

Identification of a pharmaceutical compound that partially corrects the Niemann-Pick C phenotype in cultured cells

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Abstract Niemann-Pick C (NPC) is an autosomal recessive lysosomal lipid storage disease characterized by progressive central nervous system degeneration. In cultured human NPC fibroblasts, LDL-derived cholesterol accumulates in lysosomes and endosomes, LDL-cholesterol transport from endocytic compartments to other cellular compartments is delayed, and LDL does not elicit normal homeostatic responses. Currently, there is no therapy that delays the onset of neurological symptoms or prolongs the life span of NPC children. We have developed and implemented an amphotericin B-mediated cytotoxicity assay to screen for potential therapeutic drugs that induce cholesterol movement in cultured NPC cells. NPC cells are relatively resistant to amphotericin B killing due to intracellular sequestration of cellular cholesterol. The screen was carried out using simian virus 40-transformed ovarian granulosa cells from the *npc^{mh}* mouse model of NPC disease. A library of 44,240 compounds was screened and 55 compounds were identified that promote amphotericin B-mediated killing of NPC cells. One compound, NP-27, corrected the NPC phenotype by four different measures of cholesterol homeostasis. In addition to making NPC cells more sensitive to amphotericin B, NP-27 stimulated two separate cholesterol transport pathways and restored LDL stimulation of cholesterol esterification to near normal levels.—Liscum, L., E. Arnio, M. Anthony, A. Howley, S. L. Sturley, and M. Agler. Identification of a pharmaceutical compound that partially corrects the Niemann-Pick C phenotype in cultured cells. *J. Lipid Res.* 2002. 43: 1708–1717.

Supplementary key words Niemann-Pick C • cholesterol • high throughput screen • cholesterol transport • amphotericin B

Niemann-Pick C (NPC) is an autosomal recessive lysosomal lipid storage disease (1). Historically, NPC has been considered a disease of cholesterol metabolism, since a major lipid that is stored in visceral organs is cholesterol. However, in reality it is a complex lipid storage disorder, with in-

creases in sphingomyelin, cholesterol, lysobisphosphatidic acid, neutral and acidic glycosphingolipids, and phospholipids seen in liver and spleen. The lipid storage pattern differs dramatically in the brain where gangliosides are the main storage material and cholesterol is stored to a lesser extent (2). NPC patients exhibit progressive loss of motor skills, learning difficulties, dementia, and seizures (1), all of which signal central nervous system degeneration. Onset of symptoms in the majority of NPC children is school age, and the disease is generally fatal within a decade.

NPC disease is caused by mutations in one of two genetic loci, NPC1 (3) and NPC2 (4). NPC1 and NPC2 phenotypes are clinically and biochemically indistinguishable and the two genes may encode constituents of the same lipid transport pathway. The NPC1 gene product is a 1278 amino acid membrane protein found in late endosomes (5–7). Mutations in NPC1 cause altered lipid transport kinetics and lysosomal accumulation of cholesterol and gangliosides (8–10). The biological function of NPC1 still is not clear; however, recent evidence suggests that NPC1 may have a permease activity (11). Molecular cloning of NPC2 reveals that the protein is likely to be a soluble lysosomal protein with cholesterol binding activity (4).

NPC disease does not affect lipoprotein levels or produce premature vascular disease. Thus, therapies that target hypercholesterolemia are not effective against NPC (12). Bone marrow and liver transplantation in NPC patients and an NPC mouse model have not slowed the progression of neurological symptoms (1). Currently, there is no therapy that alleviates the progressive neurodegeneration that is seen in NPC patients.

Without a defined biological function for NPC1, it is difficult to develop a cell-based, target-specific high

Abbreviations: [³H]CL-LDL, LDL labeled with [³H]cholesteryl linoleate; FCLPDS, fetal calf lipoprotein-deficient serum; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NPC, Niemann-Pick disease type C.

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Manuscript received 29 April 2002 and in revised form 26 June 2002.
DOI 10.1194/jlr.M200179.JLR200

throughput screen for therapeutic compounds that might reverse or prevent the neurodegeneration. Instead, we have developed and implemented an amphotericin B-mediated cytotoxicity assay to screen for potential therapeutic drugs that alter key aspects of the NPC phenotype. Amphotericin B is a polyene antibiotic that forms aqueous pores in sterol rich membranes, resulting in lysis and cell death. We previously found that Chinese hamster ovary cells with an NPC phenotype are resistant to amphotericin B-mediated toxicity (13), presumably due to lysosomal cholesterol storage resulting in lowered plasma membrane cholesterol content. To identify compounds that induce cholesterol movement from NPC lysosomes, a 44,240 member compound library was screened for those that cause LDL-dependent amphotericin B-mediated killing in NPC cells. The screen has identified one compound that induces specific pathways of intracellular cholesterol transport in NPC cells.

EXPERIMENTAL PROCEDURES

Materials

Sodium [^3H]acetate (100 mCi/mmol), [9,10- ^3H]oleic acid (5 Ci/mmol), cholesteryl [1- ^{14}C]oleic acid (51 mCi/mmol), [1,2- ^3H]cholesterol (45 Ci/mmol), [4- ^{14}C]cholesterol (56 mCi/mmol), and [1,2,6,7- ^3H]cholesteryl linoleate (84 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). FCS was from Hyclone (Logan, UT). Mediatech cellgro tissue culture medium was from Fisher Scientific; other tissue culture reagents were from Life Technologies, Inc. or from Sigma Chemical. Ninety-six-well tissue culture plates were from Corning. Lovastatin was from Merck Research Laboratory (Rahway, NJ) whereas pravastatin was from Bristol-Myers Squibb (Princeton, NJ). Other chemicals were from Sigma Chemical unless otherwise indicated.

Preparation of LDL, lipoprotein-deficient serum, and media

LDL was prepared by ultracentrifugation (14). Lipoprotein-deficient FCS was prepared as described, omitting the thrombin incubation (14). The following media were prepared: HD-10% FCS (a 1:1 mixture of Ham's F-12 and DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and 25 mM HEPES, pH 7.3), HD-15% fetal calf lipoprotein-deficient serum (FCLPDS) and HD-1% FCLPDS (HD-10% FCS in which the FCS was replaced with 15% or 1% fetal calf lipoprotein-deficient serum). HD-15% FCLPDS/sm is HD-15% FCLPDS supplemented with a statin (20 μM lovastatin or pravastatin to inhibit endogenous cholesterol synthesis) and 0.5 mM mevalonate (which enters the cholesterol biosynthetic pathway past the point of statin inhibition and provides essential non-steroidal isoprenoids).

Cultured cells

Normal and NPC mouse ovarian granulosa cells were generously provided by Jerome F. Strauss III (University of Pennsylvania, Philadelphia, PA) and Peter G. Pentchev (National Institute of Neurological Disorders and Stroke, National Institutes of Health). NPC cells were from the *npc^{nih}* mouse model (15). Cells were grown in a monolayer in HD-10% FCS in a humidified incubator (5% CO_2) at 37°C. Stock flasks were washed with HBSS, trypsinized and re-seeded three times per week. All test compounds identified in this study were dissolved in 100% Me_2SO

and added to the tissue culture wells at a final concentration of 1% Me_2SO . 'No Compound' controls received vehicle alone.

Cell viability assays

Cells were grown and treated as described in the figure legends. Cell viability was assessed using a colorimetric 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (16, 17) exactly as described (18).

High throughput screen

On day 0, NPC cells were seeded into 96-well plates (10,000/well) in HD-15% FCLPDS. On day 1, cells were refed HD-15% FCLPDS/sm with 25 $\mu\text{g}/\text{ml}$ LDL. During the primary screening, eighty test compounds were added per plate to columns 1–10 in a final concentration of 15 μM in 1% Me_2SO . Controls were added in the remaining two columns of the plate. After 4 h, cells were washed with HBSS and refed HD-1% FCLPDS with or without 25 $\mu\text{g}/\text{ml}$ amphotericin B. After 2 h, cells were washed twice with HBSS and refed HD-10% FCS. On day 2, cell viability was assessed using a colorimetric MTT assay. Cells were refed HD-10% FCS with 2.5 mg/ml MTT. After 2 h, the medium was aspirated and 100% Me_2SO added for 15 min. MTT cleavage was determined by reading the absorbance at 564 nm.

[^3H]oleate incorporation into cholesteryl [^3H]oleate and [^3H]triglycerides

Cells were cultured and incubated with 100 μM [^3H]oleate (20,000 dpm/nmol) as described in the legends to Fig. 5, 6, and Table 2. Cellular cholesteryl [^3H]oleate and [^3H]triglycerides were isolated as described (19). After lipids were extracted, the monolayers were dissolved in 0.1 N NaOH and aliquots removed for protein determination (20). Correction for procedural losses was made by adding [^{14}C]cholesteryl oleate (2,000 dpm, 20 μg) as an internal standard during the extraction.

Fatty acid synthesis

Cells were cultured and incubated with [^3H]acetate as described in the legend to Table 1. Cell monolayers were washed once quickly, once for 10 min, once quickly with Tris-buffered saline (50 mM Tris-Cl and 155 mM NaCl, pH 7.4), then extracted with hexane-isopropanol (3:2, v/v). Samples were saponified by the addition of 1 ml of 1 M KOH in ethanol and incubation at 80°C for 1 h. Samples were extracted with petroleum ether after which the water phase was adjusted to pH 3 by addition of 10 N HCl. The water phase was then extracted with petroleum ether and the organic extract was washed with water and dried. [^3H]fatty acids were analyzed by thin layer chromatography using silica gel 60 plates resolved with heptane-ethyl ether-etic acid (90:30:1, v/v/v). Correction for procedural losses was made by adding cholesteryl linoleate (20 μg), cholesteryl oleate (20 μg), and [^{14}C]oleic acid (2,000 dpm) as an internal standard during the hexane/isopropanol extraction.

Cholesterol synthesis

Cells were cultured and incubated with [^3H]acetate as described in the legend to Table 3. Cellular [^3H]sterol was quantified as described (21), except that thin-layer chromatography separation was performed using heptane-ethyl ether (90:60, v/v). Correction for procedural losses was made by adding [^{14}C]cholesterol (2,000 dpm, 20 μg) as an internal standard during the extraction. The endogenously labeled [^3H]sterols co-chromatograph with authentic cholesterol; however, they can be resolved by HPLC into [^3H]cholesterol (70%) and [^3H]desmosterol (30%). They are referred to as [^3H]cholesterol.

Basal esterification of plasma membrane cholesterol

Cells were cultured and incubated with [^3H]cholesterol as described in the legend to Fig. 6. Cells were washed and [^3H]cholesterol and [^3H]cholesteryl esters were isolated and quantified as described (18).

Cholesterol oxidase treatment

Cells were cultured and incubated with LDL that is labeled with [^3H]cholesteryl linoleate ([^3H]CL-LDL) as described in the legend to Fig. 7. Cells were treated with cholesterol oxidase using the method of Slotte et al. (22). Wells were then washed, lipids were extracted, and [^3H]cholesterol and [^3H]cholestenone quantified as described (23).

Statistical analysis

Statistical comparisons were made using a Student's *t*-test or Mann-Whitney test (VassarStats; <http://faculty.vassar.edu/~lowry/VassarStats.html>).

RESULTS

Development of the drug screen

The goal of this study was to identify compounds that alter the disease phenotype of cultured NPC cells. Since the biological function of NPC1 is unknown, we were unable to design a screen for compounds that would directly act on NPC1. Instead, we focused on a likely downstream effect of NPC1 activity, i.e., cholesterol transport from lysosomes to other cellular membranes. The screen for these compounds required an immortalized cell culture model for NPC disease and a biochemical assay that could be adapted for high throughput screening. The cell culture models that we used were simian virus 40-transformed mouse ovarian granulosa cell lines obtained from wild-type and *npc^{nih}* BALB/c mice (24, 25). The NPC1 gene in *npc^{nih}* BALB/c mice has a retrotransposon-like insertion that leads to prematurely truncated NPC1 protein of approximately 500 amino acids (15). The murine model exhibits a pathological and clinical phenotype resembling the classical form of the human disease and has a shortened life span (1, 15, 26). Cells cultured from the *npc^{nih}* mouse have the characteristic cholesterol storage and delayed movement of exogenous cholesterol to acyl-CoA cholesterol acyltransferase (ACAT) in the endoplasmic reticulum that is seen in human NPC fibroblasts (27).

An amphotericin B cytotoxicity assay was used to distinguish between NPC cells that express the disease phenotype and those that have had their cholesterol homeostasis restored by the action of a test compound. Polyene antibiotics, such as amphotericin B and filipin, complex with sterols and form pores, lysing cells that have a threshold amount of plasma membrane sterol (28, 29). The rationale behind the cytotoxicity assay is depicted in Fig. 1. Normal cells grown in lipoprotein-deficient serum for 24 h are relatively resistant to amphotericin B because the cholesterol content of the plasma membrane is reduced. When LDL is added to normal cells, LDL's cholesteryl esters are hydrolyzed in lysosomes. The cholesterol released is transported to the plasma membrane and renders the

cells amphotericin B sensitive (30). However, NPC cells survive amphotericin B treatment because LDL-cholesterol (LDL-C) transport to the plasma membrane is delayed (13). Our goal was to identify compounds that would promote the movement of LDL-C to the plasma membrane, rendering NPC cells amphotericin B sensitive.

We first adapted our standard amphotericin B protocol to be conducted on a smaller scale with shorter time incubations, which would permit high throughput screening. An experiment designed to illustrate amphotericin B killing of normal and NPC cells is shown in Fig. 2. In this experiment, normal and NPC cells were cultured for 24 h in HD-15% FCLPDS/sm. Growth in the absence of both endogenous cholesterol synthesis and an exogenous cholesterol supply lowers the plasma membrane cholesterol content. (Forty-eight hours of growth in this medium is needed for full amphotericin B survival of cultured cells (13); however, we needed to minimize the time required per assay.) Cells were then refed HD-15% FCLPDS/sm with or without 25 $\mu\text{g}/\text{ml}$ LDL. After 4 h, cells were either mock-treated or treated with amphotericin B. Cell survival was assessed using a colorimetric MTT assay. Normal cells grown in HD-10% FCS were killed by amphotericin B treatment (data not shown). Growth for 24 h in HD-15% FCLPDS/sm led to partial amphotericin B resistance of normal cells (Fig. 2), whereas the addition of LDL caused full amphotericin B sensitivity. NPC cells survived amphotericin B treatment, even after LDL addition. Higher concentrations of LDL and/or amphotericin B were required to confer any amphotericin B sensitivity to NPC cells (data not shown).

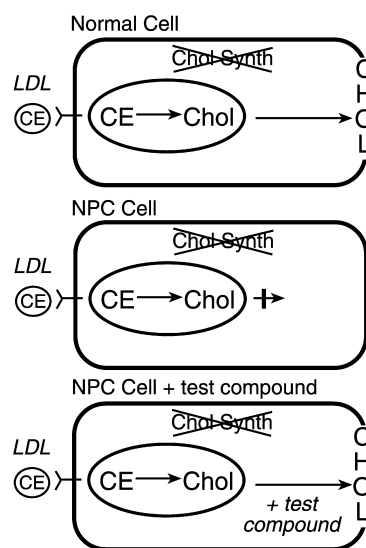


Fig. 1. Depiction of amphotericin B cytotoxicity screen. Cells are grown in HD-15% fetal calf lipoprotein-deficient serum (FCLPDS)/sm, which contains a statin to inhibit cholesterol synthesis. After 24 h, LDL is added and 4 h later the cells are treated with amphotericin B. Under these conditions, normal cells are killed because the LDL-derived cholesterol is delivered to the plasma membrane, whereas Niemann-Pick disease type C (NPC) cells survive because they have a defect in LDL-cholesterol (LDL-C) transport. Test compounds that stimulate LDL-C transport should cause NPC cells to become amphotericin B sensitive.

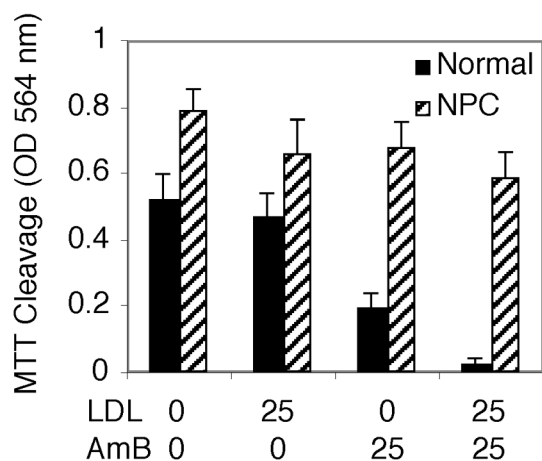


Fig. 2. Amphotericin B killing of normal and NPC cells. On day 0, cells were seeded in 96-well plates (15,000/well) in HD-10% FCS. After 6 h, cells were washed with HBSS and refed HD-15% FCLPDS/sm. On day 1, cells were refed HD-15% FCLPDS/sm with and without 25 $\mu\text{g}/\text{ml}$ LDL. After 4 h, cells were washed with HBSS and refed HD-1% FCLPDS with and without 25 $\mu\text{g}/\text{ml}$ amphotericin B (dissolved in Me_2SO). After 2 h, cells were washed and refed HD-10% FCS. On day 2, cell viability was determined using a colorimetric 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described under Experimental Procedures. Data are expressed as MTT cleaved (A564) per well and are the mean \pm S.D. of 4 or 6 wells.

Amphotericin B cytotoxicity screen

Our screen was designed to identify test compounds that stimulate LDL-C transport to the plasma membrane in NPC cells, i.e., cause amphotericin B cytotoxicity. Approximately 5,000 compounds from a primary screening deck were tested per week for a total of 44,240 compounds. The compounds were chosen to represent all

chemical classes. LDL-dependent amphotericin B-mediated cell killing in the presence of test compounds was compared with cell killing in the absence of test compounds and in the absence of amphotericin B. A histogram illustrating the frequency of the primary screening results is shown in **Fig. 3**. The cutoff threshold for the primary screen of 42,240 compounds was established and 1% of the primary test compounds that resulted in the highest percentage of killed cells were re-tested in duplicate. Next, the 64 positive compounds were re-tested at concentrations ranging from 0.032 to 100 μM and EC50s were calculated. The LDL-dependent amphotericin B-mediated killing of NPC cells in the presence of each test compound was compared with LDL-dependent amphotericin B-mediated killing of normal cells, as a gauge of the maximum killing that should occur in each assay. The EC50 curve for NP-27, one of the active compounds identified in the screening campaign, is illustrated in **Fig. 4**.

The final screen resulted in the identification of 55 compounds that caused LDL-dependent amphotericin B-mediated NPC cell killing, with EC50s ranging from 0.01 to 47.1 μM . Nine compounds were not evaluated further because they were cytotoxic. Subsequent analysis of the other compounds focused on assessing their ability to revert key aspects of the NPC biochemical phenotype.

Effect of compounds on cholesterol esterification

Cellular free cholesterol levels are kept within a tight range in normal cells by transcriptional control of cholesterol biosynthetic enzymes and the LDL receptor (LDLR) (31), as well as by activation of ACAT, an endoplasmic reticulum enzyme that catalyzes the esterification of a fatty acid to cholesterol for storage in cytoplasmic lipid droplets (32). NPC cells show no activation of ACAT activity when cellular cholesterol levels are increased by the addi-

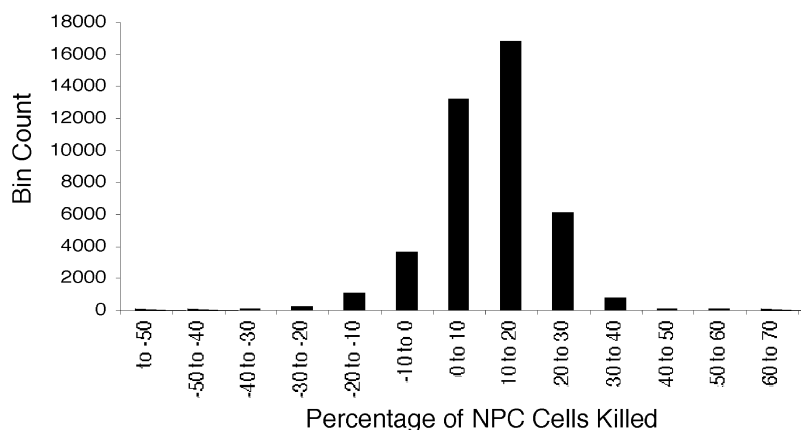


Fig. 3. Distribution of activity over the course of the high throughput screen run (42,240 primary synthetic compounds). On day 0, cells were seeded in 96-well plates (10,000/well) in HD-15% FCLPDS/sm. On day 1, cells were refed HD-15% FCLPDS/sm with 25 $\mu\text{g}/\text{ml}$ LDL. Test compounds were added to a final concentration of 15 μM in 1% Me_2SO . After 4 h, cells were washed with HBSS and refed HD-1% FCLPDS with or without 25 $\mu\text{g}/\text{ml}$ amphotericin B. After 2 h, cells were washed twice with HBSS and refed HD-10% FCS. On day 2, cell viability was assessed using a colorimetric MTT assay as described under Experimental Procedures. Data are in bins distributed between the minimum and maximum values. Each bin count on the y axis represents the number of occurrences within each bin range. The data are expressed as the percentage of NPC cells killed in the presence of amphotericin B and test compound. Each test compound is represented by a percent killed value on the chart. One percent of the test compounds that gave the highest percent killed values were chosen for further analysis after primary screening.

tion of LDL (8, 33). This aberrant response is diagnostic for NPC disease (1) and has been attributed to NPC1's proposed role in intracellular cholesterol transport (9, 34).

The effect of test compounds on basal and LDL-stimulated cholesterol esterification was assessed in normal and NPC cells. If compounds were acting by promoting the movement of endogenously-synthesized cellular cholesterol or LDL-derived cholesterol, we would expect the incorporation of [³H]oleate into cholesteryl [³H]oleate to be increased. Most of the compounds had no effect on cholesterol esterification; however, several compounds did stimulate cholesterol esterification. NP-25 had no effect on basal cholesterol esterification but increased the response to LDL in normal cells at 1.3 μ M (Fig. 5A), which was this compound's EC₅₀ in the amphotericin B assay. Unfortunately, NP-25 had no effect on cholesterol esterification in NPC cells. Two test compounds stood out in our analysis, NP-27 and NP-31. NP-27 stimulated basal cholesterol esterification in normal cells at its EC₅₀ of 1.47 μ M (Fig. 5B). NP-27 also stimulated basal esterification in NPC cells and brought the LDL-mediated response in the defective cells to that of normal cells. Higher concentrations of NP-27 were cytotoxic. NP-31 induced a significant LDL-stimulation of cholesterol esterification in NPC cells but not in normal cells (Fig. 5C).

Test compounds could cause increased cholesterol esterification by directly activating the enzyme, ACAT. How-

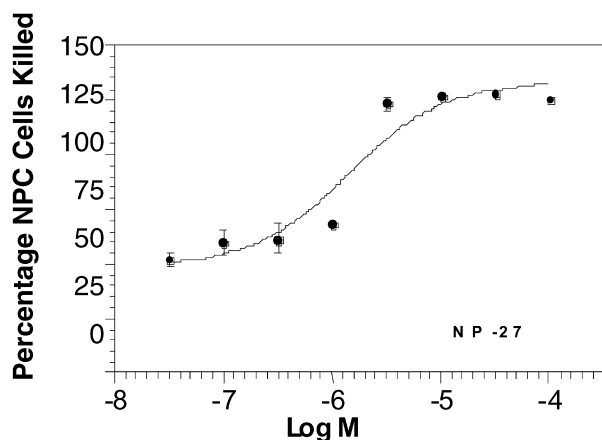


Fig. 4. Effect of NP-27 on amphotericin B cell killing in the high throughput screen. On day 0, cells were seeded in 96-well plates (10,000/well) in HD-15% FCLPDS/sm. On day 1, cells were refed HD-15% FCLPDS/sm with 25 μ g/ml LDL. Test compound was added at final concentrations ranging from 0.032 to 100 μ M in 1% Me₂SO. After 4 h, cells were washed with HBSS and refed HD-1% FCLPDS with or without 25 μ g/ml amphotericin B. After 2 h, cells were washed twice with HBSS and refed HD-10% FCS. On day 2, cell viability was assessed using a colorimetric MTT assay as described under Experimental Procedures. The data are expressed as percent of cells killed, using a calculation that compared the amphotericin B-mediated killing of NPC cells in the presence of NP-27 with the amphotericin B-mediated killing of normal cells without compound. This comparison allowed for the normalization of the test compound kill curve to the normal cell line. The EC₅₀ was calculated using the half maximum method and the result was 1.47 μ M. Data represent the mean \pm SD of duplicate wells.

ever, we found that NP-27 and NP-31 had no significant effect on in vitro ACAT activity in rat liver microsomes (data not shown). Test compounds may also appear to increase cholesterol esterification due to an effect on fatty acid metabolism, i.e., fatty acid pool size, fatty acyl-CoA formation, or triglyceride synthesis. For example, if a compound inhibited fatty acid synthesis, then the cellular fatty acid pool size would decrease and the specific activity of added [³H]oleate would not be diluted as much, resulting in in-

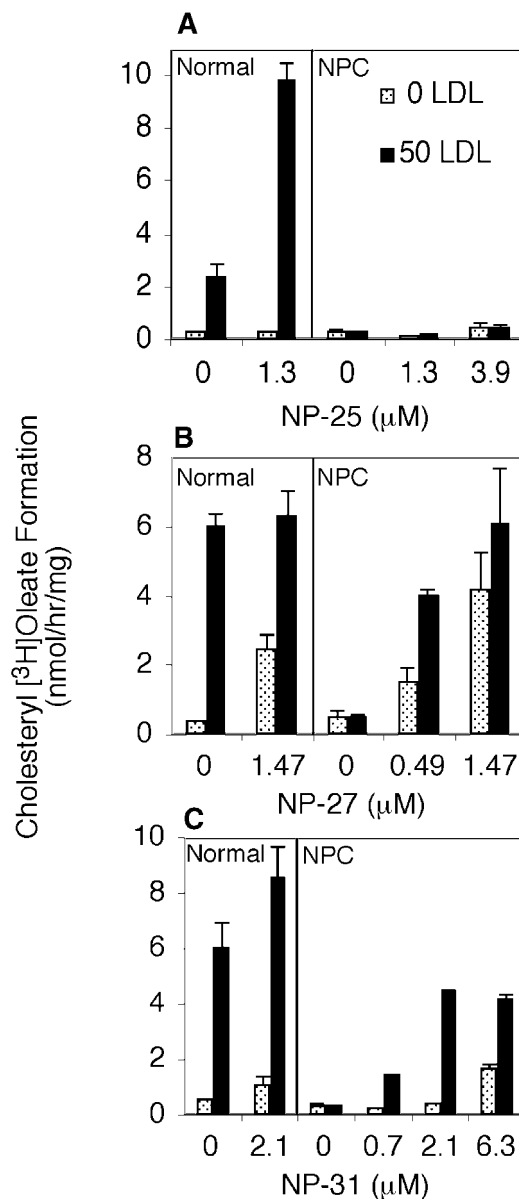


Fig. 5. Effect of NP-25 (A), NP-27 (B) and NP-31 (C) on cholesterol esterification. On day 0, normal and NPC cells were seeded in 6-well plates (25,000/well) in HD-10% FCS. The next day, cells were washed with HBSS and refed HD-15% FCLPDS. On day 2, cells were refed HD-15% FCLPDS with or without 50 μ g/ml LDL and containing the indicated concentration of compounds. After 18 h (day 3), cells were pulse-labeled with [³H]oleate for 1 h. The cellular content of cholesteryl [³H]oleate was determined as described under Experimental Procedures. Data are expressed as nmol cholesteryl [³H]oleate formed per h/mg protein and represent means \pm SD of triplicate cultures.

TABLE 1. Fatty acid synthesis in normal and NPC cells

Test Compound	³ H]Fatty Acid Synthesis	
	Normal Cells	NPC Cells
	% of 'no compound' control	
NP-27	137.8 ± 23.3	120.2 ± 21.0
NP-31	137.7 ± 32.7	135.1 ± 30.0

On day 0, cells were seeded in 12-well plates (20,000/well) in HD-10% FCS. On day 2, cells were refed HD-15% FCLPDS. On day 3, cells were refed 0.5 ml HD-15% FCLPDS and compounds were added at their EC50 (1.47 μM and 2.10 μM for NP-27 and NP-31, respectively). After 1 h, 30 μCi of [³H]acetate was added. After 2 h, cells were washed and the cellular content of [³H]fatty acids was determined as described in Experimental Procedures. The data are expressed as a percentage of the "no compound" controls, which were 416 dpm/μg and 418 dpm/μg for normal and NPC cells, respectively. The data represent the mean ± SD of three experiments.

creased cholesteryl [³H]oleate and [³H]triglyceride synthesis. However, Table 1 shows that NP-27 and NP-31 at their EC50s did not inhibit [³H]acetate incorporation into [³H]fatty acids. In fact, NP-27 and NP-31 elicited a small stimulation of fatty acid synthesis. Therefore, the increased rate of cholesterol esterification and triglyceride synthesis seen with these compounds is not secondary to an inhibition of fatty acid synthesis. NP-27 and NP-31 also elicited a stimulation of [³H]triglyceride synthesis (Table 2). Nevertheless, an effect on fatty acid metabolism cannot account for the observed stimulation of cholesterol esterification. Therefore, NP-27 appeared to increase cholesterol esterification by supplying both endogenously synthesized and LDL-derived cholesterol to the enzyme, whereas NPC-31 appeared to promote movement of LDL-derived cholesterol. Our further analysis focused on positive compounds NP-27 and NP-31.

Effect of compounds on cholesterol synthesis and transport pathways

One mechanism by which compounds might affect ACAT activity is by altering endogenous cholesterol synthesis. ACAT is allosterically activated by cholesterol (35) and we have found that statin inhibition of cholesterol

TABLE 2. [³H]oleate incorporation into [³H]triglycerides

Experiment	Test Compound	³ H]Triglyceride Formation	
		Normal Cells	NPC Cells
		nmol/h/mg	
1	None	10.5 ± 1.5	18.6 ± 5.2
	NP-27	18.0 ± 1.7	28.3 ± 5.8
2	None	11.1 ± 0.7	23.4 ± 1.7
	NP-31	18.8 ± 3.5	34.4 ± 6.4

On day 0, cells were seeded in 6-well plates (25,000/well) in HD-10% FCS. The next day, cells were washed with HBSS and refed HD-15% FCLPDS. On day 2, cells were refed HD-15% FCLPDS with or without 50 μg/ml LDL and containing compounds at their EC50 (1.47 μM and 2.10 μM for NP-27 and NP-31, respectively). After 18 h (day 3), cells were pulse-labeled with [³H]oleate for 1 h. The cellular content of [³H]triglycerides was determined as described under Experimental Procedures. The data are expressed as nmol [³H]triglyceride formed per h/mg protein and present mean ± SD of triplicate cultures.

synthesis blunts the ability of LDL to stimulate cholesterol esterification (36). Conversely, a compound that stimulates cholesterol synthesis may increase basal and/or LDL-stimulated cholesterol esterification. However, Table 3 shows that NP-25, NP-27, and NP-31, at their EC50s, had no effect on [³H]acetate incorporation into [³H]cholesterol.

Compounds might also shift cellular cholesterol pools or, in NPC cells, promote the release of stored lysosomal cholesterol. We first evaluated the effect of compounds on the basal movement of plasma membrane cholesterol to ACAT in the endoplasmic reticulum. Cultured cells incubated with [³H]cholesterol show a time-dependent incorporation of [³H]cholesterol into [³H]cholesteryl esters, reaching steady state at 10–12 h (18). Normal and NPC cells were incubated for 6 h with 1 μCi/ml of [³H]cholesterol along with compounds at their EC50. During the 6 h time interval, the cells adsorb the [³H]cholesterol and distribute it according to the cellular cholesterol pools. Figure 6 shows that, after 6 h, approximately 0.25% of the cholesterol had been metabolized to [³H]cholesteryl esters. None of the compounds had an effect on [³H]cholesteryl ester formation in normal cells. However, NP-27 stimulated [³H]cholesterol metabolism to [³H]cholesteryl esters in NPC cells 3-fold (*P* < 0.05). NP-31 had no effect in this assay.

We next evaluated the effect of compounds on the movement of LDL-derived [³H]cholesterol from lysosomes to the plasma membrane. Normal and NPC cells were incubated for 4 h with [³H]CL-LDL in the absence and presence of NP-27 and NP-31 at their EC50s. The amount of LDL-derived [³H]cholesterol that is accessible to an exogenously added cholesterol oxidase was quantified as a measure of [³H]cholesterol movement to the plasma membrane (18, 22, 23). When normal cells were incubated with [³H]CL-LDL for 4 h, 35% of the LDL-derived [³H]cholesterol was oxidizable, whereas only 20% of the [³H]cholesterol was oxidizable in NPC cells (Fig. 7). Neither NP-27 nor NP-31 had any effect on this assay in normal cells (data not shown). However, NP-27 increased the amount of LDL-[³H]cholesterol at the NPC cell plasma membrane to 33%, close to that of normal cells,

TABLE 3. Cholesterol synthesis in normal and NPC cells

Test Compound	³ H]Cholesterol Synthesis	
	Normal Cells	NPC Cells
	% of 'no compound' control	
NP-25	101.1 ± 18.0	111.8 ± 18.6
NP-27	96.9 ± 13.3	102.6 ± 24.3
NP-31	109.9 ± 14.7	111.3 ± 15.1

On day 0, cells were seeded in 12-well plates (20,000/well) in HD-10% FCS. On day 1, cells were refed HD-15% FCLPDS. On day 3, cells were refed 0.5 ml HD-15% FCLPDS and compounds were added at their EC50 (1.30 μM, 1.47 μM, and 2.10 μM for NP-25, NP-27, and NP-31, respectively). After 1 h, 30 μCi of [³H]acetate was added. After 2 h, cells were washed, cellular lipids were extracted and subjected to saponification, and the cellular content of [³H]sterol was determined as described in Experimental Procedures. The data are expressed as a percentage of the 'no compound' controls, which were 83.9 dpm/μg and 50.5 dpm/μg for normal and NPC cells, respectively. The data represent the mean ± SD of four experiments.

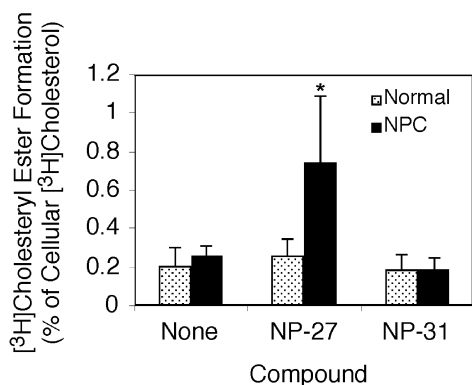


Fig. 6. Esterification of cellular [^3H]cholesterol. On day 0, normal and NPC cells were seeded in 12-well plates (20,000/well) in HD-10% FCS. On day 2, cells were refed HD-15% FCLPDS. On day 3, cells were refed HD-15% FCLPDS with or without NP-27 at 1.47 μM or NP-31 at 2.1 μM . [^3H]cholesterol (1 $\mu\text{Ci}/\text{ml}$) was added and the cells were incubated at 37°C for 6 h. Cells were then washed and the cellular content of [^3H]cholesterol and [^3H]cholesteryl ester was determined as described under Experimental Procedures. Data are expressed as the percentage of cellular [^3H]cholesterol metabolized to [^3H]cholesteryl esters and are the means \pm SD of four experiments. * $P < 0.05$ compared with “no compound” control.

whereas NP-31 had no effect. Therefore, NP-27 stimulated two separate pathways of intracellular cholesterol transport in NPC cells, but not in normal cells.

Effect of compounds on cholesterol storage and cell growth

The reduced kinetics of cholesterol movement from lysosomes to plasma membrane in NPC cells (Fig. 7) (21, 37, 38) results in lysosomal storage of free cholesterol, which can be visualized by filipin fluorescence microscopy. Filipin is a fluorescent polyene antibiotic that binds specifically to cholesterol and is used to detect cellular cholesterol pools (39). Normal cells cultured in HD-10% FCS exhibited filipin staining at the plasma membrane and in a punctate distribution, most likely representing endosomes and lysosomes, whereas NPC cells showed the intense perinuclear filipin staining characteristic of human NPC fibroblasts (data not shown). Compounds that stimulate cholesterol movement by restoring NPC1 function should cause clearance of lysosomally stored cholesterol, whereas compounds that act downstream of NPC1 may have no effect on storage.

We tested the ability of compounds to mobilize the stored cholesterol by culturing the NPC cells for 24 h in medium lacking LDL (HD-15% FCLPDS) and containing each compound at its EC50. Cholesterol storage was evaluated by fluorescence microscopy of filipin-stained cells, performed as described (40). This protocol led to no diminution of filipin fluorescence. We next tested longer incubation times with compounds at various concentrations and found that longer incubation with compounds at or above their EC50s led to significant cell death. The toxicity of NP-27 and NP-31 is shown in Fig. 8. In this experi-

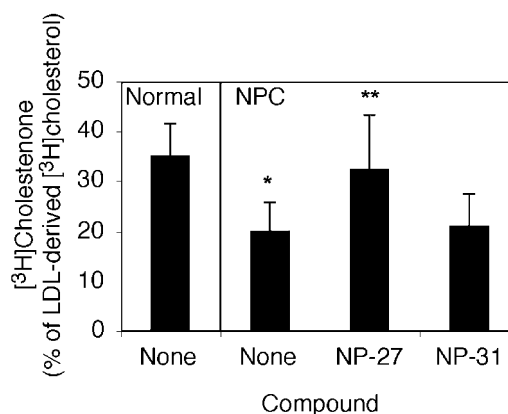


Fig. 7. Transport of LDL-[^3H]cholesterol to the plasma membrane. On day 0, normal and NPC cells were seeded in 6-well plates (30,000/well) in HD-10% FCS. On day 1, cells were refed HD-15% FCLPDS. On day 3, cells were refed HD-15% FCLPDS with or without NP-27 at 1.47 μM or NP-31 at 2.1 μM . After 30 min, 20 $\mu\text{g}/\text{ml}$ of LDL labeled with [^3H]cholesteryl linoleate ([^3H]CL-LDL) was added and the cells were incubated for 4 h. Cells were cholesterol oxidase-treated and LDL-[^3H]cholesterol conversion to [^3H]cholestenone was quantified as described under Experimental Procedures. Data represent the mean \pm S.D. of four experiments. * $P < 0.01$ compared with normal cells; ** $P < 0.05$ compared with NPC “no compound” control.

ment, normal cells were incubated for 4 days in media containing various concentrations of both compounds. NP-27 caused significant cell death at concentrations above 1 μM whereas NP-31 caused cell death at concentrations above 0.1 μM . Longer incubation times (up to 8 days) with compounds at lower, non-toxic concentrations resulted in no clearance of stored cholesterol, as visualized by fluorescence microscopy of filipin-stained cells. Compounds were equally cytotoxic in medium with and without LDL (data not shown).

DISCUSSION

NP-27 was identified in a high throughput screen for compounds that alter the biochemical phenotype of cultured NPC cells. In normal cultured cells, most of the LDL-C that is released from lysosomes is transported to the plasma membrane; from there it can cycle into the endoplasmic reticulum. However, NPC cells exhibit delayed movement of LDL-C out of lysosomes and lysosomal storage of cholesterol, which is diagnostic of NPC disease. In this study, we found that NP-27 corrected the NPC phenotype using four different measures of cholesterol homeostasis: *i*) NPC cells treated with NP-27 were more sensitive to killing by amphotericin B, *ii*) NP-27-treated NPC cells showed increased basal cholesterol esterification and near normal LDL-stimulation of cholesterol esterification, *iii*) NP-27 restored LDL-cholesterol movement from lysosomes to the plasma membrane in NPC cells, and *iv*) NP-27 stimulated plasma membrane cholesterol movement to the endoplasmic reticulum in NPC cells. The fact that NP-

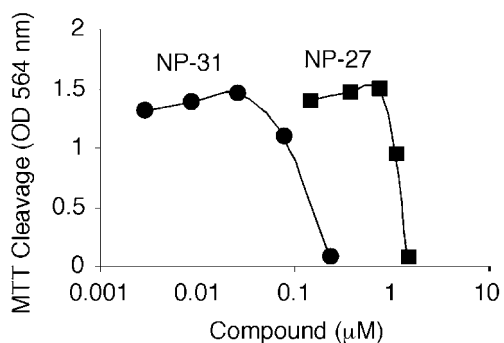


Fig. 8. Effect of NP-27 and NP-31 on cell growth. On day 0, normal and NPC cells were seeded into 96-well plates (1,000/well) in HD-10% FCS. On day 1, cells were refed HD-10% FCS with the indication concentration of compounds. On day 5, cell viability was determined using a colorimetric MTT assay as described under Experimental Procedures. Data are expressed as MTT cleaved (A564) per well and are the mean of 7 or 8 wells.

27 stimulated basal esterification indicates that the compound affects the movement of endogenously-synthesized cellular cholesterol as well as LDL-derived cholesterol.

NP-27 (**Fig. 9**) (hydrazinecarboximideamide, 2-[3-(5-nitro-2-furanyl)-1-[2-(5-nitro-2-furanyl)ethenyl]-2-propenylidene]) is a nitrofurans that is also named difurazone and nitrovin [CAS Registry # 804-36-4]. In cultured mammalian cells, the target of NP-27 action is unknown. The compound is not likely to directly activate NPC1, since NPC cells were derived from the *npc^{nih}* mouse model, which expresses a severely truncated NPC1 protein. Also, NP-27 probably does not promote non-specific cholesterol desorption from membranes, since the compound had no effect on cholesterol transport pathways in normal cells. Our data are consistent with a NP-27-induced release of lysosomal cholesterol in NPC cells, which would alter amphotericin B sensitivity of NPC cells and activate ACAT; however, the toxicity of NP-27 has precluded our ability to

detect any NP-27-induced clearance of stored lysosomal cholesterol. This may be due to the relative sensitivity of the assays. The [³H]cholesterol transport assays are capable of detecting even small, acute changes in the kinetics of cholesterol movement, whereas a major shift in lysosomal cholesterol mass may have to occur for detection by filipin fluorescence microscopy.

NPC disease is caused by mutations in either NPC1 (95%) or NPC2 (5%) genes. Individuals with NPC exhibit a vertical gaze palsy and progressive loss of motor skills, learning problems, dementia, and seizures, all of which signal central nervous system degeneration (1). NPC children often have an enlarged liver and prolonged jaundice at birth, which has allowed intensive early diagnosis in Western Europe. There, the disease frequency is estimated at one in 150,000 births (1). Onset of neurological symptoms is most common in pre-school years and affected individuals generally live into their teen years.

Currently, there is no definitive therapy for NPC disease. NPC does not affect lipoprotein levels or cause vascular disease. Nevertheless, existing therapies that target cholesterol disorders have been tested in NPC mouse models and NPC children. Erickson et al. (12) tested nifedipine and probucol on the *npc^{nih}* mouse model. Both drugs led to a reduction in liver unesterified cholesterol and increased liver esterified cholesterol, but there was no amelioration of disease onset. Similarly, introduction of a null mutation in the LDLR gene to *npc^{nih}* mice had no modifying effect on the onset of neurological symptoms (12). Patterson and colleagues found that treatment of NPC children with lovastatin, cholestyramine, and nicotinic acid resulted in a lowering of plasma cholesterol and hepatic unesterified cholesterol (41); however, they found no improvement in the clinical course of the disease (1). Also ineffective were human liver transplantation (42), bone marrow transplantation in the C57 *spm* mouse model of NPC (43), and combined bone marrow and liver transplantation in the *spm* mouse model (44). These stud-

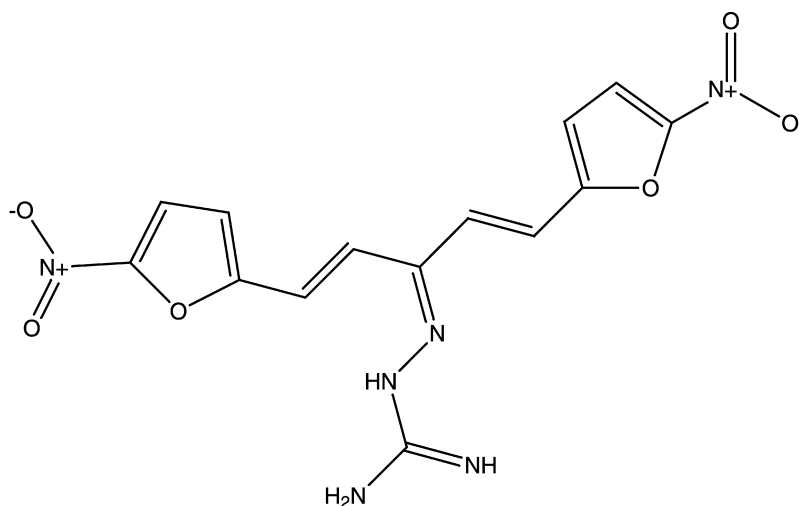



Fig. 9. Structure of NP-27. NP-27 (hydrazinecarboximideamide, 2-[3-(5-nitro-2-furanyl)-1-[2-(5-nitro-2-furanyl)ethenyl]-2-propenylidene]) is a compound of the nitrofurans class.

ies indicate that correcting the visceral disease is not enough; to be effective, a therapy for NPC must cross the blood-brain barrier. One protocol found to delay onset of neurological symptoms and increase life span of *npc^{nlh}* mice was treatment with *N*-butyldeoxyojirimycin, an inhibitor of glucosylceramide synthase (45). These results indicate that glycosphingolipid accumulation plays a role in the pathogenesis of NPC disease.

A critical test of NP-27 will be its evaluation in the NPC mouse model. That testing is precluded by the finding that the above effects all required micromolar concentrations of NP-27, which are toxic to cultured cells. To assess the action of this class of compounds on NPC disease progression, we need a structurally similar NP-27 analog that is biologically active at nanomolar concentrations. Our future work will focus on the structure activity relationship within the nitrofurans class of compounds to determine the key structural features of NP-27. 

This work was supported by grants to L.L. and S.L.S. from the Ara Parseghian Medical Research Foundation. L.L. was also supported by the National Institutes of Health (DK49564). Cell culture support was provided by the Center for Gastroenterology Research on Absorptive and Secretory Processes (NIDDK P30 DK34928). The authors gratefully acknowledge, at Bristol-Myers Squibb, Dr. Richard Gregg and Dr. John Wetterau for their continuous support during this collaboration. The authors also thank Dr. Scott Biller and Dr. Pratik Devasthale for their Structure-Activity Relationship support around the chemotypes as well as New Leads Biology during the screening campaign. Sohail Khan provided excellent assistance with the in vitro ACAT assay.

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