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CHEMICAL SCREEN TO REDUCE STEROL ACCUMULATION IN NIEMANN-PICK C DISEASE CELLS IDENTIFIES NOVEL LYSOSOMAL ACID LIPASE INHIBITORS

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SUMMARY

Niemann-Pick C disease (NPC) is a lysosomal storage disorder causing abnormal accumulation of unesterified free cholesterol in lysosomal storage organelles. High content phenotypic microscopy chemical screens in both human and hamster NPC-deficient cells have identified several compounds that partially revert the NPC phenotype. Cell biological and biochemical studies show that several of these molecules inhibit lysosomal acid lipase, the enzyme that hydrolyzes LDL-derived triacylglycerol and cholesteryl esters. The effects of reduced lysosomal acid lipase activity in lowering cholesterol accumulation in NPC mutant cells were verified by RNAi-mediated knockdown of lysosomal acid lipase in NPC1-deficient human fibroblasts. This work demonstrates the utility of phenotypic cellular screens as a means to identify molecular targets for altering a complex process such as intracellular cholesterol trafficking and metabolism.

Keywords

cholesterol accumulation; lysosomal storage organelles; lysosomal acid lipase; orlistat; NPC

INTRODUCTION

Niemann-Pick type C (NPC) disease is a genetically inherited autosomal recessive disorder that is associated with the abnormal accumulation of unesterified cholesterol and other lipids in late endosome/lysosome-like storage organelles (LSOs) [1–8]. Although the most serious clinical effects are related to neuronal degeneration in the central nervous system, other organs (e.g., liver, spleen and lungs) are also affected [1]. Most cases of NPC disease are linked to alterations in NPC1, a multi-spanning membrane protein found in late endosomes, lysosomes and the trans-Golgi network [9–12]. A small fraction of NPC cases (~5 %) present defects in NPC2, a soluble, cholesterol-binding protein targeted to the lumen of late endosomes/lysosomes (LE/LY) by a mannose-6-phosphate modification [13–15]. The precise roles of

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these two proteins are still under investigation [16,17]. NPC2 binds cholesterol in the lumen of the late endosomes and apparently plays a role in the transfer of cholesterol in a way that promotes cholesterol efflux from LE/LY. NPC1 protein also facilitates the efflux of cholesterol from LE/LY, but its exact role in this process is still not clear [18].

Esterified cholesterol is internalized via lipoprotein endocytosis and delivered to LE/LY, where hydrolysis of cholesteryl esters by lysosomal acid lipase (LAL) produces free cholesterol and fatty acids [19,20]. In normal cells, cholesterol is exported from the LE/LY and delivered to other organelles, including the plasma membrane, the endocytic recycling compartment and the endoplasmic reticulum [21]. Elevated levels of cholesterol in the ER lead to increased esterification by acyl Co-A:cholesterol acyl transferase (ACAT) [22]. Cholesterol esters formed by ACAT are stored as lipid droplets that are hydrolyzed by cytoplasmic neutral cholesterol ester hydrolase [23–26]. Cellular cholesterol can also be exported to extracellular acceptors, such as high density lipoproteins, in a process mediated by ATP-binding cassette transporters such as ABCA1 [27].

In NPC-deficient cells, LDL uptake, delivery to LE/LY, and cholesterol ester hydrolysis are normal. However, the rate of cholesterol efflux from the LE/LY is severely reduced, causing them to become LSOs [28–30]. Because of this defect in cholesterol trafficking, re-esterification of lipoprotein-derived cholesterol by ACAT is reduced [31,32]. In addition, cholesterol levels in the plasma membrane of NPC-deficient cells may be low, even though total cellular cholesterol is increased [28]. The amounts of some other lipids in the LSOs, including sphingomyelin, bis-(monoacylglycerol)-phosphate, glycosphingolipids and some phospholipids are also increased [4,33,34]. Together, these lipids form multi-layered internal whorls of membrane in the LSOs [35]. Accumulation of cholesterol in the LSOs can be visualized by staining the cells with filipin, a fluorescent polyene antibiotic [36,37]. We have used quantitative microscopy-based assays of filipin labeling in high throughput screens for compounds that reduce cholesterol accumulation in NPC1 mutant cells [38]. Under conditions similar to those we use for labeling and imaging, we found a good correlation between the intensity of filipin fluorescence in cells and the amount of unesterified cholesterol as determined by gas chromatography (GC) [39]. A similar correlation has been reported between filipin fluorescence and cholesterol levels determined by an enzymatic assay [40]. Because filipin binding to cholesterol and its fluorescence intensity can be affected by other environmental influences (*e.g.*, fluorescence quenchers), we use follow-up biochemical studies to assay cholesterol changes in cells.

No effective treatment is currently available for NPC patients [41], and proposed treatments [42–47] have yet to be shown clinically effective. Interestingly, time of clinical onset and severity of NPC symptoms varies from patient to patient. While most patients show significant abnormalities early in childhood, some patients with identical mutations have only mild symptoms that are not detected until adulthood [15,48]. This suggests that variations in genetic background and/or other factors might partially compensate for the loss of NPC1 function and allow prolonged survival. For example, it has been shown in NPC1-deficient cultured cells that transfection with Rab7, Rab8, Rab9 or Rab11 [49–54] can reduce accumulation of cholesterol in the LSOs. Additionally, introduction of a Rab9 transgene into NPC1-deficient murine models increased life span of these mice by up to 22 % [45]. Such findings indicate that various alterations in vesicular membrane traffic and lysosomal lipid composition could partially revert the NPC phenotype, although the exact mechanisms for the correction are uncertain.

In an effort to discover novel small molecule compounds that modulate cholesterol homeostasis, we conducted a search for compounds that could correct the cholesterol distribution in NPC1-deficient cells. This hypothesis-generating approach was used since the molecular mechanisms for efflux of cholesterol from LE/LY have yet to be fully elucidated.

Most importantly, this approach could lead to the identification of molecular targets not previously considered for therapy. Therefore, we developed an automated microscopy screen to identify compounds that reduce cholesterol accumulation in LSOs of Chinese hamster ovary (CHO) NPC1 mutant cells (CT60) [38] and NPC1 patient fibroblasts (GM03123; Huang, Maxfield et al., in preparation). The automated fluorescence microscopy assay employed in these screens quantifies sterol accumulation in the LSOs based on images of cells stained with filipin. From a screen of ~19,000 compounds on CT60 cells, we identified 21 compounds that reduce filipin staining of the LSOs at 10 μ M. In the case of NPC1-deficient human fibroblasts (GM03123), we examined ~40,000 compounds. We identified 234 hits that reproducibly decreased filipin labeling of LSOs by more than 3 standard deviations from the DMSO-treated controls when cells were incubated with compounds overnight at 10 μ M (Huang, Maxfield, manuscript in preparation).

Since most compounds used in our cell-based screen are novel and previously uncharacterized, a major challenge has been the identification of the molecular target(s) of effective compounds. We reasoned that analysis of general cellular mechanisms that might reduce cholesterol storage would lead to identification of molecular targets of these compounds. The decrease in cholesterol content of the LSOs in NPC-deficient cells could be explained by their effect on at least one of the following processes: 1) increase in cholesterol efflux to extracellular acceptors (e.g., high density lipoprotein), 2) decrease in the uptake or synthesis of cholesterol, 3) increase in cholesterol esterification by ACAT, or 4) decrease in hydrolysis of LDL-carried cholesteryl esters by lysosomal acid lipase (LAL). As described herein, we found that about one third of the compounds in the initial screen on CHO cells, as well as five of the compounds identified in the screen on human cells, were inhibitors of LAL. Moreover, we verified that knockdown of LAL activity using RNA interference (RNAi) would reduce cholesterol storage in LSOs.

EXPERIMENTAL PROCEDURES

Materials and Reagents

Compounds 1a4, 1a11, 1a14, 2a8, 2a9, 2a13, 2a15, 3a2, 3a6 and 3a7 were purchased from Chemical Diversity, Inc. (San Diego, CA). Compound 3a1 (orlistat) was from MicroSource Discovery Systems Inc. (Gaylordsville, CT) and 3a8 (aurintricarboxylic acid) was from Sigma Chemicals (St. Louis, MO). Compound stocks of 10 mM in dimethyl sulfoxide (DMSO) were stored at -20°C . AlexaFluor-633-N-hydroxysuccinimide ester dye, Ham's F12 cell culture medium, Minimal Essential Medium (MEM), OptiMEM (low serum optimal Minimal Essential Medium), fetal bovine serum (FBS), and Hank's balanced salt solution were obtained from Invitrogen (Carlsbad, CA). $[1-^{14}\text{C}]$ -oleic acid (50 mCi/mmol) was purchased from PerkinElmer Life Science (Boston, MA). Cholesteryl- $[4-^{14}\text{C}]$ -oleate (55 mCi/mmol) was from American Radiolabeled Chemicals, Inc. (Saint Louis, MO). Thin layer chromatography Whatman Partisil[®] LK5D plates were from VWR International, Inc. (Batavia, IL). All solvents (acetic acid, chloroform, diethyl ether, DMSO, hexane), sodium hydroxide, 99 % fatty-acid free bovine serum albumin (BSA), sodium acetate, filipin, 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES), paraformaldehyde (PFA), sodium dodecyl sulphate, tris (hydroxymethyl) aminomethane (Tris), 4-methylumbelliferyl oleate (4MUO), 4-methylumbelliferone (4MU), human pancreatic lipase and bovine milk lipoprotein lipase were from Sigma Chemicals (St. Louis, MO). Triton X-100, Tween-20 and bicinchoninic acid assay (BCA) were from Thermo Scientific (Rockford, IL). Modified Lowry protein assay was from Bio-Rad (Hercules, CA). 96- and 384-well assay plates were from Corning (Lowell, MA). The ACAT inhibitor, compound 58-035 (3-[decyldimethylsilyl]-N-[2-(4-methylphenyl)-1-phenylethyl]propanamide) [55], was a gift of Ira Tabas (Columbia University, NY). Hipertext transfection reagent, human LAL-targeting siRNA oligonucleotides (#1: CGCUACUGUCGUUAUUGAU, #2: CGUUUGCACUCAUGUCAUA, #3:

CCUGGUCUCGGAAACAUA, #4: GACUUAAGCGAGGUAUAAA) and scrambled siRNA oligonucleotides ("AllStars" negative control, cat#1027280) were from Qiagen (Valencia, CA). Purified recombinant human LAL (phLAL) was prepared as described [56].

MATLAB was from The MathWorks (Natick, MA). Compound structures were generated using ChemDraw (ChembridgeSoft, Cambridge, MA). MetaMorph and MetaExpress image analysis software were from MDS Analytical Technologies (Downington, PA).

Statistical analysis

P-values were computed using unpaired, two-tailed, Student's *t*-test.

Preparation of lipoprotein-deficient serum (LPDS) and low-density lipoprotein (LDL)

LPDS was obtained upon centrifugation of FBS adjusted to a final density of 1.2 g/ml [19]. LDL was separated from human plasma by centrifugation, based on a previously described procedure [19]. LDL was reconstituted with cholesteryl-[4-¹⁴C]-oleate according to a published procedure [57].

Cell lines

CHO NPC1 mutant cell line, CT60, was provided by Dr. T.Y. Chang (Dartmouth Medical School, Hanover, NH). TRVb1 cells are apparently normal CHO cells transfected with a human transferrin receptor [58]. Human fibroblast cell lines GM03123 (NPC1-deficient) and GM05659 (apparently normal) were obtained from Coriell Institute (Camden, NJ). CHO cells were grown in Ham's F12 medium supplemented with 1 % Penicillin/Streptomycin, 2 g/l glucose, 1.2 g/l sodium bicarbonate, pH 7.4, 10 % FBS, while fibroblasts were grown in Minimal Essential Medium (MEM) with 2.2 g/l sodium bicarbonate, pH 7.4, 10 % FBS. For the selection of TRVb1 cells 0.4 g/l G418 was used.

Evaluation of cholesteryl esters by GC in CT60 cells

The procedure described previously was followed [38]. When needed, an ACAT inhibitor (compound 58-035, 10 µg/ml) was also added to the cells [55].

Hydrolysis of cholesteryl-[4-¹⁴C]-oleate-containing LDL in CT60 and GM03123 cells

Cells were plated in Falcon 12-well plates in growth medium with 10 % FBS. After ~ 24 h incubation at 37 °C, medium was switched to one with 5 % LPDS. Following overnight incubation at 37 °C, cells were pulsed for 2 h at 37 °C in 5 % LPDS-medium containing 5 µg/ml LDL reconstituted with cholesteryl-[¹⁴C]-oleate, 10 µM compound and 10 µg/ml ACAT inhibitor (compound 58-035). Afterwards, cells were chased for 2 h at 37 °C in 5 % LPDS-medium containing 10 µM compound and 10 µg/ml ACAT inhibitor. Lipids were then extracted and separated by thin layer chromatography. Background corrected signals corresponding to cholesteryl esters relative to the sum of the signals of cholesterol and cholesteryl esters were quantified by ImageQuant (GE Healthcare, Piscataway, NJ) or MetaMorph.

In-vitro LAL activity determined with the 4MUO assay

A previously described procedure [59] was adapted as follows: 4MUO was prepared as stock solution in DMSO, then dissolved in 4 % (w/v) Triton X-100 to 0.125–1 mM final concentration. Cell extracts (TRVb1 or GM03123 cells) were prepared by lysis with Triton X-100 (1 % w/v) and passage through a 23-gauge needle. For compound inhibition assays, purified enzyme (phLAL, ~ 0.003 U/ml final concentration, 105 U/mg, 1 U of enzyme releases 1.0 µmol of 4-methylumbelliferone from 4MUO per minute at pH 5.5 at 37 °C) or cell extract was diluted in reaction buffer (200 mM sodium acetate, pH 5.5, 0.02 % Tween-20) and then

added to the pre-mixed substrate and compounds at the indicated concentrations. Reactions were stopped with 1 M Tris pH 8.0 after 30 min or read continuously at 10–15 min intervals, and a time point in the linear range of the assay was used for quantification. For LAL knock-down (siRNA) experiments, cells were grown in 96-well assay plates (Corning), washed with phosphate buffer saline (PBS) using an automated plate washer (Bio-Tek, Winooski, VT), then lysed with 50 μ l of 200 mM sodium acetate, pH 5.5, 0.02 % Tween-20, 1 % Triton X-100. The reaction was started by addition of 50 μ l 1 mM 4MUO, prepared as described above.

Reactions were incubated at 37 °C and fluorescence (355 nm excitation/450 nm emission) was monitored using a SpectraMax M2 fluorometer (MDS Inc., Toronto, Canada). Reaction times were from 30 minutes to several hours. For siRNA knock down experiments, enzymatic activity was quantified as Relative Fluorescence Units/second (RFU/sec) normalized to protein concentration as determined by the bicinchoninic acid assay (BCA, Thermo Scientific) using BSA standards. For compound inhibition experiments, enzymatic activity was quantified as background (4MUO without enzyme) corrected RFU. For cell lysates, enzyme activity was expressed relative to protein concentration determined by the modified Lowry (Bio-Rad) assay using BSA standards. IC50s (compound concentration required for 50 % inhibition of activity) for selected compounds were determined by fitting (MATLAB, Non-linear least squares Levenberg-Marquardt algorithm) dose-response curves to the rectangular hyperbola $y=m/(x+b)+c$, solved for $y = 50$, where y = normalized enzymatic activity (%), x = compound concentration (nM), and m , b , c are coefficients. All fits had $R^2 > 0.98$.

In-vitro human pancreatic lipase activity determined by the 4MUO assay

The assay was done largely as described above for LAL, except that 100 mM Tris pH 8.0 was used as reaction buffer. Final enzyme concentration was 2 U/ml, 468 U/mg, (1 U is reported by the manufacturer to release 1.0 μ mol of 2-monoglyceride from 1, 2-diglyceride per minute at pH 8.1 at 37 °C) and final 4MUO concentration was 1 mM.

In-vitro bovine lipoprotein lipase activity determined by the 4MUO assay

The assay was done largely as described above for LAL, except that 100 mM sodium phosphate pH 7.2, 150 mM NaCl, 0.5 % (w/v) Triton X-100 was used as the reaction buffer. Final enzyme concentration was 465.3 U/ml, 5770 U/mg, (1 U is reported by the manufacturer to release 1.0 nmol of *p*-nitrophenol per minute at pH 7.2 at 37 °C using *p*-nitrophenyl butyrate as substrate) and final 4MUO concentration was 1 mM.

siRNA knock down of LAL activity in GM03123 human NPC1 mutant fibroblasts

siRNA reverse-transfection protocol provided by the manufacturer (Qiagen) was adapted here. Briefly, 5 nM (final concentration) siRNA and 0.7 μ l Hiperfect per well were premixed in 25 μ l of OptiMEM media and added to the well before plating 125 μ l of GM03123 fibroblasts in 96-well optical microscopy plates (Assay plates, Corning). After incubation with the siRNA/transfection reagent complex for 5–8 h, media were replaced several times and fresh MEM media with 10 % FBS were added. Enzymatic activity and filipin staining were evaluated in matched plates ~ 72 h post-transfection.

Automated fluorescence microscopy

Filipin staining was done largely as described previously [38]. Briefly, compound-treated and control cells (DMSO-treated) were fixed with 1.5 % PFA for 20 min and then labeled with filipin (50 μ g/ml, 45 min). Images of filipin-stained cells were acquired using an ImageXpress^{MICRO} imaging system from Molecular Devices (MDS Analytical Technologies, Downingtown, PA) equipped with a 300 W Xenon-arc lamp from Perkin-Elmer (Waltham, Massachusetts, USA), a 10X Plan Fluor 0.3 NA objective from Nikon (Melville, NY), and a

Photometrics CoolSnap HQ camera ($1,392 \times 1,040$ pixels) from Roper Scientific (Tucson, AZ). Filipin images were acquired using 377/50 nm excitation and 447/60 nm emission filters with a 415 dichroic long-pass filter. Filter sets assembled in Nikon filter cubes were obtained from Semrock (Rochester, NY). Plates were transported from plate hotels using a CRS CataLyst Express robot from Thermo Fisher Scientific (Waltham, MA). Images were acquired at four sites per well using 2×2 binning. Sites were centered in the wells with 200 μm spacing between sites. Each site was individually focused using a high-speed laser autofocus comprised of a 690 nm diode laser and a dedicated 8-bit CMOS camera. 696×520 pixel images were acquired at 12 intensity bits per pixel. Each pixel was $1.25 \times 1.25 \mu\text{m}$ in the object plane.

Image-analysis assay: LSO compartment ratio assay

Images of filipin-stained cells were analyzed using MetaExpress image-analysis software and a custom-designed analysis assay called the “LSO compartment ratio assay” which has been described in detail [38]. Briefly, each image was first corrected for shading and background. Then, two different thresholds were applied to the filipin images. A low threshold was set to define the total area of the cells in the field, whereas the high threshold was set to identify the bright, filipin-stained, perinuclear LSO regions of the cells. The LSO compartment ratio describes the total filipin intensity above the high threshold divided by the cell area (μm^2) as defined by the low filipin threshold.

Confocal Microscopy, Neutral lipid staining and colocalization with LE/LY

Human LDL was labeled with AlexaFluor-633-N-hydroxysuccinimide ester (Invitrogen) following manufacturer’s protocol. GM05659 and GM03123 cells were incubated for ~1 day in growth medium supplemented with 10 % LPDS, and then kept for another day in medium with 100 $\mu\text{g}/\text{ml}$ AlexaFluor-633 LDL $\pm$ 10 μM compound 3a2. Cells were then chased for ~1 h in growth medium with 10 % FBS $\pm$ 10 μM compound 3a2, fixed with PFA, and stained with LipidTox Green (Invitrogen, 1:3000 dilution) for ~1 h.

Confocal images were acquired on a Zeiss LSM510 laser scanning confocal microscope with a plan-apochromat 63X (1.4 NA) oil immersion objective. LipidTOX Green was visualized with 488 nm excitation/505–530 BP emission filter and AlexaFluor-633 LDL was visualized with 633 nm excitation/650 LP emission filter.

RESULTS

Initially, we examined the effects of the 21 compounds selected in a screen on NPC1 mutant CHO cells [38] on intracellular cholesterol pathways. Most of these 21 small molecules did not exhibit a cytotoxic effect on mutant CHO cells treated for 24 h at 10 μM , as described previously [38]. Moreover, the cholesterol-lowering effect of these compounds identified by the fluorescence microscopy assay was validated with an analytical chemical method involving GC separation and quantification of solvent-extracted cellular lipids [38].

We investigated the effects of the compounds on cholesterol efflux from cells, cholesterol uptake via lipoprotein endocytosis (data not shown), esterification by ACAT, and the amount of cholesteryl esters in the cells. The compounds did not show a uniform profile in terms of their effects on these processes, suggesting that the compounds might act *via* several different mechanisms.

Biochemical and enzymatic assays of the compounds from the CT60 screen

We noted that seven compounds (1a4, 1a11, 1a14, 2a8, 2a9, 2a13 and 2a15; structures in Suppl. Fig. 1) increased the cholesteryl ester levels in the cells to at least 150 % of the control value (Fig. 1A). One of the possible causes for this increase could be decreased hydrolysis of LDL-

derived cholesteryl esters by LAL. In order to test this possibility we assayed the effects of these compounds on hydrolysis of LDL-associated cholesteryl esters in LE/LY. Cells were incubated with human LDL reconstituted with cholesteryl-[^{14}C]-oleate, in the presence of both the selected compounds and ACAT inhibitor (58-035) to prevent cholesterol re-esterification (Fig. 1B). All seven compounds inhibited hydrolysis of cholesteryl esters in CT60 cells as compared to control, DMSO-treated cells. LAL is the only enzyme that hydrolyzes lipoprotein-derived cholesteryl esters [20, 60], indicating that the compounds reduced LAL activity in treated CHO cells. We verified that most of these compounds inhibited LAL activity in extracts of CHO cells using hydrolysis of 4-methylumbelliferyl oleate (4MUO) as an assay [59] (Fig. 1C). Six compounds (1a4, 1a11, 2a8, 2a9, 2a13, and 2a15) were found to be inhibitors of CHO LAL, and one compound (1a14) was only weakly effective. Compound 1a14 was also the most cytotoxic hit compound from the initial screen [38]. The apparent IC₅₀s for these compounds are listed in Table 1A.

Another possible way for these compounds to elevate cholesteryl ester levels would be by increased delivery of cholesterol to ACAT, the enzyme that esterifies cholesterol for storage in lipid droplets. We tested whether these compounds increased the incorporation of [^{14}C]-oleic acid into cholesteryl-[^{14}C]-oleate, as a measure of esterification of cholesterol by ACAT. All seven compounds actually caused a *decrease* of [^{14}C]-oleic acid incorporation into cholesteryl esters as compared to the control (Suppl. Fig. 2A). The effect of compounds on cholesterol esterification by ACAT was additionally tested by treating the cells with [^{14}C]-cholesterol and quantifying [^{14}C]-cholesteryl-oleate formed before and after addition of the compounds to the cells. Once again, all seven selected compounds decreased esterification (data not shown). These results showed that the increase in cholesteryl ester was not caused by increased esterification by ACAT. To further verify that ACAT was not required for the increase in cholesteryl ester caused by these compounds, for four of the compounds we quantified the amount of cholesteryl ester in the cells upon treatment with a hit compound in the presence or absence of an ACAT inhibitor. Cells treated with the selected compounds (1a4, 1a11, 2a8, 2a15) and ACAT inhibitor (58-035) showed much higher levels of cholesteryl ester than the DMSO-treated control cells that received only the ACAT inhibitor (Suppl. Fig. 2B). Moreover, for most of the compounds the amount of cholesteryl ester in the cells treated with compound only was not much different from that found in cells treated with both compound and the ACAT inhibitor, confirming that increased esterification by ACAT is not the mechanism responsible for the increase of ester levels in compound-treated cells. Additionally, CT60 cells, in the absence or presence of the ACAT inhibitor, also showed decreased levels of free cholesterol when treated with the selected compounds as compared to DMSO-treated controls (Suppl. Fig. 2C).

Effects on human NPC1-deficient cells of the CHO-selected compounds

Several of the 21 compounds that were selected in our screen of CT60 NPC1-defective CHO cells were also effective in reducing filipin labeling (and cholesterol accumulation) in an NPC1 mutant human cell line, GM03123 (data not shown). However, none of the seven compounds that inhibited LAL in the CHO mutant cells were effective in reducing cholesterol accumulation in GM03123 cells. Consistent with this species specificity, compounds 1a11, 2a8, 2a9 and 2a13 did not significantly reduce hydrolysis of LDL-delivered [^{14}C]-cholesteryl ester (Suppl. Fig. 3A). While compounds 1a4, 1a14 and 2a15 did have a small, yet statistically significant ($p < 0.05$) effect, this was probably insufficient in magnitude to significantly reduce cholesterol accumulation in human NPC1-deficient cells. Additionally, none of these compounds inhibited LAL activity in purified recombinant human LAL expressed in *P. pastoris* (phLAL [56], Suppl. Fig. 3B) or lysates from human cells (Suppl. Fig. 3C). This indicated that LAL is divergent enough between hamsters and humans to make our selected compounds inactive against the human enzyme.

RNAi experiments corroborate effect of LAL inhibition

Although the compounds selected from the screen of NPC1-deficient CHO cells were ineffective in human NPC1-mutant cells, the results did indicate that reduced LAL activity would reduce the accumulation of cholesterol in LSOs. To determine whether reduced LAL activity would reduce cholesterol storage in human NPC-deficient cells, we employed RNAi gene silencing. Four different siRNA's were effective in knocking down LAL expression, as verified by an LAL activity assay (Suppl. Fig. 4B). Two of these siRNA's (#1 and #2, as identified in the Methods) also significantly reduced cholesterol accumulation in LSOs, as verified by filipin staining (Suppl. Fig. 4A). Combination of the three most effective siRNA's (#1, 2 and 4) resulted in reduction to ~ 35% of control LAL activity 72 hours after transfection (Fig. 2A). A similar 4MUO activity value (~ 20 % of wild type levels) has been reported [61] for human fibroblasts deficient in LAL, indicating that our knock down approached near maximal levels. This RNAi-mediated decrease in LAL activity caused a significant reduction in free cholesterol accumulation in LSOs (Fig. 2B), demonstrating that reduced LAL activity also diminishes cholesterol accumulation in human NPC1 mutant cells.

Chemical Screen on human NPC1-deficient cells identifies additional LAL inhibitors

While these studies were in progress, a screen of compound libraries using a human NPC1 mutant cell line, GM03123, was carried out (Huang, Maxfield et al., in preparation). We tested the compounds that were found to be most effective in reducing cholesterol accumulation in these human NPC1-defective cells for inhibition of purified recombinant human enzyme, pHAL [56]. Five of these compounds (structures in Suppl. Fig. 5) were found to be effective inhibitors of pHAL (Fig. 3A). None of these five compounds had any significant effect on cell viability as shown in Suppl. Fig. 6. Compound 3a1 is orlistat (tetrahydrolipstatin, Alli®, Xenical®), a well known inhibitor of various lipases, including LAL [59,62]. Compound 3a8 is aurintricarboxylic acid, which has been reported to affect numerous cellular processes such as DNA replication [63] and apoptosis [64]. Three remaining compounds (3a2, 3a6, 3a7) are structurally related thiadiazole carbamates. These novel inhibitors of LAL are effective in the mid-nanomolar range. Apparent IC₅₀s for these compounds are shown in Table 1B. We also tested these compounds against CHO-derived LAL from cell lysates. As shown in Figure 3B, compound 3a1 is still a very potent inhibitor of CHO LAL, whereas compounds 3a2, 3a6 and 3a7 are only effective in the micromolar range. Compound 3a8 is ineffective (IC₅₀ >10 µM) against CHO LAL, again demonstrating species differences in inhibition of LAL.

Effects of novel LAL inhibitors are verified on both human and CHO NPC mutant cells

The reduction of filipin staining of GM03123 NPC1-deficient human fibroblasts upon treatment with compounds 3a2 and 3a7 is shown in Figure 4, and the dose dependent effects of compounds 3a1, 3a2, 3a6, 3a7 and 3a8 on corresponding LSO values are shown in Figure 5A. It appears that compounds 3a1, 3a2, and 3a7 can significantly reduce cholesterol accumulation in LSOs down to sub-micromolar range, whereas compound 3a6, a less potent inhibitor of LAL, also appears to be less effective in reducing cholesterol accumulation in LSOs. Compound 3a8, the least potent inhibitor of human LAL studied here, had only marginal effects on cholesterol accumulation. Compounds 3a1 and 3a2 were also tested in the LSO assay on CT60 (CHO NPC1 mutant) cells, and their effects again correlate well with *in vitro* LAL inhibition data. Compound 3a1, a very potent inhibitor of CHO-derived LAL (Fig. 3B), shows marked reduction in LSO values of CT60 cells, down to 10 nM (Fig. 5B). On the other hand, compound 3a2, being significantly less effective in inhibiting CHO-derived LAL, reduces cholesterol accumulation only slightly at 10 µM.

Subsequently, we verified that inhibition of LAL by compound 3a2 leads to accumulation of neutral lipids (stained with LipidTOX Green) that partially overlap with LE/LY marked with AlexaFluor-633 LDL, as shown in Figure 6 and Suppl. Fig. 7, for GM05659 (normal cells)

and GM03123 cells (NPC1 mutant cells), respectively. Treatment with 3a2 seems to cause the morphology of the LDL-containing LE/LY to change somewhat. In the fluorescence microscope these compartments appear elongated and slightly larger. This observation would be consistent with published electron micrographs of cells deficient in LAL, which exhibit an enlarged lysosome compartment with altered shapes [65].

Time-course studies of enzyme inhibition

It has been reported that tetrahydrolipstatin (compound 3a1) is a reversible inhibitor of LAL [59]. We examined the time course of inhibition by compounds 3a1, 3a2, 3a6 and 3a7. At inhibitor concentrations that produced a partial effect after 5 minutes, there was an increase in inhibition with a 2 hour preincubation for compounds 3a2, 3a6 and 3a7 (Fig. 7). However, this effect was only partial even though there was a large molar excess of inhibitor. This could reflect a slowly reversible inhibition or breakdown of the inhibitors producing more potent inhibitors. No time dependence of inhibition was observed for compound 3a1, as expected. The detailed mechanisms of inhibition were not examined further in this study.

Human pancreatic and bovine lipoprotein lipase assays reveal compound specificity

To determine whether the LAL inhibitors discovered in this study can discriminate between different lipases, we tested their effects on human pancreatic lipase and bovine milk lipoprotein lipase. As shown in Figure 8, compounds 3a2, 3a6, 3a7 and 3a8 (10 μ M) had only small effects on pancreatic lipase activity or lipoprotein lipase, indicating specificity for LAL. On the other hand, compound 3a1 (orlistat) caused a significant reduction in activity of both these enzymes, as expected [66–73].

DISCUSSION

Small molecule compounds studied in this work have been shown to decrease overall cholesterol levels and cholesterol in the LSOs of NPC1-deficient CHO CT60 [38] and/or human GM03123 cells. The goal of this study was to determine which one of the numerous, previously unconsidered for NPC disease therapy, cholesterol homeostasis processes was affected by the hit compounds. In this paper we show that the decrease of cholesterol in NPC1-defective cells treated with several of the compounds is due to impaired hydrolysis of cholesteryl esters by LAL.

Quantification by GC of cholesteryl ester levels in compound-treated CT60 (Fig. 1A) cells and inhibition of hydrolysis of LDL-delivered [14 C]-cholesteryl esters in CT60 cells (Fig. 1B) indicated that seven hits from the CT60 screen increased cholesteryl ester levels by inhibiting LAL activity. This finding was confirmed using an *in vitro* assay of hydrolysis of a fluorogenic substrate by TRVb1 (CHO) cell lysates (Fig. 1C).

Since cholesteryl ester levels in compound-treated cells could be higher due to increased sterol esterification in the endoplasmic reticulum by ACAT, we examined this possibility by performing [14 C]-oleate incorporation assays as well as assays employing ACAT inhibitor (Suppl. Fig. 2). No role for ACAT-mediated increase in cholesteryl ester levels was found in compound-treated cells.

To our surprise, these seven compounds did not reduce the free cholesterol detected by filipin in a human NPC1 mutant cell line (GM03123; Huang, Maxfield et al, in preparation). Most of these compounds also did not affect [14 C]-cholesteryl ester hydrolysis (Suppl. Fig 3A). Consistent with this lack of effect, the compounds did not inhibit LAL activity in lysates of GM03123 cells (Suppl. Fig. 3C) or purified enzyme (phLAL, Suppl. Fig. 3B). Thus, the compounds identified in the CT60 screen pointed to a potential molecular target in NPC CHO

mutant cells (*i.e.*, LAL), but the compounds themselves were not useful for inhibition of human LAL. This surprising selectivity points to a presumed divergence of the two LAL enzymes. A further study of this structure-activity relationship was not pursued as the sequence of *C. griseus* (Chinese hamster) LAL is not known yet.

To determine if reduction of LAL activity in human cells would reduce the cholesterol accumulation in LE/LY, we used siRNA's to knock down the expression of human LAL in GM03123 NPC1-deficient fibroblasts. We observed a significant decrease in filipin labeling of the LE/LY in the siRNA treated cells as compared to those treated with a non-targeting, scrambled, control siRNA ~3 days after transfection (Figure 2). This indicated that chemical inhibition of human LAL should also reduce cholesterol accumulation in human NPC-deficient fibroblasts.

While this work was in progress, we completed preliminary analysis of a screen of ~40,000 compounds on the human GM03123 NPC1-defective fibroblasts (Huang, Maxfield et al., in prep.). We tested the compounds that were most effective in reducing filipin labeling of these cells for inhibition of human LAL. We found five compounds that inhibited pH-LAL activity (Fig. 3A). One of these, compound 3a1, is tetrahydrolipstatin ((S)-((S)-1-((2S,3S)-3-hexyl-4-oxooxetan-2-yl)tridecan-2-yl) 2-formamido-4-methylpentanoate), a well-characterized inhibitor of pancreatic lipase [62,66–73] that can also inhibit other lipases [59,62]. Another, compound 3a8, is aurintricarboxylic acid, which has been found to have numerous biological effects such as inhibition of translation initiation [74], DNA replication [63], apoptosis [64], and many others, but we are not aware of any prior reports of its inhibition of LAL or any other lipase. The three remaining compounds found to inhibit human LAL (3a2, 3a6, and 3a7) are structurally closely related thiadiazole carbamates that resemble substrate analogs and are novel inhibitors of both human and hamster LAL.

A PubChem BioAssay [75] search of reported activities for these compounds (conducted on 23 January 2009) reveals that compound 3a6 (PubChem Compound ID # 655946) has been reported active only in two assays: Primary High Throughput Screening (HTS) assay for chemical inhibitors of *E. coli* RNA polymerase (Assay ID 559, 31.4 % inhibition at 10 μ M; 3a2 and 3a7 were not tested in this assay) [76] and Primary HTS assay for chemical inhibitors of TNF α stimulated E-Selectin expression (Assay ID 1246, 76.8 % inhibition at 2 μ M; 3a2 and 3a7 tested negative in this assay). Compound 3a2 (PubChem Compound ID # 651937) has been reported active only in one assay: Primary Cell-based HTS Assay for Inhibitors of Wee1 Degradation (Assay ID 1321, 8.9 % activation compared to MG132 at 5 μ M; 3a6 and 3a7 tested negative in this assay). Compound 3a7 (PubChem Compound ID # 648404) has not been yet reported active in any PubChem assays. Currently, we can see no apparent link between these reported activities and the reduction of cholesterol storage in NPC1-deficient cells treated with these compounds. All three compounds inhibit human LAL at sub-micromolar concentrations and CHO-derived LAL at micromolar concentrations (Fig. 3) and are effective in reducing cholesterol storage in LSOs of both human (Fig. 4, 5A) and CHO (Fig. 5B) NPC-deficient cells in concentration ranges that correlate well with their ability to inhibit LAL (Fig. 3). This supports our hypothesis that inhibiting LAL correlates with reduction in cholesterol accumulation in LSOs of NPC mutant cells. Moreover, we show that compound 3a2 causes increase in neutral lipid accumulation in both apparently normal (GM05659, Fig. 6) and NPC1-deficient (GM03123, Suppl. Fig. 7) human fibroblasts. The observed accumulation of neutral lipids partially overlaps with LE/LY, as would be expected as the result of LAL inhibition. Only partial overlap is observed since LipidTOX stains all neutral lipids in the cell including those present in lipid droplets along with cholesteryl esters and triglycerides present in LE/LY. In addition, we determined that compounds 3a2, 3a6 and 3a7 are also relatively specific in that they do not inhibit human pancreatic lipase (Fig. 8A) or bovine lipoprotein lipase (Fig. 8B).

In order for the IC₅₀ values reported in Table 1 to be truly meaningful (*i.e.*, correlate with an apparent K_i value), compounds would need to be reversible inhibitors. We attempted to address this question by pre-incubating our compounds with pH₄LAL, prior to reaction start. It appears that while compound 3a1, as expected from previous studies [59], does not inactivate LAL in a time dependent fashion, compounds 3a2, 3a6, and 3a7 do show some time dependence (Fig. 7). However, this time dependence is significantly smaller than what one would expect for inhibition by a completely irreversible inhibitor, which should follow a rapid exponential decay. Compounds 3a2, 3a6 and 3a7 appear to structurally resemble LAL substrates, thus allowing for a possibility that they can also be hydrolyzed by LAL, further complicating reaction kinetics. More detailed studies of any potential compound hydrolysis products by mass spectrometry and detailed studies of time dependence effects on compound inhibition of LAL might reveal a more complete mechanism of action of these novel inhibitors of LAL.

It is unclear whether *partial* inhibition of LAL can be part of a therapeutic regime for NPC disease. If an excess of free cholesterol in LE/LY is part of the basis for cell toxicity, then slower production of free cholesterol in these organelles may be helpful. The free cholesterol in the LSOs of NPC-deficient cells does exchange slowly with other pools of free cholesterol [29,77], so slower production of free cholesterol would reduce the steady state level of cholesterol in the LSOs. This is precisely what we have observed in tissue culture cells when we inhibited LAL or knocked down expression of LAL by siRNA. Complete inhibition of LAL would be unfavorable since this can lead to Wolman's disease, a recessive lysosomal storage disorder caused by LAL deficiency [78–80]. Cell viability data (Suppl. Fig. 6) indicate that at least in this tissue culture fibroblast model cell growth is not affected by treatment with LAL inhibitors.

Moreover, chemical inhibition of LAL activity can be helpful in cellular studies of the lysosomal breakdown of cholesteryl esters and triglycerides. There are other inhibitors of LAL (*e.g.*, tetrahydrolipstatin, or diethyl *p*-nitrophenyl phosphate (E600) [59,62], and 4-PDMP (D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) [81]), but tetrahydrolipstatin and E600 also inhibit other lipases [66,69,70,72,82–84], whereas 4-PDMP was reported [81] to inhibit bis-(monoacylglycerol)-phosphate stimulation of LAL activity, but does not appear to have an effect in the absence of bis-(monoacylglycerol)-phosphate[21].

There are advantages to cell-based screens in that compounds selected can be shown to be non-toxic and to work in a biological context – at least at the cellular level. A significant challenge is then to identify the molecular targets that are affected by the compounds. As a first step, we carried out general biochemical analyses of the effects of the selected compounds on key steps in cholesterol transport and metabolism such as LAL-mediated degradation of cholesteryl esters. Findings presented in this manuscript demonstrate that by reducing LAL activity *via* either chemical inhibition or siRNA-mediated decrease in expression we can partially correct the NPC1 defect in both human cells derived from patients and model systems such as CT60 cells. From a more general perspective, this work exemplifies how phenotypic screens can be used to identify potential pathways for therapy and novel compounds that affect them.

Supplementary Material

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Abbreviations

LAL	lysosomal acid lipase
ACAT	acyl Co-A:cholesterol acyl transferase
BSA	bovine serum albumin
CHO	Chinese hamster ovary
DMSO	dimethyl sulfoxide
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid
FBS	fetal bovine serum
GC	gas chromatography
LPDS	lipoprotein-deficient serum
LE	late endosomes
LY	lysosomes
LDL	low-density lipoprotein
LSO	late endosome/lysosome-like storage organelles
NPC	Niemann-Pick disease type C
PBS	phosphate buffer saline
PFA	paraformaldehyde
FC	free cholesterol
CE	cholesteryl ester
4MUO	4-methylumbelliferyl oleate
pHLAL	recombinant (<i>P. pastoris</i>) human LAL
HTS	High Throughput Screening

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Figure 1. Effect of compounds on NPC1 mutant and wild type CHO cells

(A) Quantification of cholesteryl ester levels by GC in CT60 cells treated with compounds. CT60 cells were treated with compounds (10 μM) overnight at 37 $^{\circ}\text{C}$ and cellular lipids were extracted. Total cholesterol (after saponification) and free cholesterol were measured, and the difference between these values was taken as the amount of cholesteryl ester. The content of cholesteryl ester in the cells per μg cell protein was determined for compound-treated cells and compared to the levels in control, DMSO-treated cells. (Referencing all values to the solvent-treated cells allowed us to pool data from separate experiments for statistical analysis.) For solvent-treated (control) CT60 cells, the mean value was 0.042 ± 0.004 μg cholesteryl ester/ μg cell protein. For compound-treated cells, cholesteryl esters levels are presented as fractions of the control value and are shown as mean $\pm$ S.E. (* $p < 0.01$). Two to four independent experiments were conducted ($6 \leq n \leq 12$ per condition). (B) Hydrolysis of cholesteryl-[^{14}C]-oleate-containing LDL in CT60 cells treated with compounds. CT60 cells were incubated overnight at 37 $^{\circ}\text{C}$ in 5 % LPDS medium and then pulsed for 2 h with 5 $\mu\text{g}/\text{ml}$ cholesteryl-[^{14}C]-oleate-LDL, compounds (10 μM) and 10 $\mu\text{g}/\text{ml}$ ACAT inhibitor (compound 58-035). Afterward, cells were chased for 2 h at 37 $^{\circ}\text{C}$ in 5 % LPDS-medium with compounds (10 μM) and 10 $\mu\text{g}/\text{ml}$ ACAT inhibitor. Lipids were extracted, and cholesteryl-[^{14}C]-oleate was measured as a fraction of the total radiolabeled cholesterol. For DMSO-treated cells, the value was 0.37 ± 0.03 . Experimental data of compound-treated cells are displayed as fractions of the control value and reflect averages $\pm$ S.E from three experiments (* $p < 0.001$, when compared

to control; $6 \leq n \leq 9$ per condition). (C) Lysosomal acid lipase activity in presence of compounds from the CHO cell screen. Triton X-100 cell extracts (~ 0.2 mg/ml) from TRVb1 cells were mixed with compounds at indicated final concentrations, and the reaction was started by adding 4MUO substrate. Enzymatic activity in the linear range of the assay at 37°C was quantified as background corrected 4-methylumbelliferone fluorescence, normalized to DMSO control average value for a particular experiment. Data reflect averages $\pm$ S.E from two independent experiments ($4 \leq n \leq 12$ per condition).

Figure 2. siRNA knock-down of LAL

siRNA-mediated decrease in LAL expression as quantified by the 4MUO activity assay is illustrated in (A) and corresponding decrease in LSO values, as measured by the filipin staining and quantified by the LSO assay, is shown in (B). Data were obtained ~3 days post treatment with siRNA/Hiperfect complexes. LSO values were quantified using a specific threshold to select the LSOs and then normalized to the untreated control average value for a particular experiment. GM05659 cells are apparently normal fibroblasts. Enzymatic activity at 37 °C was quantified as background corrected increase in 4-methylumbelliferone fluorescence as a function of time (RFU/sec), normalized to protein concentration as measured by bicinchoninic acid assay (OD₅₆₂), and subsequently normalized to the untreated control average value for a particular experiment. Data reflect averages $\pm$ S.E from five independent experiments (n ~ 40, per condition).

Figure 3. Lysosomal acid lipase activity in presence of compounds from the human NPC mutant cells screen

Compounds from GM03123 fibroblast screen (3a1, 3a2, 3a6, 3a7 and 3a8) show dose dependent inhibition of human purified enzyme, pHAL (A) and CHO (TRVb1) cell lysates (B). Human purified enzyme (pHAL, 0.003 U/ml, 105 U/mg) or Triton X-100 cell extracts (0.4–1.0 mg/ml) from TRVb1 cells was mixed with compounds at indicated final concentrations, and the reaction was started by adding 4MUO substrate. Enzymatic activity at 37 °C was quantified as background corrected 4-methylumbelliferone fluorescence, normalized to DMSO control average value for a particular experiment. Data reflect averages $\pm$ S.E from two independent experiments (n=28 for DMSO, n=6 for each compound (A); n=16 for DMSO, n=6 for each compound (B)).

A. DMSO **B. 10 μ M 3a2** **C. 10 μ M 3a7**

Figure 4. Filipin images of GM03123 cells treated with LAL inhibitors show decreased cholesterol accumulation in LSOs

Cells were plated in 384-well plates in growth medium, and the next day treated with DMSO (A), 10 μ M 3a2 (B), or 10 μ M 3a7 (C) in screening medium (MEM, 5.5 % FBS, 10 mM HEPES, 2.2 g/L sodium bicarbonate, pH 7.4). After an overnight incubation, cells were washed with PBS, fixed with PFA and stained with filipin. 10X magnification images were obtained using ImageXpress^{MICRO} automated imaging system. Images were corrected for shade and background. Bar = 100 μ m. $\gamma=0.74$ was used to generate this figure.

A. GM03123 cells

B. CT60 cells

Figure 5. LSO assay quantification of the effect of LAL inhibitors 3a1, 3a2, 3a6 and 3a7 on filipin-stained cholesterol in NPC mutant human fibroblasts (GM03123) and CHO (CT60) cells
 GM03123 cells (A) or CT60 cells (B) were seeded in two 384-well plates in growth medium. After one day, compounds in screening medium were added to achieve final concentrations of 10 μ M, 1 μ M, 0.1 μ M and 0.01 μ M in 12 different wells per plate (total n=24/condition) and allowed to incubate overnight. Cells were washed with PBS, fixed with PFA and stained with filipin. 10X magnification filipin images were acquired and processed as described previously [38]. LSO ratio values reflecting cholesterol accumulation were calculated as described previously [38] and were normalized to control (DMSO-treated) average values. Data reflect averages $\pm$ S.E (when visible) from one representative experiment (n = 24 per compound condition).

A. GM05659 cells treated with compound 3a2 **B. GM05659 cells treated with DMSO (control)**

Figure 6. Effect of LAL inhibition with compound 3a2 on the accumulation of neutral lipids in normal human fibroblasts

GM05659 cells were grown in growth medium with 10 % LPDS for 1 day, then treated with either 10 μ M compound 3a2 for an additional day (A) or with DMSO (B), all in presence of 10 % LPDS and 100 μ g/ml human LDL labeled with AlexaFluor-633 (red). Cells were then fixed with PFA and stained with LipidTOX (green). Confocal image stacks were acquired on a Zeiss LSM510 confocal microscope, and single optical sections of representative images are shown. For each channel, the same brightness and contrast settings were used to display images of compound-treated and control cells. Bar = 20 μ m.

Figure 7. Time-course studies of compound pre-incubation with LAL

Compounds 3a1, 3a2, 3a6, and 3a7 were tested for time dependence in their inhibition of human purified enzyme, phLAL (0.003 U/ml, 105 U/mg). phLAL was incubated at room temperature for either ~ 2 hours or ~ 5 minutes with compounds at 50 nM final concentration, and the reaction was started by adding 4MUO substrate. Enzymatic activity at 37 °C was quantified as background corrected 4-methylumbelliferone fluorescence, normalized to DMSO control average value for a particular experiment. Data reflect averages $\pm$ S.E from two independent experiments, except for compound 3a1, where data from only one experiment are shown ($3 \leq n \leq 17$).

Figure 8. Human pancreatic lipase and bovine milk lipoprotein lipase activity assays in the presence of compounds selected from the human NPC mutant cells screen

Purified enzymes, human pancreatic lipase (2 U/ml, 468 U/mg) (A) and bovine milk lipoprotein lipase (465.3 U/ml, 5770 U/mg) (B), were mixed with 10 μ M compounds, and the reaction was started by adding 4MUO substrate. Enzymatic activity at 37 $^{\circ}$ C was quantified as background corrected 4-methylumbelliferone fluorescence, normalized to the DMSO control average value for a particular experiment. Data reflect averages $\pm$ S.E from two independent experiments ($5 \leq n \leq 12$ per condition).

Table 1
IC₅₀ values for inhibition of LAL

Apparent IC₅₀s for selected compounds against CHO cell lysates (**A**) and phLAL (**B**) were determined by fitting (MATLAB, Non-linear least squares Levenberg-Marquardt algorithm) dose-response curves (obtained with 4MUO assay) to the rectangular hyperbola $y=m/(x+b)+c$, for $y=50$, where y = normalized enzymatic activity [%] and x = compound concentration [μ M], and m , b , c are coefficients. All fits had $R^2 > 0.98$.

Table 1A	
Compound name	IC₅₀ [μM]
1a4	<0.54
1a11	1.71
1a14	>20
2a8	0.63
2a9	0.73
2a13	0.90
2a15	13.41
Table 1B	
Compound name	IC₅₀ [μM]
3a1	0.168
3a2	0.121
3a6	0.421
3a7	0.220
3a8	1.339