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

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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 $P<0.05$ (*), $P<0.01$ (**), $P<0.001$ (***).

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