Identification and characterization of prescription drugs that change levels of 7-dehydrocholesterol and desmosterol

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Abstract Regulating blood cholesterol (Chol) levels by pharmacotherapy has successfully improved cardiovascular health. There is growing interest in the role of Chol precursors in the treatment of diseases. One sterol precursor, desmosterol (Des), is a potential pharmacological target for inflammatory and neurodegenerative disorders. However, elevating levels of the precursor 7-dehydrocholesterol (7-DHC) by inhibiting the enzyme 7-dehydrocholesterol reductase is linked to teratogenic outcomes. Thus, altering the sterol profile may either increase risk toward an adverse outcome or confer therapeutic benefit depending on the metabolite affected by the pharmacophore. In order to characterize any unknown activity of drugs on Chol biosynthesis, a chemical library of Food and Drug Administration-approved drugs was screened for the potential to modulate 7-DHC or Des levels in a neural cell line. Over 20% of the collection was shown to impact Chol biosynthesis, including 75 compounds that alter 7-DHC levels and 49 that modulate Des levels. Evidence is provided that three tyrosine kinase inhibitors, imatinib, ponatinib, and masitinib, elevate Des levels as well as other substrates of 24-dehydrocholesterol reductase, the enzyme responsible for converting Des to Chol. Additionally, the mechanism of action for ponatinib and masitinib was explored, demonstrating that protein levels are decreased as a result of treatment with these drugs.—Wages, P. A., H-Y. H. Kim, Z. Korade, and N. A. Porter. Identification and characterization of prescription drugs that change levels of 7-dehydrocholesterol and desmosterol. J. Lipid Res. 2018. 59: 1916–1926.

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Maintenance of blood cholesterol (Chol) levels through deliberate change in diet or the use of pharmacotherapy is frequently undertaken to improve health. For example, the results from a meta-analysis of statin use in human populations aimed to lower total Chol levels showed a decreased mortality associated with cardiovascular disease (1). More recently, a debate has arisen concerning the effect of statins on the CNS with clinical observations showing both positive and negative outcomes on neurological disorders as a result of statin use (2). Indeed, it is not surprising that modifying lipid metabolism could impact neurological health, because the brain consists of more than 25% of all the Chol in the human body. Additionally, because Chol cannot freely cross the blood-brain barrier, the CNS is required to maintain a separate pool of Chol that is independent from that of the rest of the body. Whether the neurological changes associated with statin use are caused by reducing Chol levels or by another pleiotropic mechanism is not known (3).

Statins target Chol formation at the transformation promoted by HMG-CoA reductase (HMGCR), the first committed step of biosynthesis (4). But there are over a dozen subsequent steps required to produce the final product (4), see Fig. 1. Drugs that modulate any of these subsequent steps will perturb sterol homeostasis and provide a potential approach to reduce Chol levels. For example, a compound that inhibits 24-dehydrocholesterol reductase...
Novel small molecule modulation of cholesterol metabolism

(DHCR24) would reduce Chol levels, but a consequence of such an approach would also be to increase levels of the substrate for DHCR24, desmosterol (Des). By the same token, inhibitors of 7-dehydrocholesterol reductase (DHCR7) would increase 7-dehydrocholesterol (7-DHC) at the expense of Chol.

While there may be instances in which manipulation of post-lanosterol (Lan) sterol levels is therapeutically desirable (5), these potential therapies are clinically tempered by the fact that perturbing sterol homeostasis during fetal and neuronal development will likely have pathological consequences (6). A number of known syndromes (Fig. 1) are the result of defects in genes that encode the enzymes involved in the Chol biosynthetic pathway. Two such recessive disorders that clinically present with hypocholesterolemia, Smith-Lemli-Opitz syndrome and desmosterolosis, are caused by mutations in the DHCR7 and DHCR24 genes, respectively.

The severity of Smith-Lemli-Opitz syndrome is directly linked to specific mutations in the gene that encodes DHCR7, the enzyme responsible for the conversion of 7-DHC to Chol (7–9). Over 150 known pathogenic mutations of DHCR7 have been reported (9–11), and a carrier frequency up to 3% has been suggested (12). Patients with defective DHCR7 that survive in utero, clinically present with craniofacial deformities and CNS malformations, and are often diagnosed with neurobehavioral deficits (6). Even though low Chol levels may impair normal neuronal development by disrupting cell division and the development of myelin sheathes (13), elevated 7-DHC levels may also contribute to the pathological outcomes. The 7-DHC has the potential to induce cell stress responses because it is highly oxidizable and can contribute to the exaggerated formation of free radicals that are known to damage nucleotides, proteins, and other lipids (14, 15). In support of the pathological potential of targeting DHCR7, it has been demonstrated that chemically modulating 7-DHC and Chol levels with the known DHCR7 inhibitor, AY-9944, leads to numerous developmental and behavioral deficits in rodent models (16, 17).

Desmosterolosis, a syndrome resulting from defects in DHCR24, is an even more rare syndrome with only a handful of reported cases (18). DHCR24 is known to act on multiple metabolites within the Chol biosynthetic pathway (Fig. 1) with some tissue specificity. The brain, for example, is reported to have an affinity to metabolize zymosterol (Zym) (19). Any defect or inhibition of DHCR24 function would ultimately lead to reduced levels of all sterols that are immediate products of that enzyme, including Chol, with a corresponding increased level of Des. It should be noted that there is growing interest in the role of Des in translational science because this sterol has been linked to a variety of neurological and cardiovascular disorders, as well as increased susceptibility to viral infections (20–22). Furthermore, it has been hypothesized that specifically targeting Des levels could confer regulation over inflammatory signaling processes in macrophages (23, 24).

There have been several studies that have individually identified a handful of small molecules that modulate levels of 7-DHC and Des in cell culture. Recently a neuroblastoma cell line [Neuro2a (WT-N2a)] and a Dher7-deficient derivative of that cell [Dher7-deficient Neuro2a (D7-N2a)] have been used to identify active compounds in a National Institutes of Health library of clinically relevant molecules (25, 26). High-throughput screening methods developed in those studies permitted us to evaluate a collection of Food and Drug Administration (FDA)-approved prescription drugs more relevant to public health. A majority of these compounds had not been previously assessed for their potential beneficial or detrimental impact on human health by compromising Chol biosynthesis. Here, we report the outcome of that study; a screen of a chemical library of 1,003 FDA-approved drugs in which more than 100 compounds were found to

Fig. 1. Chol biosynthetic pathway. Chol and key sterol precursor structures are shown with relevant enzymes indicated in blue. Shown in red are the syndromes caused by inborn errors of Chol biosynthesis at the associated step in Chol biosynthesis.
have a significant effect on levels of 7-DHC or Des in WT-N2a or D7-N2a cells.

MATERIALS AND METHODS

Materials

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich (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 (Monroe, NC). Natural and isotopically labeled sterol standards used in this study are available from Kerafast, Inc. (Boston, MA). Other materials were purchased as follows: 10% NuPage NoveX Bis-Tris® precast mini gel (Invitrogen, Grand Island, NY), PVDF membrane and Simply Blue (Bio-Rad, Hercules, CA), IRDye® 800CW streptavidin (925-32230; Li-Cor, Lincoln, NE), and blocking buffer (Rockland, Gilbertsville, PA or Odyssey blocking buffer). Antibodies of DHC24 (anti-seladin-1, ab40490) and actin (ab8299) were purchased from Abcam and Santa-Cruz, respectively. Appropriate secondary antibodies (anti-mouse and anti-rabbit) that can be visualized at either 700 nm or 800 nm were purchased from either Li-Cor or Life Technology.

Cell culture

The neuroblastoma cell line, WT-N2a, was purchased from American Type Culture Collection (Rockville, MD). N2a cells were maintained in DMEM supplemented with 1-glutamine, 10% FBS (VWR/Seradigm, Radnor, PA), and penicillin/streptomycin at 37°C and 5% CO₂. D7-N2a cells were generated as previously described (27). All cells were subcultured every 4-5 days up to ten passages, and the culture medium was changed every 2 days.

Chemical screening and exposures

All screening experiments were conducted in the Vanderbilt University High-Throughput Screening Facility as previously described (25, 26). Briefly, 2.5 nl of each compound (dissolved in DMSO) from the FDA-approved Drug Library (Selleck Chemicals, Houston, TX) were deposited in a single well of a black 384-well plate (0.2 mg per well). The plates were sealed with Easy Pierce heat sealing foil (Thermo Scientific; AB-1720), followed by 20 min of agitation at room temperature. The sealed plates were kept at −80°C until LC-MS analysis. For verification experiments, WT-N2a and D7-N2a cells were seeded onto black 96-well plates (Greiner Bio-One) in DMEM supplemented with 1-glutamine and 10% FBS (VWR/Seradigm) at 0.75 × 10⁴ WT-N2a cells per well and 1.00 × 10⁴ D7-N2a cells per well. Lead-hit chemical exposure plates containing exposures at 2× were created by depositing known concentrations of chemical on a 96-well plate backfilled to 40 nl with DMSO using Labcyte Echo 550/555 followed by the addition of 200 µl of DMEM supplemented with 1-glutamine and N-2 supplement (Thermo Fisher). After 24 h from initial seeding, medium on cells was replaced with 50 µl of DMEM supplemented with 1-glutamine and N-2 supplement (Thermo Fisher) followed by the addition of 50 µl of exposure medium from the lead-hit chemical master plate, as indicated for each experiment. After an additional 24 h, 50 µl of medium were collected for cytokotoxicity analysis, as determined by LDH release with the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Subsequently, cells were prepared for mass spectrometric analysis as with the 384-well plates, but to each well in the 96-well plate the internal standards were added (10 µl of stock solution in methanol: 0.087 nmol of d₇-Chol, 0.033 nmol of d₇-7-DHC, 0.25 nmol of 13C₃-Des, and 0.23 nmol of 13C₃-Lan) and methanol (100 µl). The plate was gently shaken on an orbital shaker for 20 min at room temperature to lyse the cells and extract the sterols. The supernatant was transferred to a PTAD-predeposited 96-well plate (0.2 mg per well). The plates were sealed with Easy Pierce heat sealing foil (Thermo Scientific; AB-1720) and allowed to react for 30 min at room temperature. The sealed plates were kept at −80°C until LC-MS analysis.

LC-MS analysis

The scaled plates were analyzed on an Acquity UPLC system equipped with an ANSI-compliant well plate holder. The sterols (10 µl injection) were analyzed on an UPLC C18 column (Acquity UPLC BEH C18, 1.7 µm, 2.1 × 50 mm) with 100% methanol (0.1% ν/v acetic acid) mobile phase at a flow rate of 500 µl/min and runtime of 1.2 min. A TSQ Quantum Ultra tandem mass spectrometer (Thermo Fisher) was used for MS detections, and data were acquired with a Finnigan Xcalibur software package. Selected reaction monitoring (SRM) of the PTAD derivatives was acquired in the positive ion mode using atmospheric pressure chemical ionization. MS parameters were optimized for the 7-DHC-PTAD adduct and were as follows: auxiliary nitrogen gas pressure at 55 psi and sheath gas pressure at 60 psi; discharge current at 22 µA and vaporizer temperature at 342°C. Collision-induced dissociation was optimized at 12 eV under 1.0 mTorr of argon. The SRM transitions of precursors (sterol with PTAD moiety) metabolite m/z → product ions m/z included: 7-DHC m/z 560 → 365, d₇-7-DHC: m/z 567 → 372, Des m/z 592 → 365, Lan m/z 634 → 602, 13C₃-Des m/z 595 → 368, and 13C₃-Lan m/z 637 → 605. Pseudo-SRM transitions of m/z 369 → 369 and m/z 376 → 376 were utilized to monitor Chol and d₇-Chol, respectively, because Chol does not react with PTAD. Final sterol numbers are reported as nanomoles per million cells.

GC-MS analysis

The remaining undericladized 100 µl of the supernatant of the verification experiments from each sample in each well were transferred to vials and concentrated on a SpeedVac concentrator for GC-MS analysis (Agilent Technologies; 7890B). To each vial, N,Obis(trimethylsilyl)-trifluoroacetamide (50 µl) was added, vortexed well, and allowed to react for 30 min. The sample (5 µl) was injected onto the column (SPB-5, 0.25 mm, 0.52 mm × 30 m) with a temperature program of 220–300°C (5 min) at 20°C/min and helium flow rate of 2.0 ml/min. The data were collected in full

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Immunoblot analysis

N2a-cells were grown in 60 mm plates (Genesee Scientific) for 24 h in complete medium followed by incubation with chemicals (100 nM) dissolved in DMSO in N-2-supplemented medium for 24 h. Experimental conditions were optimized so that at the conclusion of the experiment, ~1.5 × 10^6 cells were harvested for immunoblot analysis. Cells were washed twice in ice-cold 1× PBS, lysed with lysis buffer, and then normalized by protein concentration as determined by DC™ protein assay kit (Bio-Rad). Samples were then resolved by 10% NuPAGE Novex BisTris® gel and then transferred to PVDF membrane. The transferred proteins were incubated with antibodies of DHCR24 and actin, (1:1,000) overnight in the cold room at 4°C. Alexa Fluor 680®-labeled secondary anti-rabbit or anti-mouse was used to detect target proteins. Immunoreactive proteins were visualized using Image Studio (version 5.2) (Li-Cor).

Statistics

Statistical analyses were performed with Prism software (GraphPad, La Jolla, CA) in combination with spreadsheet-based software including Excel® (Microsoft®, Redmond, WA). P values <0.05 were considered significant; specific analyses used are indicated with each set of data and experiment. In addition, we calculated screening window coefficients, “Z” and “Z score”. (28) These factors reflect the assay dynamic range and data variation associated with the measurements. Unless otherwise noted, at least three replicates of all experiments were undertaken.

RESULTS

High-throughput screening of FDA drugs targeting Chol biosynthesis

One thousand and three FDA-approved drugs were screened in WT-N2a and D7-N2a cells for their potential to affect late-stage Chol biosynthesis. The use of a high-sensitivity UPLC-MS-(SRM) analytical protocol and a high-throughput screening platform permitted the rapid analysis of 7-DHC, Des, Lan, and Chol following a 24 h exposure to the FDA drugs at 1 μM (29). In addition to the library compounds, a number of positive controls were utilized during the screening procedure to assess the functional health and activity of the Chol biosynthetic pathway in both neural cell lines. These controls included simvastatin (to inhibit HMGCR), econazole (to inhibit Cyp51), and aripiprazole (to inhibit DHCR7). Lead-hit chemicals were determined by utilizing the 24 vehicle control wells (0.01% DMSO) on each plate as a reference for each compound’s exposure effect resulting in a Z score. The screen was conducted in duplicate for each cell line. The outcome of the high throughput screen across both cell lines demonstrated that over 200 of the compounds in the library impacted Chol biosynthesis significantly, as determined by a Z score found to be greater than or less than three standard deviations from the mean, i.e., Z greater than +3 or Z less than −3 (see the supplemental material). For additional comparison, WT- and D7-N2a cells were exposed to 100 nM AY-9944 and 1,000 nM triparanol to demonstrate inhibition of DHCR7 and DHCR24 with prototypical Chol biosynthesis inhibitors, respectively (supplemental Fig. S1). When Z scores were calculated for these compounds at their optimal effect, AY-9944 exposed to WT-N2a cells resulted in a Z score of 160.1 for an increase in 7-DHC levels, whereas triparanol exposed to D7-N2a cells resulted in a Z score of 3.7 with respect to Des.

FDA drugs elevate 7-DHC levels

Of the lead hits, 75 of the drugs altered the levels of 7-DHC in one or both cell lines (Table 1). Compounds that reduced 7-DHC levels (Z score less than −3) were found exclusively in those experiments that used D7-N2a, while drugs that increased 7-DHC with Z scores greater than +3 were found, for the most part, in the WT-N2a cultures. With one exception, the most potent drugs found in WT-N2a were found to have Z scores greater than +3 in the D7-N2a cells. Nine compounds in the library were very potent, increasing levels of 7-DHC with a Z score greater than 100. Of these nine drugs, haloperidol, aripiprazole, and domiphen had previously been reported to affect 7-DHC levels in cell culture studies and/or in vivo (30). Six of the compounds (nebivolol, rotigotine, iloperidone, ziprasidone, dyclonine, and penfluridol) had not been previously linked to elevated 7-DHC. In order to verify the results of the initial screen, we examined these six potent drugs in cultures of WT-N2a and D7-N2a cells across a range of concentrations from 6 to 1,000 nM. No cytotoxicity was found in any of these exposures (data not shown) and the experiments confirmed the effect on 7-DHC for each of the six drugs at 1,000 nM in the WT-N2a cell line (Fig. 2). Nebivolol and rotigotine had a significant effect on sterol homeostasis in WT-N2a cells at concentrations as low as 60 nM, while dyclonine affected sterol levels at 125 nM (Fig. 2A, B, E). In all exposures in which 7-DHC was significantly elevated, Des levels were significantly reduced and Chol levels unchanged. Although the drugs perturbed sterol homeostasis, they did not affect the total sterol cellular levels (Chol + 7-DHC + Des) measured for any of the drugs at any of the concentrations tested. The six drugs that had a Z score of more than 100 in WT-N2a cells were also cultured with the cell line deficient in Dhcr7, D7-N2a, but no significant perturbation of sterol levels was found even at the highest concentrations of the drugs tested, 1,000 nM (see supplemental Fig. S2).

FDA drugs elevating Des levels

All lead-hit compounds identified in the screens that increased Des levels in WT- and D7-N2a cells had a Z score less than 12, see Table 2. This is in marked contrast to the lead hits that elevate 7-DHC, which had Z scores as high as +20. Ten compounds were found to increase levels of Des in both the WT- and D7-N2a cells (Table 2) under the conditions of the high-throughput screen exposure paradigm.
These drugs were subjected to further study to determine the nature of the perturbation on sterol levels and to examine their dose-response effect in relation to Chol homeostasis. Chol levels were significantly lowered by all drugs in the WT-N2a cell line at the 62 nM exposure, except for vandetanib and darifenacin, which only lowered Chol levels at the highest exposure of 1,000 nM (supplemental Fig. S3). Des levels were elevated in WT-N2a cells in at least one dose for all tyrosine kinase inhibitors (TKIs) (imatinib, masitinib, ponatinib, and vandetanib) and steroids (pregnenolone and progesterone) tested (supplemental Fig. S3). When these compounds are exposed to D7-N2a cells, the absolute levels of Des increase or, in a few cases, the fraction of Des in the sterols measured was found to increase (supplemental Fig. S4). Chol levels did not change significantly as a function of drug concentration, but the total sterols measured (Chol + 7-DHC + Des) decreased with increasing drug concentration, principally because of a loss of 7-DHC in the cells (supplemental Fig. S4). Subsequent GC-MS studies in both WT-N2a and D7-N2a provide additional insight, as illustrated in Tables 3 and 4, respectively, with the TKIs, masitinib and ponatinib, and the steroid, pregnenolone. While Des levels were elevated by each drug at 62 nM in the WT-N2a cells (Table 3), the most significant change observed was in the D7-N2a cells as an increase of 7-dehydrodesmosterol (7-DHD), a precursor to both Des and 7-DHC, see Fig. 1. These results confirmed the previous conclusions that the steroid, pregnenolone, impacts DHCR24 function (31). Additionally, the two TKIs utilized in this experiment also appear to impact the function of DHCR24, which would be similar to previous findings associated with another TKI, imatinib (25). In support of this notion, both ponatinib and masitinib significantly caused elevated levels of 24-DHL in addition to 7-DHD and Des in the D7-N2a cell line (Table 4), wherein all three sterols are known to be substrates of DHCR24 (Fig. 1).

Masitinib and ponatinib impact DHCR24 protein expression

It is possible that these DHCR24-active compounds cause an elevation of Des by altering transcription of the gene DHCR24. There are contradictory reports that TKIs, including imatinib, increase transcription of DHCR24 (32, 33), thus we examined the effect of TKIs on Dhcr24 mRNA expression. We utilized a uniform dose of 100 nM for all compounds, as this concentration is lower than the majority of the Cmax of TKIs in humans (34). No significant change in mRNA levels was observed in the WT-N2a cells (supplemental Fig. S5). We then measured DHCR24 protein levels to provide an additional perspective by conducting DHCR24 immunoblot analyses at the same drug exposure times that were used for determination of sterol levels. Cells exposed to pregnenolone had significantly lower DHCR24 protein levels compared with vehicle control in WT-N2a cells (Fig. 3A). Masitinib and ponatinib decreased protein expression as well (Fig. 3A). When D7-N2a cells were similarly tested, only ponatinib was observed to lower DHCR24 protein expression (Fig. 3B); however, it should be noted that the D7-N2a cell line had significantly elevated levels of DHCR24 protein levels compared with its WT-N2a

| TABLE 1. Lead-hit compounds in FDA-approved chemical library altering levels of 7-DHC |
|-------------------------------|-------------------------------|------------------------------|
| Drug                          | Z score WT-N2a | D7-N2a |
| Dyclonine                     | 109.4           | 233.6 |
| Dronedarone                   | 46.6            | 223.8 |
| Pramoxine                     | 26.7            | 220.4 |
| Vitamin D3                    | 12.9            | 202.8 |
| Alverine                      | 12.9            | 163.7 |
| Buflomedil                    | 12.6            | 112.0 |
| Nafamostat                    | 9.4             | 112.0 |
| Mirabeegrone                  | 8.9             | 105.2 |
| Esmolol                       | 8.8             | 97.2  |
| Clorprenaline                 | 7.3             | 77.4  |
| Alexidine                     | 6.3             | 72.3  |
| Tetracaine                    | 6.1             | 62.8  |
| Droperidol                    | 5.2             | 50.1  |
| Levetiracetam                 | 5.2             | 47.0  |
| Camylofin                     | 5.0             | 34.5  |
| Clavirex                      | 4.5             | 17.0  |
| Articaine                     | 4.5             | 13.5  |
| Betaxolol                     | 4.2             | 8.9   |
| Sulfamethizole                | 4.0             | 6.3   |
| Geniposide                    | 3.8             | 3.9   |
| Butenafine                    | 3.7             | 3.6   |
| Dapoxetine                    | 3.3             | 3.7   |
| Anfencim                      | 3.3             | 3.0   |
| Zalooprofen                   | 3.2             | 3.6   |
| Terbinafine                   | 3.1             | 3.4   |
| Tropisetron                   | 3.1             | 3.9   |

| Drug                          | Z score WT-N2a | D7-N2a |
| Bromhexine                    | 3.8             | 6.2   |
| Econazole                     | -3.0            | 9.3   |
| Hydroxyzine                   | -3.1            | 4.5   |
| Sorafenib                     | -3.1            | 8.1   |
| Ciclopirox                    | -3.3            | 5.5   |
| Bortezomib                    | -3.3            | 6.6   |
| Imatinib                      | -3.4            | 7.3   |
| Progestriol                   | -3.4            | 6.1   |
| Trimebutine                   | -3.4            | 3.5   |
| Cephalosporin                 | -3.5            | 3.9   |
| Amorolfine                    | -3.5            | 5.5   |
| Sulconazo上海                   | -3.6            | 3.6   |
| Tamoxifen                     | -3.7            | 3.1   |
| Rosuvastatin                  | -3.9            | 3.7   |
| Masitinib                     | -3.9            | 3.0   |
| Mevastatin                    | -4.1            | 3.6   |
| Pregnenolone                  | -4.1            | 3.6   |
| Clomiphene                    | -4.1            | 3.9   |
| Toremifene                    | -4.1            | 3.9   |
| Bazedoxifene                  | -4.1            | 3.8   |
| Estàra                       | -4.1            | 4.2   |
| Fenticonazole                 | -4.2            | 4.2   |
| Emetine                      | -4.2            | 4.2   |
| Butoconazole                 | -4.2            | 4.2   |
| Fluvastatin                   | -4.2            | 4.2   |
| Simvastatin                   | -4.2            | 4.2   |
| Pitavastatin                  | -4.3            | 4.2   |
counterpart (supplemental Figs. S5, S6) and, as such, is likely confounding the effect of masitinib and pregnenolonone on DHCR24 expression in this particular cell line. In contrast, imatinib had no statistically significant impact on the expression of DHCR24 in either cell line, suggesting that this TKI may have an alternate means to regulate DHCR24 activity.

**DISCUSSION**

**High-throughput screening for compounds that target Chol biosynthesis**

Multiple analytical methods were used to screen compounds in a chemical library of FDA-approved drugs in two variants of the N2a cell line: the background cell type or “WT” (WT-N2a) and a Dicer7-deficient cell line (D7-N2a). Our primary analytical protocol makes use of a derivatization procedure (PTAD) to provide sensitive, selective, and rapid assays of the penultimate sterols in the Chol biosynthetic pathway, Des and 7-DHC. This method was adapted to the use of high-throughput screening instruments to semi-automate the experimental procedures from cell culture to analysis. The protocol provides absolute levels of Des, 7-DHC, and Chol normalized to cell count in UPLC/MS runs of 1 min, making possible the ability to screen commercial chemical libraries for drugs that impact Chol biosynthesis. For the data presented here, less than 3 days of mass spectrometer instrument time were used to provide the lead-hit prioritization of compounds that affect some step in Chol biosynthesis, from HMGCR to DHCR7 or DHCR24. A first-pass screen using the PTAD protocol identifies an initial lead-hit list, which can then be verified by the use of established analytical methods to provide a more complete sterol profile. These established methods (35, 36) consume more instrument time making them less amenable for use with high throughput screening. It should be noted that the experimental conditions of the screening procedures do involve the use of a medium with a known quantity of Chol, a known component of serum. Thus follow-up multiday experiments involving the conditioning of cells to serum-free, and thus Chol-free, medium were conducted to minimize confounding sources of Chol detected in the analysis.

N2a cells are exceptionally useful for screening purposes because they are relatively rich in Chol (16 nmol per million cells) and Des (2.5 nmol per million cells), but 7-DHC is found at much lower levels (0.1 nmol per million cells). The PTAD method uses a multiple reaction mass spectrometric method that is particularly selective for 7-DHC, making it possible to readily identify library compounds that affect DHCR7 by detecting increases in the levels of 7-DHC. Analysis of 7-DHC in D7-N2a cells provides complimentary information to the screens of compounds in WT-N2a cells. D7-N2a cells have much higher levels of 7-DHC (4 nmol per million cells) than N2a and lower levels of Des (1 nmol per million cells) than the WT cell line (supplemental Fig. S1). A compound that affects any of the transformations that precede the formation of 7-DHC in Chol biosynthesis is detected by a reduction in
the levels of 7-DHC present in the D7-N2a cells. Thus, com-
ounds that inhibit HMGCR are readily detected by a de-
crease in levels of 7-DHC in D7-N2a cells without a
compensatory increase in Chol. In a similar way, DHCR24
inhibitors decrease levels of 7-DHC in these cells, as do
compounds that affect other transformations between
HMGCR and DHCR24.

In this study, one in five of the FDA-approved drugs
screened was identified to have an impact on Chol biosyn-
thesis in some manner. A number of the lead-hit com-
pounds are well-known, such as the statins, which inhibit
HMGCR (37), or azole fungicides (38) that target Cyp51.
Additionally, twenty-one of the lead hits identified in this
screen, including the antipsychotic, aripiprazole (30), were
similarly identified in a screen of a National Institutes
of Health clinical collection chemical library (25, 26) with
similar Z scores (R2 > 0.95). It is nevertheless of interest
that 82 of the drugs that were identified in this study had
an effect on levels of Des or 7-DHC that was previously
unreported. It is also of interest to put the percentage of
hit compounds found in this screen in perspective with
drugs that are being routinely prescribed. Utilizing the
2015 Medical Expenditure Panel Survey (39), we conclude
that 27 of the top 200 most prescribed drugs affect Chol
biosynthesis and, of this set of drugs, 13 target either
DHCR7 or DHCR24 (Fig. 4). It should also be noted that a
number of the top 200 drugs have yet to be tested for their
effect on sterol biosynthesis, including compounds with
high abuse potential that are not readily available for
screening purposes (39).

The potential risks of elevated levels of 7-DHC
Perturbing DHCR7 activity has been a known human
health risk since the 1960s when the DHCR7 inhibitor, AY-
9944, was demonstrated to be teratogenic in rodent models
(17). Since that time, a number of other chemicals have
been identified as DHCR7 inhibitors and, similarly, their
use is cautioned and/or altogether discontinued during

| Drug            | Z score | Drug          | Z score | Drug            | Z score |
|-----------------|---------|---------------|---------|-----------------|---------|
| Amfenac         | 11.7    | Imatinib      | 9.4     | Bazedoxifene    | 7.9     |
| Sulfamethizole  | 9.5     | Pregnenolone  | 10.3    | Clomiphene      | 4.8     |
| Gefitinib       | 8.8     | Masitinib     | 8.0     | Orphenadrine    | 4.2     |
| Bosutinib       | 8.3     | Progestosterone| 8.8     | Tolmetin        | 3.3     |
| Natamycin       | 4.7     | Vanketanide   | 5.5     | Idoxuridine     | 3.3     |
| Carmofur        | 4.3     | Ponatinib     | 4.7     | Rosuvastatin    | −3.0    |
| Asenapine       | 4.1     | Clemastine    | 3.7     | Cabozantinib    | −3.1    |
| Zaltoprofen     | 4.1     | Raloxifene    | 3.5     |                 | 8.0     |
| Dutasteride     | 4.0     | Darifenacin   | 3.0     |                 | 3.0     |
| Teniposide      | 3.9     | Toremifene    | 3.0     |                 | 6.2     |
| Mepivacaine     | 3.9     | Simvastatin   | −3.2    |                 | −4.0    |
| Fulvranst        | 3.7     | Mevastatin    | −3.9    |                 | −3.6    |
| Etoposide       | 3.6     | Sulconazole   | −4.0    |                 | −3.1    |
| Methylprednisolone | 3.6   | Amoroffine    | −4.5    |                 | −3.9    |
| Estradiol cypionate | 3.4 | Fluvastatin | −4.6    |                 | −4.2    |
| Mercaptopurine  | 3.4     | Fenticonazole | −4.6    |                 | −4.0    |
| Topotecan       | 3.3     | Butoconazole  | −4.7    |                 | −4.1    |
| Amodipidine     | 3.1     | Pitavastatin  | −4.7    |                 | −4.2    |
| Domiphen        | −3.0    |               |         |                 |         |
| Citrimumium     | −3.2    |               |         |                 |         |
| Trimebutine     | −3.5    |               |         |                 |         |
| Bifonazole      | −3.6    |               |         |                 |         |
| Econazole       | −3.9    |               |         |                 |         |
| Clofazimazole   | −4.0    |               |         |                 |         |

Data are expressed as mean ± SEM. Zyme, zymostenol; Lath, lathosterol; n.d., not detected.

*Significant difference to control, P < 0.05.
pregnancy (30). In addition to increased risk of birth defects and perturbed fetal development, there is also the potential for DHCR7 inhibitors to pose increased health risks for adult patients as well. The 7-DHC is one of the most highly oxidizable organic molecules and it accumulates upon administration of a DHCR7 inhibitor (40, 41). The increased level of 7-DHC may not in itself pose immediate toxicity, but it seems likely that 7-DHC could prime a system for a severe insult when an oxidative stress is initiated. The 7-DHC molecules are readily oxidized to form highly reactive oxysterols that have diverse biological activities and that can readily form adducts to proteins (42). In addition, DHCR24 activity and protein stability have been shown to be posttranslationally regulated by two distinct tyrosine residues (46), and it is possible that these specific tyrosine residues are posttranslationally regulated by two distinct tyrosine residues (46), and it is possible that these specific studies of the effects of these drugs on development during pregnancy should be undertaken to understand their potential risk.

### Aspects to consider in the regulation of Des levels

Of the 49 compounds identified to potentially impact Des levels, focus was directed toward the TKIs, as many of them elevated Des levels in both the WT- and D7-N2a cell line screens. Of particular interest were the drugs masitinib and ponatinib, as they were shown to be potent modulators of multiple biomolecules associated with DHCR24 beyond Des, including 7-DHD and 24-DHL. The effect of these two TKIs is likely translatable to human populations, as the concentrations utilized in this project for masitinib and ponatinib are similar to their known C_{max} values of 51 nM and 145 nM, respectively (44, 45). In contrast, the other TKI utilized in this study, imatinib, may be even more efficacious, as it can be tolerated in humans at blood concentrations in the micromolar range (34). The only other set of compounds that affected Des levels in both cell lines were the progesterin steroids (progesterone and pregnenolone), which have previously been reported to affect DHCR24 activity (31), a conclusion that our findings support. However, DHCR24 activity and protein stability have been shown to be posttranslationally regulated by two distinct tyrosine residues (46), and it is possible that these specific drugs and perturbed fetal development, there is also the potential for DHCR7 inhibitors to pose increased health risks for adult patients as well. The 7-DHC is one of the most highly oxidizable organic molecules and it accumulates upon administration of a DHCR7 inhibitor (40, 41). The increased level of 7-DHC may not in itself pose immediate toxicity, but it seems likely that 7-DHC could prime a system for a severe insult when an oxidative stress is initiated. The 7-DHC molecules are readily oxidized to form highly reactive oxysterols that have diverse biological activities and that can readily form adducts to proteins (42). In addition, DHCR24 activity and protein stability have been shown to be posttranslationally regulated by two distinct tyrosine residues (46), and it is possible that these specific studies of the effects of these drugs on development during pregnancy should be undertaken to understand their potential risk.

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residues may be the targets of the TKIs examined in our study. Future studies with in vivo models seem to be called for because regulating DHCR24 activity may have some therapeutic benefits. In this regard, targeting Des levels as a therapy for neurodegeneration has been proposed, and masitinib is being repurposed as an adjuvant therapy for amyotrophic lateral sclerosis (47) and Alzheimer’s disease (AD) (48–50). Evidence suggests that masitinib is beneficial to patients with mild to moderate AD by normalizing neuronal signaling to support dendritic health (48). Yet, there are many findings that demonstrate that AD patients have perturbed sterol profiles (51) characterized by low Des levels in neuronal tissues (52, 53). This study provides evidence that masitinib is a potent elevator of Des by lowering DHCR24 protein expression levels. These findings suggest that masitinib may, in addition to the already determined pharmacological effects in AD patients, also normalize sterol homeostasis and provide additional physiological benefits in these patients. If this is indeed the case, then it may prove beneficial to identify means to normalize Des levels in all AD patients as adjuvant therapy.

**SUMMARY AND CONCLUSIONS**

Chol is an essential biological molecule, and when pathologies lead to elevations or reductions of it or one of its precursors, there can be severe health consequences. Thus, it is essential to determine regulatory mechanisms of maintaining sterol homeostasis. This is a prerequisite for evaluating the risk of altering sterols or using pharmacotherapy to normalize levels of sterols. This work has sought to accomplish this goal by identifying a more complete pharmacopoeia of Chol biosynthesis disruptors that should prove useful for researchers, translational scientists, and clinicians alike. Additionally, the results of the study provide a chemical tool set to begin exploring the mechanistic involvement of the entire Chol biosynthetic pathway in a number of disorders, including AD.

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**Fig. 4.** Common prescription drugs identified as Chol biosynthetic active compounds. Shown are the top 27 prescription drugs determined to inhibit Chol biosynthesis at the indicated enzyme (blue). Next to each drug in parentheses is the rank of drug utilization in the US based on the number of individuals with a purchase of that drug (39). Drugs identified beyond the current screen are indicated with a star from either screening experiments (26) or targeted studies, as in the case of verapamil (54) and amiodarone (55).
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