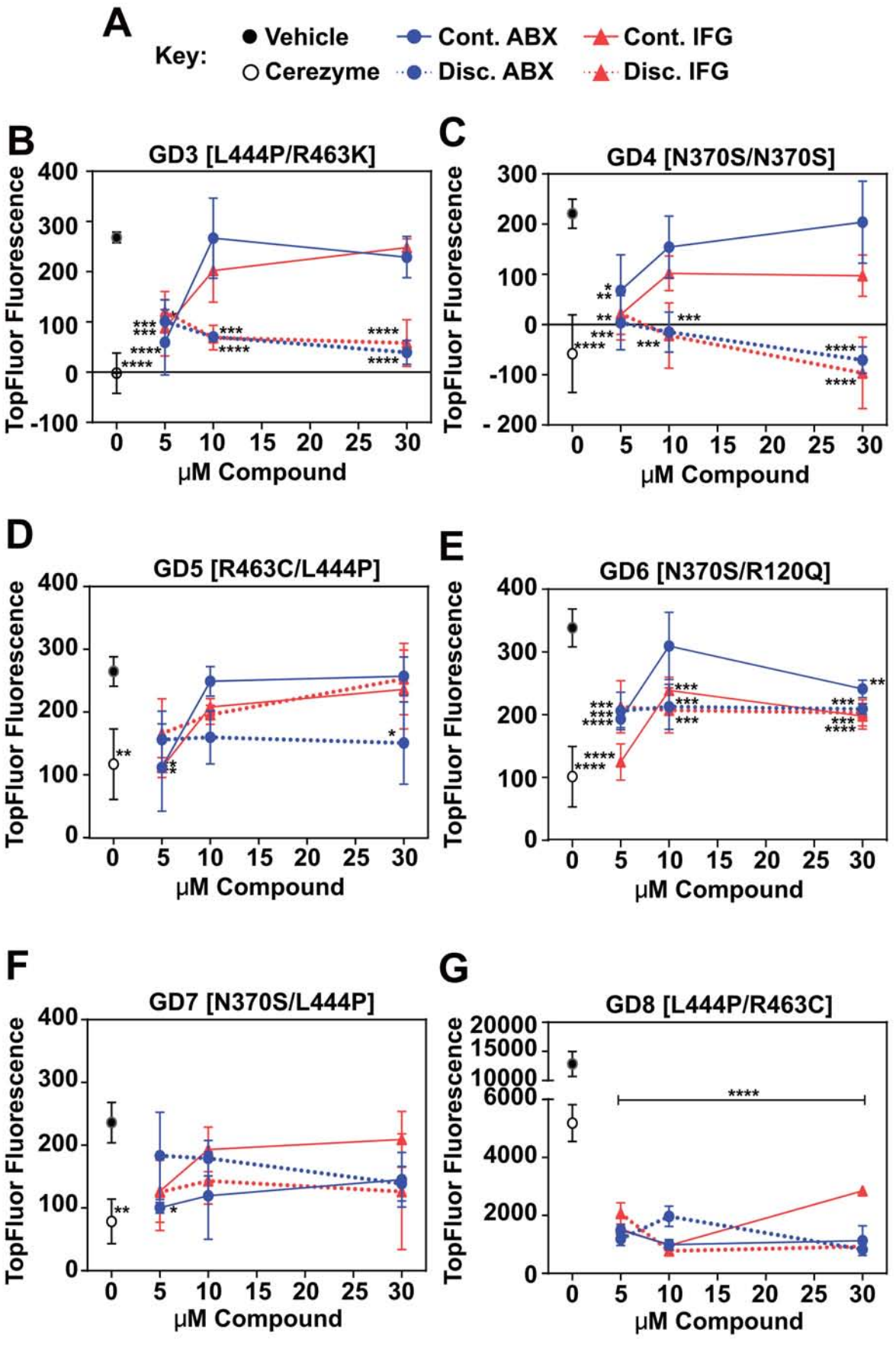
Figure legends:

Figure 1. PBMC-derived macrophage model to assess functional impact of GCase-specific inhibitory chaperone compounds. A-C) Fibroblasts from a GD patient [*GBA*1 N370S/del] were treated with increasing doses (0-50µM) of isofagomine or ambroxol for six days before harvesting. **A)** Western blot of GCase protein levels and GAPDH loading control from whole cell lysates. **B)** Dose-response curves of densitometrically quantified GCase protein levels. Boxed points are outliers removed due to observable toxicity. **C)** Dose-response curves of GCase activity using whole cell lysates from ambroxol- or isofagomine-treated GD fibroblasts. Boxed points are outliers removed due to observable toxicity **D-G)** Patient-derived monocytes were isolated using a Percoll gradient and CD14+ magnetic beads then differentiated into PBMC-derived macrophages using GM-CSF. Erythroblast ghosts were generated by hypo-osmotic lysis. Unlabeled erythroblast ghosts were added to the macrophages for phagocytosis at assay set up (day 0) and at 48hrs (day 2), to saturate the intracellular glycolipid pool. Twenty-four hours before assay readout (day 4) erythroblast ghosts labelled with TopFluor-glucosylceramide (GlcCer) were added to the macrophages. Remaining TopFluor-GlcCer levels in PBMC-derived macrophages were read out at 485/528nm using a spectrophotometer (12). **D)** Fluorescence activated cell sorting analysis showing enrichment of CD68+ve population of differentiated macrophages compared with CD14/CD11b+ve monocytic precursors. **E)** Transmitted light micrographs showing representative PBMC-derived macrophages from normal donor control (left) and a GD patient (middles) and erythroblast ghosts (right). **F)** Confocal micrographs of propidium iodide (PI)-labelled fixed PBMC-derived macrophages (red) and TopFluor-labelled erythroblast ghosts (green) after 24 hours incubation with TopFluor-GlcCer-labelled erythroblast ghosts. **G)** Schematic representation of erythroblast ghost delivery and compound treatment protocols. On day four, compounds were either (i) replenished as part of a continuous protocol (Cont.), or (ii) removed for the 24hr period of TopFluor-GlcCer-labelled erythroblast ghost delivery in a discontinuous protocol (Discont.). **H)** Two different GD PBMC-derived macrophage samples were subjected to 10µM isofagomine in the Continuous protocol and GCase activity (black bars) and TopFluor-GlcCer (grey bars) measured and expressed as fold change DMSO vehicle control (dotted line at 1). Scale bar in E and F = 50µM.

Figure 2. Comparison of differential drug dosing protocols revealed positive effect of GCase inhibitory chaperone compounds on lysosomal GCase function. GD PBMC-derived macrophages were treated with 5, 10 or 30µM ambroxol (ABX, blue) or isofagomine (IFG, red) or DMSO vehicle control (black filled circles) either continuously (Cont.; solid lines) for five days

or with discontinuation of treatment (Discont.; dashed lines) 24hrs before readout of TopFluor fluorescence (excitation at 485 and emission at 528nm). Continuous treatment with 0.4U Cerezyme (open circles) for five days was used as a control. **A)** Key to compound treatment conditions (see Fig. 1G for schematic representation of dosing schedules). **B-G).** Quantification of TopFluor fluorescence in PBMC-derived macrophages derived from six different GD patients that harbored different combinations of *GBA1* mutant alleles as shown. All samples were assayed in triplicate. Graphs show mean and standard deviation. Data were analyzed by One-way ANOVA followed by Dunnett's multiple comparisons test. P-values are *=p≤0.05; **=p≤0.01; ***=p≤0.001, compared to DMSO control.


A
[μM]: isofagomine
ambroxol
GCase
GAPDH
B
GCase Protein
Fold change
IFG
ABX
compound [M]
C
GCase Activity
FU
IFG
ABX
compound [M]
D
Monocytes
Macrophages
% of max
FL1-H CD14
FL1-H CD68
FL1-H CD11b
CD marker
Isotype ctl
E
Differentiated macrophages (10d)
HC donor GBA [WT/WT]
GD donor GBA [N370S/ L444P]
Erythroblast ghosts GBA [WT/WT]
F
Macrophages + Erythroblast ghosts
PI
TopFluor
Merge
G
Erythroblast ghosts:
unlabelled
TopFluor-GlcCer labelled
Day: 0 1 2 3 4 5 End
Drug
Cont.: + + +
Discont.: + + -
H
10μM isofagomine
Fold DMSO
GCase Activity
TopFluor
N370S/ N370S
L444P/ N370S


A
Key:
Vehicle
Cerezyme
Cont. ABX
Disc. ABX
Cont. IFG
Disc. IFG
B
GD3 [L444P/R463K]
C
GD4 [N370S/N370S]
D
GD5 [R463C/L444P]
E
GD6 [N370S/R120Q]
F
GD7 [N370S/L444P]
G
GD8 [L444P/R463C]
TopFluor Fluorescence
µM Compound

Welsh et al. Supplementary Materials and Methods

Patient blood samples and genotyping

Patient-derived macrophages were generated from monocytes isolated from blood samples from a total of 10 patients with type 1 GD. Control macrophages were generated from blood obtained from healthy volunteers (Cambridge Biosciences). All samples were collected with informed consent under the 10_H0720_21 ethics approved clinical protocol. The genotypes of donors were obtained at patient admission by sequencing all exons of GBA, as described (20).

Isolation of patient-blood monocytes and macrophage differentiation

Peripheral blood mononuclear cells from controls and type 1 GD patients were isolated using Histopaque gradient (Sigma, 10771) and monocytes were purified using magnetic monocyte enrichment beads (CD14 magnetic beads, Milteny Biotech). Macrophages were differentiated from purified monocytes using GM-CSF (50ng/ml) (R&D Systems) in RPMI1640 medium, supplemented with 10% FCS (Invitrogen). Differentiation medium was refreshed every other day and macrophages were harvested on day ten.

TopFluor-GlcCer assay and compound treatment of patient-derived macrophages

Differentiated macrophages were harvested using PBS (MgCl and CaCl-free) (Thermo Fisher, 14190169) and seeded in black 96-well plates (Corning, CZ405) with a density of 20,000 macrophages per well. To enhance glucosylceramide (GlcCer) storage, patient-derived macrophages were fed with Gaucher erythroblast ghosts, prepared as described (21). A proportion of the ghosts were fluorescently labelled by incubation with *N*-[11-dipyrromethene borondifluorideundecanoyl]-D-glucosyl-β1-1′-D-erythro-sphingosine [C11 TopFluor glucosylceramide (glucosylceramide-Bodipy) (Avanti Polar Lipids)] for 30 min at 37°C. Treatment with unlabelled Gaucher erythroblast ghosts lasted for 5 days, changing medium every 2 days. Macrophages phagocytose 9 to 12 ghosts daily; hence the quantity of ghosts added was based on the number of macrophages present. On day 5, the TopFluor-GlcCer labelled Gaucher erythroblast ghosts were added for 24 hours. Cells were then washed with PBS and resuspended in OptiMEM (without phenol red). Fluorescence was measured at 485/528nm on Synergy HT spectrometer (BIO-TEK).

Chaperone treatment of fibroblasts

Treatment with ambroxol hydrochloride (ambroxol) (Sigma-Aldrich, A9797) or isofagomine D-tartrate (isofagomine) (Cayman Chemical, 16137) started when cells were 50% confluent in 10 cm plates. Macrophages were treated with vehicle (dimethyl sulfoxide) or respective chaperone on days 0, 2, and 4.

Western Blot

Cell lysates were prepared in lysis buffer (Citric acid 50mM, K_2HPO_4 176mM, Sodium Taurocholate 10mM, Tween 20 0.01%, and Triton X-100 1%). Samples were ran on 4-20% Bis-Tri midi gel (Invitrogen) in MOPs running buffer. Gels were blotted onto nitrocellulose membrane (Amersham 10600048) and probed with anti-GBA antibody (Sigma G4171) or anti GAPDH (Cell Signalling 2118) and anti-rabbit HRP conjugate (Jackson Immuno Research 711-0350152) or (GE healthcare NA934) respectively. Membranes were developed using ECL Western Blotting Substrate (Thermo 32106) on X-ray film (Fujifilm).

FACS analysis

Isolated monocytes and differentiated macrophages underwent immunophenotyping. Monocytes and macrophages were washed in PBS and stained with CD11b and CD14 (monocyte markers), as well as CD68 (macrophage marker) (all obtained from Milteny Biotech). Unstained cells were used as negative control. Staining was also performed using an isotype control for each marker (Milteny Biotech). Cells were analysed with FlowJo software (LLC) using a Becton Dickinson instrument.

GCase activity assay

GCase activity was measured in fibroblast or macrophage cell lysates in McIlvaine citrate-phosphate buffer (pH 5.4) with 10 mM sodium taurochlorate and 5 mM 4–methylumbelliferyl–β–D–glucopyranoside as substrate at 37 °C as previously described (22). The reaction was stopped with 0.25 M glycine (pH 10.4) and 4–methylumbelliferone fluorescence measured on a plate reader (excitation, 360 nm; emission 460 nm) Synergy HT spectrometer (BIO-TEK). Data were expressed as nmol 4–methylumbelliferone/h/mg protein.

Confocal microscopy

Patient-derived macrophages were plated on black-walled 96-well plates with optimetric bottom (Corning) and fed with erythroblast ghosts. Cells were resuspended in PBS and confocal images were acquired with a Nikon Eclipse Ti microscope using a 488nm argon and a ultraviolet laser. Images were acquired using a 40x extra-long working distance objective.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Significance was determined by Data from two groups or more were analysed by one-way ANOVA with Dunnett's multiple comparisons test. Data are presented as means +/- standard deviation. Significance levels between patient-macrophages and different conditions were set when $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).

1. Stone DL, Tayebi N, Orvisky E, et al. Glucocerebrosidase gene mutations in patients with type 2 Gaucher disease. Hum Mutat. 2000;15(2):181-188.

2. von der Heul C, Kroos MJ, de Jeu-Jaspars CM, von Eijk HG. The uptake of iron by reticulocytes. The influence of purification of the ghosts on iron-containing components in the ghost suspension. Biochim Biophys Acta. 1980;601(3):572-583.

3. Gegg ME, Burke D, Heales SJ, et al. Glucocerebrosidase deficiency in substantia nigra of parkinson disease brains. Ann Neurol. 2012;72(3):455-463.