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

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 killing due to intracellular sequestration of cellular cholesterol, 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.

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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 increases 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 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]cholesterol linolate; 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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throughput screen for therapeutic compounds that might reverse or prevent the neurodegeneration. Indeed, 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), [3H]cholesterol (56 mCi/mmole), and [1,2,6,7-3H]cholesterol linolate (84 Ci/mmole) 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 µg/ml streptomycin and 25 mM HEPES, pH 7.3), HD-15% fetal calf lipoprotein-deficient serum (FCLPDS) and HD-1% FCLPDS (HD-15% FCLPDS/sm with 25 µg/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% MeSO. Controls were added in the remaining two columns of the plate. After 4 h, cells were washed with HBSS and re-fed HD-1% FCLPDS with or without 25 µg/ml amphotericin B. After 2 h, cells were washed twice with HBSS and re-fed HD-10% FCS. On day 2, cell viability was assessed using a colorimetric MTT assay. Cells were re-fed HD-10% FCS with 2.5 mg/ml MTT. After 2 h, the medium was aspirated and 100% MeSO added for 15 min. MTT cleavage was determined by reading the absorbance at 564 nm.

**[3H]Olate 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 [14C]cholesterol 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-ether-acetic acid (90:30:1, v/v/v). Correction for procedural losses was made by adding cholesteryl linoleate (20 µg), cholesteryl oleate (20 µg), and [14C]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 [14C]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 $[^{3}H]$cholesterol as described in the legend to Fig. 6. Cells were washed and $[^{3}H]$cholesterol and $[^{3}H]$cholesteryl esters were isolated and quantified as described (18).

Cholesterol oxidase treatment

Cells were cultured and incubated with LDL that is labeled with $[^{3}H]$cholesterol linoleate ($[^{3}H]$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 $[^{3}H]$cholesterol and $[^{3}H]$cholestenone were 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 model that we used was simian virus 40-transformed mouse ovarian granulosa cell lines obtained from wild-type and npe$^{nh}$ BALB/c mice (24, 25). The NPC1 gene in npe$^{nh}$ 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 npe$^{nh}$ 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 plus a statin (to inhibit endogenous cholesterol synthesis) 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 μg/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).
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 addition...
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 μM (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 μM (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. However, 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. 4](image-url) 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 μg/ml LDL. Test compound was added at final concentrations ranging from 0.032 to 100 μM in 1% Me2SO. After 4 h, cells were washed with HBSS and refed HD-1% FCLPDS with or without 25 μg/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 μM. Data represent the mean ± SD of duplicate wells.

![Fig. 5](image-url) 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 with or without 50 μg/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 ± SD of triplicate cultures.
TABLE 1. Fatty acid synthesis in normal and NPC cells

| Test Compound | Normal Cells | NPC Cells |
|---------------|--------------|-----------|
|               | [3H]Acetate Incorporation | [3H]Cholesterol Synthesis |
| % of ‘no compound’ control | nmol/h/mg | % of ‘no compound’ control | nmol/h/mg |
| 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 EC50s (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.

cells were pulse-labeled with [3H]oleate for 1 h. The cellular content of their EC50s did not inhibit [3H]acetate incorporation.

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 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]cholesterol ester formation in normal cells. However, NP-27 stimulated [3H]cholesterol metabolism to [3H]cholesterol 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 2. [3H]oleate incorporation into [3H]triglycerides

| Experiment | Test Compound | Normal Cells | NPC Cells |
|------------|---------------|--------------|-----------|
|            | [3H]Triglyceride Formation | nmol/h/mg | nmol/h/mg |
| 1          | None          | 10.5 ± 1.5   | 18.6 ± 5.2 |
|            | NP-27         | 18.0 ± 1.7   | 23.8 ± 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 in Experimental Procedures. The data are expressed as nmol [3H]triglyceride formed per h/mg protein and present mean ± SD of triplicate cultures.

TABLE 3. Cholesterol synthesis in normal and NPC cells

| Test Compound | Normal Cells | NPC Cells |
|---------------|--------------|-----------|
|               | [3H]Cholesterol Synthesis | % of ‘no compound’ control | nmol/h/mg |
| NP-25         | 107.1 ± 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.

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wheras 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 experiment, normal cells were incubated for 4 days in medium 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-
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 nitrofuran 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 npcnih 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 npcnih 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 npcnih 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-
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 npe<sup>ahl</sup> mice was treatment with N-butyloxyoxynojirimycin, 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 nitrofuran class of compounds to determine the key structural features of NP-27.

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REFERENCES

1. Patterson, M. C., M. T. Vanier, K. Suzuki, J. A. Morris, E. Carstea, E. B. Neufeld, J. E. Blanchette-Mackie, and P. G. Pentchev. 2001. Niemann-Pick Disease Type C: a lipid trafficking disorder. In The Metabolic and Molecular Bases of Inherited Disease. D. Valle, editor. Vol. III, 8th edition. McGraw-Hill, New York. 3611–3633.

2. Zervas, M., K. Dobrenis, and S. U. Walkley. 2001. Neurons in Niemann-Pick disease type C accumulate gangliosides as well as unesterified cholesterol and undergo dendritic and axonal alterations. J. Neuropathol. Exp. Neurol. 60: 89–64.

3. Carstea, E. D., J. A. Morris, K. G. Coleman, S. K. Lofus, D. Zhang, C. Cummings, J. Gu, M. A. Rosenfeld, W. J. Pavan, D. B. Krizman, J. Nagle, M. H. Polymeropoulos, S. L. Sturley, Y. A. Ioannou, M. E. Higgins, M. Comly, A. Cooney, A. Brown, C. R. Kaneski, E. J. Blanche-Mackie, N. K. Dwyer, E. B. Neufeld, T.-Y. Chang, L. Lissner, M. R. O’Neill, O. P. v. Diggelen, M. Elleder, M. C. Patterson, R. O. Brady, M. T. Vanier, P. G. Pentchev, and D. A. Tagele. (1997). Niemann-Pick C1 disease gene: homology to mediators of intracellular cholesterol homeostasis. Science. 277: 228–231.

4. Naureckiene, S., D. E. Sleat, H. Lackland, A. Fensom, M. T. Vanier, R. Wattiaux, M. Jadot, and P. Lobe. 2000. Identification of HEI as the second gene of Niemann-Pick C disease. Science. 290: 2298–2301.

5. Watari, H., E. J. Blanchette-Mackie, N. K. Dwyer, J. M. Glick, S. Patel, E. B. Neufeld, R. O. Brady, P. G. Pentchev, and J. P. Strauss 3rd. 1999. Niemann-Pick C1 protein: obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. Proc. Natl. Acad. Sci. U.S.A. 96: 805–810.

6. Neufeld, E. B., M. Wastney, S. Patel, S. Suresh, A. M. Cooney, N. K. Dwyer, C. F. Roff, K. Ohno, J. A. Morris, E. D. Carstea, J. P. Incardona, J. P. Strauss 3rd, M. T. Vanier, M. C. Patterson, R. O. Brady, P. G. Pentchev, and E. J. Blanchette-Mackie. 1999. The Niemann-Pick C1 protein resides in a vesicular compartment linked to retrograde transport of multiple lysosomal cargo. J. Biol. Chem. 274: 9627–9635.

7. Higgins, M. E., J. P. Davies, F. W. Chen, and Y. A. Ioannou. 1999. Niemann-Pick C1 is a late endosome-resident protein that transiently associates with lysosomes and the trans-Golgi network. Mol. Genet. Metab. 68: 1–13.

8. Pentchev, P. G., E. J. Blanchette-Mackie, and L. Liscum. 1997. Biological implications of the Niemann-Pick C mutation. Subcellular Biochemistry. 28: 457–451.

9. Liscum, L. 2000. Niemann-Pick type C mutations cause lipid traffic jam. Traffic. 1: 218–225.

10. Puri, V., R. Watanabe, M. Dominguez, X. Sun, C. L. Wheatley, D. L. Marks, and R. E. Pagano. 1999. Cholesterol modulates membrane traffic along the endocytic pathway in sphingolipid storage diseases. Nat. Cell Biol. 1: 386–388.

11. Davies, J. P., F. W. Chen, and Y. A. Ioannou. 2000. Transmembrane molecular pump activity of Niemann-Pick C1 protein. Science. 290: 2295–2298.

12. Erickson, R. P., W. S. Garver, F. Camargo, G. S. Hossain, and R. A. Heidenreich. 2000. Pharmacological and genetic modifications of somatic cholesterol do not substantially alter the course of CNS disease in Niemann-Pick C mice. J. Inherit. Metab. Dis. 23: 54–62.

13. Dahl, N. K., K. L. Reed, M. A. Daunais, J. R. Faust, and L. Liscum. 1992. Isolation and characterization of Chinese hamster ovary cells defective in the intracellular metabolism of LDL-derived cholesterol. J. Biol. Chem. 267: 1889–1896.

14. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. Methods Enzymol. 98: 241–262.

15. Lofus, S. K., J. A. Morris, E. D. Carstea, J. Z. Gu, C. Cummings, A. Brown, J. Ellison, K. Ohno, M. A. Rosenfeld, D. A. Tague, P. G. Pentchev, and W. J. Pavan. 1997. Murine model of Niemann-Pick C disease: Mutation in a cholesterol homeostasis gene. Science. 272: 229–235.

16. Hossmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65: 55–63.

17. Korting, H. C., S. Schindler, A. Hartinger, M. Kerscher, T. Angerhofer, and H. I. Maibach. 1994. MTT assay and neutral red release assay: Relative role in the prediction of the irritancy potential of surfactants. Life Sci. 53: 533–540.

18. Underwood, K. W., N. L. Jacobs, A. Howley, and L. Liscum. 1998. Evidence for a cholesterol transport pathway from lysosomes to endoplasmic reticulum that is independent of the plasma membrane. J. Biol. Chem. 273: 4266–4274.

19. Liscum, L., and J. R. Faust. 1987. Low density lipoprotein (LDL)-mediated suppression of cholesterol synthesis and LDL uptake is defective in Niemann-Pick type C fibroblasts. J. Biol. Chem. 262: 17002–17008.

20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.

21. Liscum, L., R. M. Ruggiero, and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niemann-Pick Type C fibroblasts. J. Cell Biol. 108: 1625–1636.

22. Slote, J. P., G. Hedstrom, S. Rannstrom, and S. Ekman. 1989. Effects of sphingomyelin degradation on cell cholesterol oxidizability and steady-state distribution between the cell surface and the cell interior. Biochim. Biophys. Acta. 985: 90–96.

23. Underwood, K. W., B. Andremariam, G. L. Williams, and L. Liscum. 1996. Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport. J. Lipid Res. 37: 1556–1568.

24. Amsterdam, A., I. Hanukoglu, B. S. Suh, D. Keren-Tal, D. Pfeh-Dujowich, R. Sprengel, H. Rennert, and J. F. Strauss. 1992. J. Steroid Biochem. Mol. Biol. 43: 875–884.

25. Gu, J. Z., E. D. Carstea, C. Cummings, J. A. Morris, S. K. Lofus, D. Zhang, K. G. Coleman, A. M. Cooney, M. E. Combi, L. Fandino, C. Roff, D. A. Tague, W. J. Pavan, P. G. Pentchev, and M. A. Rosenfeld. 1997. Substantial narrowing of the Niemann-Pick C candidate interval by yeast artificial chromosome complementation. Proc. Natl. Acad. Sci. U.S.A. 94: 7738–7783.

26. Liu, Y., T. F. Wu, R. Wada, E. B. Neufeld, K. A. Mullin, A. C. Howard, P. G. Pentchev, M. T. Vanier, K. Suzuki, and R. I. Proia. 2000. Allelation of neuronal ganglioside storage does not improve the clinical course of the Niemann-Pick C disease mouse. Hum. Mol. Genet. 9: 1087–1092.
27. Pentchev, P. G., M. E. Comly, H. S. Kruth, S. Patel, M. Proestel, and H. Weintroub. 1986. The cholesterol storage disorder of the mutant BALB/c mouse. A primary genetic lesion closely linked to defective esterification of exogenously derived cholesterol and its relationship to human type C Niemann-Pick disease. J. Biol. Chem. 261: 2772–2777.

28. Saito, Y., S. M. Chou, and D. F. Silbert. 1977. Animal cell mutants defective in sterol metabolism: A specific selection procedure and partial characterization of defects. Proc. Natl. Acad. Sci. USA. 74: 5730–5734.

29. Hidaka, K., H. Endo, S. Akiyama, and M. Kuwano. 1978. Isolation and characterization of amphotericin B-resistant cell lines in Chinese hamster cells. Cell. 14: 415–421.

30. Kieler, M., J. Martin, M. Segal, and D. Kingsley. 1983. Amphotericin B selection of mutant Chinese hamster cells with defects in the receptor-mediated endocytosis of low density lipoprotein and cholesterol biosynthesis. Proc. Natl. Acad. Sci. USA. 80: 5607–5611.

31. Brown, M. S., and J. L. Goldstein. 1999. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc. Natl. Acad. Sci. USA. 96: 11041–11048.

32. Chang, T. Y., C. C. Chang, and D. Cheng. 1997. Acyl-coenzyme A:cholesterol acyltransferase. Annu. Rev. Biochem. 66: 613–638.

33. Pentchev, P. G., R. O. Brady, E. J. Blanchette-Mackie, M. T. Vanier, E. D. Carstea, C. C. Parker, E. Goldin, and C. F. Roff. 1994. The Niemann-Pick C lesion and its relationship to the intracellular distribution and utilization of LDL cholesterol. Biochim. Biophys. Acta. 1225: 235–243.

34. Liscum, L., and J. J. Klansel. 1998. Niemann-Pick disease type C. Curr. Opin. Lipidol. 9: 131–135.

35. Cheng, D., C. C. Y. Chang, X-m. Qu, and T.Y. Chang. 1995. Activation of acyl:coenzyme A:cholesterol acyltransferase by cholesterol or by oxysterol in a cell-free system. J. Biol. Chem. 270: 685–695.

36. Liscum, L., and J. R. Faust. 1989. The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3β-[2-(diethylamino)ethoxy]androstan-5-en-17-one. J. Biol. Chem. 264: 11796–11806.

37. Argoff, C. E., M. E. Comly, J. Blanchette-Mackie, H. S. Kruth, H. T. Pye, E. Goldin, C. Kaneski, M. T. Vanier, R. O. Brady, and P. G. Pentchev. 1991. Type C Niemann-Pick disease: cellular uncoupling of cholesterol homeostasis is linked to the severity of disruption in the intracellular transport of exogenously derived cholesterol. Biochem. Biophys. Acta. 1096: 319–327.

38. Neufeld, E. B., A. M. Cooney, J. Pitka, E. A. Dawidowicz, N. K. Dwyer, P. G. Pentchev, and E. J. Blanchette-Mackie. 1996. Intracellular trafficking of cholesterol monitored with a cyclodextrin. J. Biol. Chem. 271: 21604–21613.

39. Blanchette-Mackie, E. J., N. K. Dwyer, L. M. Amendt, H. S. Kruth, J. D. Butler, J. Sokol, M. E. Comly, M. T. Vanier, J. T. August, R. O. Brady, and P. G. Pentchev. 1988. Type-C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes. Proc. Natl. Acad. Sci. USA. 85: 8022–8026.

40. Jacobs, N. L., B. Andemamariat, K. W. Underwood, K. Panchalingam, D. Sternberg, M. Kielian, and L. Liscum. 1997. Analysis of a Chinese hamster ovary cell mutant with defective mobilization of cholesterol from the plasma membrane to the endoplasmic reticulum. J. Lipid Res. 38: 1973–1987.

41. Patterson, M. C., A. M. Di Bisceglie, J. J. Higgins, R. B. Abel, R. Schiffmann, C. C. Parker, C. E. Argoff, R. P. Grewal, K. Yu, and P. G. Pentchev. 1993. The effect of cholesterol-lowering agents on hepatic and plasma cholesterol in Niemann-Pick disease type C. Neurology. 43: 61–64.

42. Gartner, J. C., Jr., J. Bergman, J. J. Malatack, B. J. Zitelli, R. Jaffe, J. B. Watkins, B. W. Shaw, S. Iwatsuki, and T. E. Starzl. 1986. Progression of neurovisceral storage disease with supranuclear ophthalmoplegia following orthotopic liver transplantation. Pediatrics. 77: 104–106.

43. Sakiyama, T., M. Tsuda, M. Owada, K. Joh, S. Miyawaki, and T. Kitagawa. 1983. Bone marrow transplantation for Niemann-Pick mice. Biochem. Biophys. Res. Commun. 113: 605–610.

44. Yasumizu, R., S. Miyawaki, K. Sugiura, T. Nakamura, Y. Ohnishi, R. A. Good, Y. Hamashima, and S. Ikehara. 1990. Allogeneic bone marrow-plus-liver transplantation in the C57BL/KsJ spm/spm mouse, an animal model of Niemann-Pick disease. Transplantation. 49: 759–764.

45. Zervas, M., K. L. Somers, M. A. Thrall, and S. U. Walkley. 2001. Critical role for glycosphingolipids in Niemann-Pick disease type C. Curr. Biol. 11: 1283–1287.