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Inhibitors of 7-Dehydrocholesterol Reductase: Screening of a Collection of Pharmacologically Active Compounds in Neuro2a Cells

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Abstract

A small library of pharmacologically active compounds (the NIH Clinical Collection) was assayed in Neuro2a cells to determine their effect on the last step in the biosynthesis of cholesterol, the transformation of 7-dehydrocholesterol (7-DHC) to cholesterol promoted by 7-dehydrocholesterol reductase, *DHCR7*. Of some 727 compounds in the NIH Clinical Collection, over 30 compounds significantly increased 7-DHC in Neuro2a cells when assayed at 1 μ M. Active compounds that increased 7-DHC with a *Z*-score of +3 or greater generally gave rise to modest decreases in desmosterol and increases in lanosterol levels. Among the most active compounds identified in the library were the antipsychotic, antidepressant, and anxiolytic compounds that included perospirone, nefazodone, haloperidol, aripiprazole, trazodone, and buspirone. Fluoxetine and risperidone were also active at 1 μ M, and another 10 compounds in this class of pharmaceuticals were identified in the screen at concentrations of 10 μ M. Increased levels of 7-DHC are associated with Smith-Lemli-Opitz syndrome (SLOS), a human condition that results from a mutation in the gene that encodes *DHCR7*. The SLOS phenotype includes neurological deficits and congenital malformations, and it is linked to a higher incidence of autism spectrum disorder. The significance of the current study is that it identifies common pharmacological compounds that may induce a biochemical presentation similar to SLOS. Little is known about the side effects of elevated 7-

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.6b00054. Structures of compounds that are active at 1 and 10 μ M in Neuro2a cells and a list of compounds that are active at 1 μ M in *Dhcr7*-deficient Neuro2a cells (PDF)

Notes

The authors declare no competing financial interest.

DHC postdevelopmentally, and the elevated 7-DHC that results from exposure to these compounds may also be a confounder in the diagnosis of SLOS.

Graphical abstract

INTRODUCTION

The inhibition of cholesterol biosynthesis has been of longstanding interest.¹ The development of statins, the biggest selling pharmaceuticals of all time, is one of the triumphs of the modern pharmaceutical industry.² These compounds inhibit the transformation of acetate into mevalonate, controlling the entire biosynthesis pathway, as shown in Figure 1. The inhibition of subsequent steps in the cholesterol biosynthesis pathway has also been explored as a strategy for controlling cholesterol levels, but this approach has generally failed. Many compounds that inhibit late-stage sterol transformations promoted by 7-dehydrocholesterol reductase (DHCR7), for example, have failed as drugs because of the teratogenicity of these compounds.³ AY9944 [*trans*-1,4-bis(2-chloro-benzylaminomethyl)] is one example of a small molecule teratogen that inhibits the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol and 7-dehydrosmosterol (DHD) to desmosterol (Des) as shown in Figure 1. Cholesterol levels are significantly reduced, and 7-DHC levels are increased in animals exposed to AY9944. Exposure of pregnant animals to this compound results in miscarriage, preterm births, and severe physical deformities of any offspring that survive.³

These AY9944-treated rats serve as a rodent pharmacological model for Smith-Lemli-Opitz syndrome (SLOS),⁴⁻⁹ a human condition that results from mutations in the gene that encodes DHCR7.¹⁰⁻¹⁷ This recessive disorder is biochemically characterized by both higher levels of 7-DHC and lower levels of cholesterol than is considered normal in all tissues. The range of severity in SLOS is wide, and it depends on the *DHCR7* mutation inherited, over 150 of which have been reported.^{18,19} The consequence of the genetic defect is a broad spectrum of phenotypes that include congenital malformations and neurological deficits, with more than 50% of SLOS cases receiving the diagnosis of autism spectrum disorder.^{20,21} There is an unusually high carrier frequency for this recessive disorder. Recent studies estimate that about 1% of the population carry one copy of a mutant *DHCR7* gene (1 in 30 to 1 in 100).^{17,22}

While small molecule inhibitors of DHCR7 have been the object of extensive pharmacological investigation and have been used to create rodent models for SLOS,⁴⁻⁷ it has only been in recent years that a measurable link between prescribed pharmaceuticals in a

population and the perturbation of sterol homeostasis has been reported. In a recent study,²³ medical records of patients with elevated plasma 7-DHC, but a false-positive SLOS diagnosis, were linked to prescribed aripiprazole, an atypical antipsychotic or trazodone, an antidepressant. The authors of this study note that both aripiprazole and trazodone are listed as Category C drugs during pregnancy,²³ which means that reproduction studies have shown an adverse effect on the fetus but that potential benefits may warrant use of the drug in pregnant women despite potential risks.

Given the links between 7-DHC, the effect of small molecules on DHCR7, and the devastating human disorder SLOS, it seems important to know whether other prescribed pharmaceuticals affect this important biochemical transformation. This question stimulated us to initiate a search for small molecules of clinical importance that affect cholesterol biosynthesis at the DHCR7 step. We report here the results of a screen of some 730 compounds in the NIH Clinical Collection, 5% of which at 1 μ M give rise to a significant increase of 7-DHC in Neuro2a cells.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich Co (St. Louis, MO). HPLC grade solvents were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). All cell culture reagents were from Mediatech (Manassas, VA), Life Technologies (Grand Island, NY), and Greiner Bio-One GmbH (Monroe, NC). Standard α_7 -Chol was purchased from Medical Isotopes, Inc., and α_7 -7-DHC, $^{13}\text{C}_3$ -Lan, and $^{13}\text{C}_3$ -Des were synthesized as previously described.^{24,25}

Cell Cultures and the Screening Method: Neuro2a

The neuroblastoma cell line Neuro2a was purchased from American Type Culture Collection (Rockville, MD). Neuro2a were maintained in DMEM supplemented with L-glutamine, 10% fetal bovine serum (FBS; Thermo Scientific HyClone, Logan, UT), and penicillin/streptomycin at 37 °C and 5% CO_2 . Chemicals were deposited in a 384 cell culture plate (Greiner Bio-One) using a Labcyte Echo 550/555 (Sunnyvale, CA) liquid handler equipped in the Vanderbilt University High-throughput Screening Facility. Neuro2a cells were trypsinized, counted, resuspended in DMEM medium with reduced serum (0.5% FBS), and dispensed to 384-well plates (on top of predeposited compounds) using Multidrop Combi (ThermoScientific). Plating density was $5 \times 10^4/\text{cm}^2$. After 24 h of incubation at 37 °C and 5% CO_2 , Hoechst dye (Molecular Probes) was added using Multidrop Combi and incubated at room temperature for 30 min in the dark. Cells were imaged using ImageXpress Micro XL (Molecular Devices, Sunnyvale, CA) with a 10 $\times$ objective. Following imaging, the medium was removed, and 80 μ L of MeOH containing internal standards (0.0165 nmol for α_7 -7-DHC, 0.125 nmol for $^{13}\text{C}_3$ -Des, and 0.115 nmol for $^{13}\text{C}_3$ -Lan/well) was added using the Multidrop Combi. The 384-well plate was placed on a rotary shaker for 20 min at room temperature and then centrifuged for 10 min using a Sorvall swing rotor. The supernatant was transferred by an automated liquid handler Agilent Velocity 11 Bravo to a 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD)-predeposited plate (Waters #186002631, 100 μ L

flat bottomed). The method of analysis is based on a derivatization of 7-DHC in a Diels–Alder reaction with PTAD, and the details of the derivatization procedure have been previously described.^{25,26} The plates were immediately sealed with Easy Pierce Heat Sealing Foil (ThermoScientific AB-1720), followed by 20 min of agitation at room temperature. The sealed plates were kept in –80 °C until LC-MS analysis.

LC-MS (SRM) Analysis

The sealed plates were placed in an Acquity UPLC system equipped with ANSI-compliant well plate holder. Ten microliters was injected onto the column (Acquity UPLC BEH C18, 1.7 μm , 2.1 $\times$ 50 mm) with 100% MeOH (0.1% acetic acid) mobile phase for 1 min runtime at a flow rate of 300 $\mu\text{L}/\text{min}$. The monitored transitions included 7-DHC 560 $\rightarrow$ 365, d_7 -7-DHC 567 $\rightarrow$ 372, desmosterol 592 $\rightarrow$ 365, $^{13}\text{C}_3$ -desmosterol 595 $\rightarrow$ 368, lanosterol 634 $\rightarrow$ 602, and $^{13}\text{C}_3$ -lanosterol 637 $\rightarrow$ 605 with retention times of 0.8, 0.5, and 0.6 min, respectively.

Lipid Extraction, Separation, and GC-MS Analyses of Sterols in Cells

Verification of the hits obtained from the initial screening was carried out with varying concentrations of the compounds of interest. In addition, a more complete sterol profile was determined. The experiment was carried out in 96 well plates as described above for the 384 well plates. After counting cells on ImageXpress Micro XL (10X objective), the medium was removed, the cells were washed with PBS, and the buffer was removed. The plates were stored at –80 °C. To each well was added 10 μL of BHT/TPP solution (2.5 mg of TPP and 1 mg of BHT in 1 mL of EtOH), 10 μL of internal standard solution (0.87 nmol for d_7 -Chol, 0.033 nmol for d_7 -7-DHC, 0.25 nmol for $^{13}\text{C}_3$ -Des, and 0.23 nmol for $^{13}\text{C}_3$ -Lan/well), and 200 μL of MeOH. The plate was placed on a rotary shaker for 20 min at room temperature. An aliquot (100 μL) of the supernatant was transferred to a PTAD-predeposited plate, sealed with Easy Pierce Heat Sealing Foil, followed by 20 min of shaking at room temperature, and analyzed by LC-MS as described above. The flow rate was increased to 500 $\mu\text{L}/\text{min}$ and run time to 1.5 min to include cholesterol analysis (Chol 369 $\rightarrow$ 369 and d_7 -Chol 376 $\rightarrow$ 376). The remaining sample in each well was transferred to vials and concentrated on a SpeedVac concentrator for GC-MS analysis. To each vial was added *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, 50 μL), vortexed well, and allowed to react for 30 min. The sample (5 μL) was injected onto the column (SPB-5, 0.25 μm , 0.32 mm $\times$ 30 m) with a temperature program of 220–300° (5 min) at 20°/min and helium flow rate of 2.0 mL/min. The data were collected in full scan mode and the following ions extracted for quantitation relative to d_7 -Chol: 458 for cholesterol, zymosterol, lathosterol; 456 for zymosterol, dehydrolathosterol; 349 (M-105) for dehydrodesmosterol; 393 (M-105) for lanosterol; 395 (M-105) for dihydrolanosterol; 486 for dimethylzymosterol; and 465 for d_7 -cholesterol. Desmosterol and 7-DHC were not analyzed by GC-MS because they coelute. The remaining sterol intermediates were not detected. All data were normalized to cell count.

Statistical Analysis

All calculations of variability, standard deviation (SD), and normalized SD were performed in Microsoft Excel or GraphPad Prism. In addition, we calculated screening window

coefficients, “Z” and “Z’-Score”.²⁷ These factors reflect the assay dynamic range and data variation associated with the measurements.

RESULTS

Recent advances in methods for sterol analysis now permit the screening of modest-sized libraries of pharmaceutical agents to identify compounds that alter cellular levels of 7-DHC and other cholesterol precursors. These methods rely on a sterol derivatization with the dienophile 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) and a UPLC-MS protocol that permits the assay of several sterols in a run-time of less than a minute.^{25,26} Deuterium and ¹³C labeled sterols, synthesized in our laboratories, facilitate the analysis of sterols in cell culture by standard isotope dilution methods. Our analytical protocol thus includes the addition of known amounts of isotopically labeled *d*₇-cholesterol, *d*₇-7-DHC, and ¹³C₃-desmosterol to a lysate of cells in culture that have been previously exposed to a pharmaceutical agent for 24 h. Determination of 7-DHC and desmosterol levels by UPLC-MS analysis with simultaneous selected reaction monitoring (SRM) for endogenous sterols and exogenous isotopically labeled standards along with HTS cell counting of a 384-well plate provides a readout of compound activity on cholesterol biosynthesis at the 7-DHC → cholesterol or desmosterol → cholesterol steps. Lanosterol was also determined in our analysis by comparison of endogenous lanosterol levels with added ¹³C₃-lanosterol. Picomolar levels of 7-DHC, desmosterol, and lanosterol could be measured, among other sterols, making analysis of a few thousand cells or 20 μ L of human plasma relatively straightforward. Neuro2a cells, a mouse neuroblastoma cell line frequently used in the neuroscience community to model neuronal cells, were used in this study. The cells double in about 24 h, and they can be readily adapted for use in a 96- or 384-well format.

The ~730 compounds in the NIH Clinical Collection were distributed on three 384-well plates. Each plate also had 24 control wells with DMSO only, and there were 10 wells each for reference compounds that included aripiprazole (100 nM) and pitavastatin (100 nM). Duplicates of the three plates were prepared, and the six plates were analyzed by the UPLC-MS method. The reliability of the screen on each plate was assessed by the determination of *Z*’ values by comparison of 7-DHC levels measured in the multiple control and aripiprazole wells.²⁸ Figure 2 shows the 7-DHC levels in the DMSO control and 100 nM aripiprazole wells for the six plates along with the calculated *Z*’s, all of which fall in the 0.2–0.6 range generally considered acceptable for an HTS screen but which suggest rescreening of hits for validation.²⁸ In order to maximize the number of hits of potentially active compounds, we chose a library concentration of 1 μ M for our standard 384-well screens, an order of magnitude higher than the “aripiprazole standard” used to determine *Z*’s but a concentration that is still pharmacologically relevant.

Figure 3 presents the results of a 384-well plate screen at 1 μ M of 320 of the compounds in the NIH Clinical Collection. Neuro2a cells were treated with compounds for 24 h and then counted automatically, sterols were determined by UPLC-MS, and the cellular level of 7-DHC in nanomol/million cells was then converted to a *Z*-score²⁸ so that multiple plates could be compared.

Aripiprazole was found to reproducibly increase levels of 7-DHC and reduce levels of desmosterol in each plate analyzed, while pitavastatin reduced levels of all sterols analyzed; see Figure 3 in which aripiprazole data are shown in red and pitavastatin in green. These observations are consistent with these drugs acting on DHCR7 and HMGCR, respectively, in the cholesterol biosynthetic pathway. Analysis of the data for the duplicate screen of the entire NIH Clinical Collection indicated that some 34 compounds in the library were found to increase 7-DHC levels above a threshold Z-score of +3. One of the compounds identified as being active in the initial 384-well screen (Captopril) was not validated in the triplicate rescreen of these hits. Several of the 34 compounds identified in the screen were antipsychotic, antidepressant, or anxiolytic medications, an observation that led us to separately screen all compounds in the collection that have antipsychotic, antidepressant, or anxiolytic properties. This screening was done in triplicate at 1 and 10 μM on 96-well plates, and three additional compounds were identified that had a significant effect on 7-DHC levels at 1 μM in Neuro2a cells.

The 37 compounds that gave significant increases in 7-DHC in Neuro2a cells at 1 μM (Z-score $> +3$) are presented in Figure 4 in the order of decreasing 7-DHC levels found in the assay, and the structures of these compounds are presented in Supporting Information (Table S1). Figure 4 also presents compounds in the Collection that affect levels of desmosterol (Figure 4B) and lanosterol (Figure 4C) in Neuro2a cells. Background desmosterol levels in Neuro2a cells are high, and the increase in desmosterol caused by small molecules in the library is attenuated compared with the increases observed for 7-DHC and lanosterol, both of which have very low background levels. This is shown in Figure 4 in which the maximum desmosterol Z-score difference for treated and untreated Neuro2a cells is less than +10 (Figure 4B), while Z-scores for 7-DHC and lanosterol (Figures 4A and C) are as high as +300.

Most of the compounds that significantly increase 7-DHC levels in Neuro2a cells (shown in Figure 4A) also show a modestly elevated level of lanosterol as well as a reduced level of desmosterol after treatment. This is shown in Figure 5 for triplicate experiments in which Neuro2a cells were assayed for sterols after exposure to 1 μM of drug for 24 h. Five of the six compounds shown in the Figure, perospirone, aripiprazole, trazodone, ifenprodil, and risperidone give results typical of most of the hit compounds shown in Figure 4A. In contrast to most of the compounds, 1 μM nefazodone gives rise to a sterol profile in which desmosterol is also elevated as a result of the incubation. Of the active compounds identified in the 384-well screen shown in Figure 4A, only nefazodone, dofetilide, and fluoxetine give rise to elevated levels of both 7-DHC and desmosterol, all other compounds show a small decrease in desmosterol levels. Cholesterol in Neuro2a cells is only marginally affected as a result of a 1 μM exposure to any of the hit compounds shown in Figure 4A during the course of a 24 h incubation.

When exposure concentrations of antidepressant/antipsychotic medications in Neuro2a are increased to 10 μM , elevated 7-DHC with a Z-score greater than 3 is found for treatments with 10 additional drugs. In the order of decreasing activity, the compounds found to be active at 10 μM are bupropion, sertraline, nortriptyline, clomipramine, maprotiline, amitriptyline, desipramine, amoxapine, imipramine, and mosapride, and their structures are

presented in Table S2. It should be noted that there is associated toxicity with incubations at 10 μ M concentrations for most of these compounds, as seen by a decrease in cell numbers for the treated cells compared to the controls after 24 h. This is in contrast to the experiments at 1 μ M where toxicity was not an issue for the active compounds, and cell numbers were unaffected by any of the compounds screened at this concentration. Fluoxetine (prozac) and sertraline (zoloft) show significant toxicity at 10 μ M with particularly low cell numbers found in Neuro2a after 24 h.

At the same time that the NIH Clinical Collection was screened in Neuro2a cells, a duplicate screen of the same library was carried out with *Dhcr7*-deficient Neuro2a cells that have been used as a cellular model for SLOS. 7-DHC levels in these *Dhcr7*-deficient cells are significantly higher than that in control Neuro2a, and they have previously proven useful in screens to identify small molecules that decrease levels of 7-DHC in cell culture.²⁵ Small molecules that interrupt the biosynthesis at the lanosterol or zymosterol intermediate stages, for example (see Figure 1), suppress levels of 7-DHC and can be identified from the *Dhcr7*-deficient cells in culture.

Screens of the NIH Clinical Collection with *Dhcr7*-deficient cells identified several compounds in the library that caused a significant increase of 7-DHC in this cell line. Table 1 presents a list of drugs that increase levels of 7-DHC only in control Neuro2a and in both control and *Dhcr7*-deficient Neuro2a cells. Thirteen compounds were active in both cell lines with Z scores greater than +3 (99.9% confidence) for both, while 24 compounds caused a significant increase of 7-DHC only in Neuro2a cells. Ten compounds increased 7-DHC in *Dhcr7*-deficient Neuro2a cells alone (see Table S3), but Z-scores were only modest, and the compounds showed little activity in control Neuro2a cells.

DISCUSSION

SLOS and SLOS Pharmacological Rodent Models

7-Dehydrocholesterol accumulates in tissues and fluids of patients with Smith-Lemli-Opitz syndrome, a recessive neurodevelopmental disorder caused by mutations in the gene encoding 3-hydroxysterol-reductase (DHCR7; EC 1.3.1.21). Although the exact mechanism of the syndrome is not understood, cholesterol deficiency and/or the buildup of 7-DHC is generally thought to be critical to the pathobiology of SLOS. An animal model of SLOS⁴⁻⁸ has been developed by treating pregnant rats with AY9944, a selective inhibitor of DHCR7, the same enzyme that is affected in SLOS. Levels of 7-DHC are elevated, and cholesterol is reduced in tissues and fluids of the AY9944 rat pups, and the same biochemical hallmarks that are found in the *Dhcr7*-null mouse. 7-DHC derived oxysterols have also been isolated from the brain, liver, and serum from the *Dhcr7*-null mouse and the AY9944 rat, both of which have been used extensively to study the phenotype and pathogenesis of SLOS.²⁹

We reason that any pharmacological perturbation that gives rise to a sterol profile similar to that found in SLOS will likely have significant negative neurodevelopmental consequences, similar to the syndrome. The AY9944 rat model for SLOS is an exhibit in support of this notion and suggests that an effort is warranted to identify small molecules that increase 7-DHC levels and alter sterol homeostasis. Human exposure to environmental agents or

pharmaceuticals that increase 7-DHC and reduce cholesterol levels will likely have serious health consequences, particularly during pregnancy. Indeed, a recent clinical study finding that elevated plasma 7-DHC is associated with prescribed aripiprazole and trazodone “provides some information that may be relevant to human risk for teratogenic effects of these drugs”, according to the authors of that investigation.²³

The structures of AY9944, aripiprazole, and trazodone are presented in Figure 6. The three compounds do have similar features, including a central diamine substructure and terminal chloroaromatic units, and they each cause a significant increase in 7-DHC in Neuro2a cell cultures at 10 nM and above.²⁵ A more substantive understanding of the structural elements that are essential for activity in this screen is the topic of ongoing studies in our laboratory and will be reported in due course.

Screening Methods for 7-DHC

Powerful methods have been developed for the analysis of sterols, including the biosynthetic precursors to cholesterol shown in Figure 1.^{30–32} We have recently developed a protocol for 7-DHC analysis that involves derivatization of the conjugated diene in a Diels–Alder reaction with the dienophile PTAD.²⁵ The procedure when applied to a library screening involves the exposure of an appropriate cell to the library of small molecules, cell lysis in methanol with added isotopically labeled standards, followed by reaction of 7-DHC and its isotopic counterpart with PTAD at room temperature for 30 min. The PTAD-7-DHC adduct can be readily detected by selected reaction monitoring (SRM) in a triple quadrupole mass spectrometer after reverse phase chromatography with an isocratic methanol solvent. Background levels of 7-DHC can be readily detected in 2000 Neuro2a cells, and an added benefit of the method is the detection of “ene” type PTAD products of desmosterol, lanosterol, and any sterol in the Bloch biosynthesis pathway that has a 24 double bond if the appropriate isotopic standards are available.²⁶ Indeed, efforts are ongoing to synthesize the isotopic standards for 24 dehydrosterols like zymosterol and dehydrodesmosterol, but these compounds were not available at the time of this study. While the PTAD method does not permit a truly “high throughput” screen, it currently makes possible an assay of lanosterol, desmosterol, 7-DHC, and cholesterol in cells exposed to a small library such as the NIH Clinical Collection.²⁵

We note that our initial 384-well screen failed to identify three compounds as “actives” that were found in a subsequent screen that was limited to the 33 antipsychotic medications in the library. While two of the new hits had only modest activity, the third compound (nefazodone) was among the most potent compounds found in the library. The initial screen also gave a false-positive identification to one of the 34 hits (Captopril) found in the library. A greater understanding of structure–activity may help direct future screening strategies by using chemical structure as a predictor of likely hits or raise questions about false discovery for compounds in a library. At any rate, structural considerations, replicate analyses, and extensive validation are suggested when using this relatively new methodology. If a number of hits are identified in a compound class in a broad screen, for example, an abundance of caution suggests that a follow-up based upon structure or compound class would be a prudent investment of additional effort.

Pharmaceutical Agents That Affect 7-DHC in Neuro2a

It is of note that prominent among the list of active compounds are several prescribed antipsychotic, antidepressant, and anxiolytic medications. Perospirone, nefazodone, haloperidol, aripiprazole, and trazodone are particularly potent in control Neuro2a cells with melperone, fluoxetine, and risperidone showing less but still significant potency. The effect of active compounds on 7-DHC levels in Neuro2a cells is proportionately greater than the effect observed in the *Dhcr7*-deficient cell line; see Table 1. Aripiprazole as low as 50 nM, for example, can cause an increase in 7-DHC in control Neuro2a from 0.2 ng/ μ g protein at background to ~25 ng/ μ g protein, at nearly the same levels that are found in *Dhcr7*-deficient cells.²⁶ 7-DHC levels are, of course, significantly higher in the *Dhcr7*-deficient cells than in control Neuro2a, and the added effect of a drug on deficient cells is only modest, presumably since they already have a compromised biosynthetic pathway at the 7-DHC $\rightarrow$ cholesterol step. This leveling effect in activity in *Dhcr7*-deficient cells compared to control cells is observed for all of the compounds shown in Table 1, although all of the compounds but one (CGS 12066B) that increase 7-DHC levels in control Neuro2a cells still increase levels of this sterol in the *Dhcr7*-deficient cells, albeit by an attenuated amount.

We have previously reported that several antipsychotic medications act on DHCR24 (trifluoperazine, perphenazine, doxepin, and procholorperazine) and the 8 $\rightarrow$ 7 isomerase (fluphenazine, perphenazine, and hydroxyzine) in *Dhcr7*-deficient Neuro2a cells,²⁵ and other laboratories have reported that haloperidol, clozapine, risperidone, and ziprasidone alter cholesterol metabolism³² in HepG2 and HL-60 cells. At the higher concentrations used in these studies (between 5 and 25 μ M),³² multiple enzymes of the biosynthesis pathway are affected by these medications. We also found activity for haloperidol and risperidone at 1 μ M in Neuro2a cells and significant effects on cholesterol biosynthesis for haloperidol at concentrations as low as 10 nM.²⁵ In Neuro2a cells at 10 μ M and above, we find that cytotoxicity becomes a major issue for many of these agents.

Desmosterol and Lanosterol in Neuro2a

The screening method provides information about the effect of compounds in the NIH Clinical Collection on desmosterol and lanosterol in Neuro2a. Desmosterol levels in Neuro2a are relatively high, compared to that of other sterol precursors to cholesterol, and increases in desmosterol are smaller than those found for lanosterol and 7-DHC; see Figure 4. We note, however, that imatinib and progesterone are found to have the highest Z-scores in this study, and they also act as inhibitors of DHCR24 in *Dhcr7*-deficient Neuro2a cells and are reported to have this activity in other cell lines.³³ It is also noteworthy that of those compounds that are found in our screen to increase levels of lanosterol in Neuro2a cells, most have a common “azole” substructure that is characteristic of fungicides. The azole substructure acts on the P450 demethylase that removes the C-14 methyl group of lanosterol, and compounds that affect this enzyme cause a buildup of the precursor substrate, lanosterol.³⁴ Indeed, seven of the eight “most actives” in the lanosterol panel in Figure 4C belong to this group of pharmaceuticals that inhibit the C14-demethylase.

Consequences of Exposures That Lead to Elevated 7-DHC

The biochemical link between the devastating genetic disorder SLOS and the pharmacological model for that condition based on the small molecule AY9944 raises the possibility that human exposure to compounds that increase 7-DHC levels such as AY9944 (see Figure 4 and Table 1) will exert similar pathophysiological consequences. Indeed, it seems appropriate to raise a number of issues based on the data at hand. Among the questions of potential interest are the following: Are there common pathologies associated with the “active” compounds identified in our screen? Is there evidence that pathologies associated with SLOS find analogy in exposures to small molecules like the “actives” identified in our screen? Is there, in effect, an aripiprazole, trazodone (or other prescribed pharmaceutical) rodent model for SLOS, similar to that of AY9944? Do environmental agents outside of the Pharmacopeia that show “activity” in our screen pose a threat? Are the 1–3% of the population who are carriers of a SLOS mutation more vulnerable to exposure to “active” small molecules that affect the conversion 7-DHC → cholesterol than a control individual?

While studies to directly answer the questions presented above have not been carried out, there is some circumstantial evidence that can be cited. Nefazodone, trazodone, and buspirone, three of the most potent compounds found in our screen, are known to cause mitochondrial dysfunction and loss of cellular viability.³⁵ The severity of the exposure-associated toxicities mirrors the compound potency found in our screen with the activity decreasing from nefazodone to trazodone and buspirone. Nefazodone and trazodone were found to reduce mitochondrial membrane potential and cause a decrease in cellular glutathione levels, one conclusion being that these compounds impose an oxidative stress on the exposed cells. In point of fact, 7-DHC is highly prone to free radical chain oxidation reactions,^{36–38} and the pathology of SLOS has been linked to oxidative stress that results from this susceptibility.^{24,39–44} 7-DHC is the most reactive lipid toward free radical chain oxidation studied to date, and this inherent reactivity for peroxidation fits with the notion that oxidative stress, free radical peroxidation, and systemic redox status are altered when elevated levels of this sterol are found in biology. Any human exposure that leads to elevated levels of 7-DHC in tissues, for example, the drugs identified in our screen, will likely be linked to a change in lipid redox status as a result of that exposure. Indeed, the vulnerability of 7-DHC to peroxidation and the biological activity of its derived oxysterols were principal reasons that we set out to develop methods to screen for 7-DHC in relevant cell systems. Any perturbation of sterol homeostasis that results in an elevated cellular level of 7-DHC, such as exposure to nefazodone or other active compounds, will likely be associated with increased lipid peroxidation and oxidative stress.

Another example of a common pathological response to “active” compounds found in our screen is the loss of retinal function that has been associated with the AY9944-exposed rat as well as in instances of vision loss linked to both aripiprazole⁴⁵ and nefazodone⁴⁶ usage. The AY9944 rats undergo selective photoreceptor degeneration, and their responsiveness to light stimulation is markedly reduced. Electrophysiological analysis of the rats showed that the timing of both rod and cone responses to photic stimulation was significantly delayed. It is of some interest then, that a loss of rod retinal function has been reported for SLOS patients

in recent studies.^{47,48} For all cases cited above, including retinopathies associated with aripiprazole and nefazodone, the primary locus of neural damage is retinal and is likely to have resulted from an induced neurotoxicity.

Finally, we mention that prenatal exposure to certain medications has been associated with an increased risk of autism spectrum disorder (ASD), a condition that is also common to SLOS patients.^{20,21,49} Although the association between the use of antidepressants, antipsychotics, and anxiolytic medications and ASD is still controversial, recent studies have concluded that second and/or third trimester use of drugs in these categories are statistically associated with an increased risk of ASD.^{50,51} While the ASD phenotype is broad and the causes of the disorder remain unknown, the parallel between SLOS, a single gene mutation that causes a disruption of sterol homeostasis, and small molecules that also perturb the cholesterol biosynthetic pathway and their corresponding associations with ASD seems worthy of additional investigation. In this regard, an interplay between genetics and environment risk factors during the prenatal period may well play a role in some neurodevelopmental disorders,^{52–54} and it seems reasonable to suggest that the 1% of the population who are carriers of a SLOS mutation may be particularly vulnerable to exposure to compounds that affect the conversion of 7-DHC → cholesterol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

7-DHC	7-dehydrocholesterol
Des	desmosterol
Lan	lanosterol
Chol	cholesterol
PTAD	4-phenyl-1,2,4-triazoline-3,5-dione
SLOS	Smith-Lemli-Opitz syndrome
DHCR7	7-dehydrocholesterol reductase
MS	mass spectrometry

APCI	atmospheric pressure chemical ionization
SRM	selected reaction monitoring
UPLC-MS	ultrahigh pressure liquid chromatography–mass spectrometry
HPLC-UV	high pressure liquid chromatography–ultraviolet spectroscopy
MeOH	methanol
NMR	nuclear magnetic resonance
TIC	total ion current
FBS	fetal bovine serum
DMEM	Dulbecco's modified Eagle's medium
PBS	phosphate buffered saline
DMSO	dimethyl sulfoxide
HTS	high throughput screening

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Figure 1.
Small molecules inhibit late-stage transformations in cholesterol biosynthesis.

Figure 2. Nanomoles of 7-DHC in control (DMSO) and aripiprazole (100 nM) wells on six 384-well plates.

Figure 3.

Z-Score for 7-DHC in Neuro2a cells treated for 24 h with compounds in the NIH Clinical Collection. Positive controls, 100 nM of aripiprazole in red, and pitavastatin in green are shown at the left and +3 and -3 Z-score cutoffs in blue. The Z-score of zero is set by the analysis of 20 control DMSO wells.

Figure 4.

Compounds in the NIH Clinical Collection that give a Z-Score of greater than +3 at 1 μ M when Neuro2a cells are treated with the compound for 24 h. (A) For 7-DHC, the Z-score is shown as a log plot. * indicates compounds found on a triplicate rescreen. Captopril did not validate in a subsequent rescreen. HTFMT = histamine trifluoromethyl toluidide; TFMPP = *N*-(3-trifluoromethylphenyl)piperazine. TPP: 1-[3-(2,4,6-trimethoxybenzoyl)propyl]pyrrolidinium chloride. (B) For desmosterol. (C) For lanosterol, the Z-score is shown as a log plot. Z-scores shown are averages of assays on duplicate plates.

Figure 5.

Typical 7-DHC, desmosterol (Des), and lanosterol (Lan) profiles for compounds in the NIH Clinical Collection that give a Z-score of greater than +3 at 1 μ M when Neuro2a cells are treated with the compound for 24 h. (A) Perospirone; (B) aripiprazole; (C) nefazodone; (D) trazodone; (E) ifenprodil; (F) risperidone. Sterol profiles for A–D are typical for screening hits. The exceptions are nefazodone, dofetilide, and fluoxetine that give profiles like nefazodone.

Figure 6. Structures of AY9944, aripiprazole, and trazodone, each of which causes significant increases in 7-DHC in Neuro2a cells.

Table 1

Compounds at 1 μ M That Increase 7-DHC Only in Control Neuro2a or in Both Control and *Dhcr7*-Deficient Neuro2a Cells^a

significant activity only in Neuro2a (N2a)														
active in both Neuro2a (N2a) and <i>Dher7</i> -deficient Neuro2a (Def)														
drugs	Z scores			FDA	drugs	Z scores			FDA	drugs	Z scores			
	N2a	Def				N2a	Def				N2a	Def		
perospirone ^b	287	5.2		NR	nefazodone ^b	227	0.9		C	cisapride ^c	10	3.0		C
HTFMT	231	3.4		Ex	trazodone ^b	171	2.9		C	lofexidine	9	0.2		NR
haloperidol ^b	223	3.7		C	ifenprodil	110	2.4		NR	TPP	8	1.1		Ex
aripiprazole ^b	216	3.4		C	donepezil ^b	40	1.9		C	captopril	7	0.4		D
vesamicol	99	6.9		Ex	procaine	39	0.1		C	fluoxetine ^b	6	0.6		C
bupirone ^b	94	5.5		B	clobenpropit	30	0.5		Ex	formoterol	5	0.5		C
nafadotride	50	3.8		Ex	melperone ^b	28	2.7		NR	CGS 12066B	4.5	-1.8		Ex
naftopidil	47	3.4		NR	pergolide ^c	28	1.8		Ex	esmolol	4	1.2		C
oxybutynin	21	7.3		B	dofetilide	20	0.6		C	salmeterol	4	0.7		C
E-4031	19	3.2		Ex	loxoprofen	19	0.2		NA	risperidone ^b	4	0.6		C
altanserlin	6	3.6		Ex	lobeline	13	2.3		NR	droperidol ^b	3	0.1		C
isoxsuprine	3.1	4.2		C	pirenerone ^b	13	0.4		NA					
acebutolol	3.0	3.1		C	TFMPP	13	0.4		Ex					

^aHTFMT = histamine trifluoromethyl toluidide; TFMPP = *N*-(3-trifluoromethylphenyl)piperazine; TPP = 1-[3-(2,4,6-trimethoxybenzoyl)propyl]-pyrrolidinium chloride; FDA pregnancy indication.^bAntipsychotic, antidepressant or anxiolytic properties; NA, no information; NR, international use, not recommended during pregnancy, or data not available; Ex, not approved for use, withdrawn, or experimental.^cWithdrawn in the US.