Inhibition of lysosomal phospholipase A2 predicts drug-induced phospholipidosis

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Abstract Phospholipidosis, the excessive accumulation of phospholipids within lysosomes, is a pathological response observed following exposure to many drugs across multiple therapeutic groups. A clear mechanistic understanding of the causes and implications of this form of drug toxicity has remained elusive. We previously reported the discovery and characterization of a lysosome-specific phospholipase A2 (PLA2G15) and later reported that amiodarone, a known cause of drug-induced phospholipidosis, inhibits this enzyme. Here, we assayed a library of 163 drugs for inhibition of PLA2G15 to determine whether this phospholipase was the cellular target for therapeutics other than amiodarone that cause phospholipidosis. We observed that 144 compounds inhibited PLA2G15 activity. Thirty-six compounds not previously reported to cause phospholipidosis inhibited PLA2G15 with IC₅₀ values less than 1 mM and were confirmed to cause phospholipidosis in an in vitro assay. Within this group, fosinopril was the most potent inhibitor (IC₅₀ 0.18 μM). Additional characterization of the inhibition of PLA2G15 by fosinopril was consistent with interference of PLA2G15 binding to liposomes. PLA2G15 inhibition was more accurate in predicting phospholipidosis compared with in silico models based on pKa and ClogP, measures of protonation, and transport-independent distribution in the lysosome, respectively. In summary, PLA2G15 is a primary target for cationic amphiphilic drugs that cause phospholipidosis, and PLA2G15 inhibition by cationic amphiphilic compounds provides a potentially robust screening platform for potential toxicity during drug development.

Supplementary key words Acyltransferase • 1-O-acylceramide • lysosome • phospholipase A2 group XV • drug-induced phospholipidosis • drug toxicity • cationic amphiphilic drugs • drug development • high-throughput screening • amiodarone

Phospholipidosis is the excess storage of phospholipids within lysosomes. Drug-induced phospholipidosis (DIP), in distinction to inherited forms of lysosomal phospholipid accumulation such as those associated with disorders such as Niemann–Pick C disease, represents an acquired lysosomal disorder (1, 2). DIP most often involves the lung, liver, or kidney where it is associated with pulmonary fibrosis, hepatic steatosis or steatohepatitis, and acute or chronic kidney injury, respectively. Phospholipidosis often, but not always, results from exposure to basic cationic amphiphilic drugs (CADs). DIP is measured experimentally by use of in vitro or in vivo assays and is often observed in clinical settings. It is among the most common forms of drug toxicity as it is associated with exposure to more than 50 FDA-approved agents. When DIP is detected in preclinical screening studies, an otherwise promising compound may be abandoned. If DIP is found in patients under treatment with a specific drug, then the therapeutic is often discontinued. Research on DIP has been dominated by three overarching questions. First, what are the mechanisms responsible for DIP? Second, what chemical properties of a candidate compound can be used to predict phospholipidosis and used as a guide for further development? Third, what significant short- and long-term toxicities are the specific consequences of DIP?

With regard to the first question, several mechanisms have been proposed as the basis DIP. These include the stimulation of phospholipid synthesis (3), the direct binding of CADs to lysosomal phospholipases with inhibition of these enzymes by competitive or allosteric mechanisms (4), the inhibition of lysosomal trafficking to lysosomes (5), the displacement of phospholipases from the lysosomal membrane with secondary degradation by lysosomal proteases (6), and the binding of CADs to phospholipids with prevention of their degradation (7). Lysosomal phospholipase A1, A2, and C activities have been previously associated with DIP. However, to date only three phospholipases are known to be lysosome-based. They include acid sphingomyelinase (8), phospholipase D3 (9), and lysosomal phospholipase A2 (10).

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With regard to the second question, efforts to predict DIP have generally followed two strategies. The first approach has employed analyses in which the physical properties of drugs are correlated with empirically observed phospholipidosis (11–14). The second strategy has used the development of novel in vitro assays that can be applied to the screening of individual drug candidates or chemical libraries to predict phospholipidosis potential. These assays include those that detect lipid accumulation in cell lines or that specifically measure lysosome associated lipid biomarkers such as bis(monoacylglycerol)phosphate (15) or gene expression profiling (16). The assessment of these various in silico and in vitro strategies is limited by the absence of proof of a mechanism responsible for DIP.

With regard to the third question, a determination of the pathological significance of phospholipidosis has been limited by the lack of identification and characterization of a specific target or targets of compounds that cause DIP. Identifying the cellular target or targets responsible for DIP as distinguished from toxicities resulting from separate off-target effects would represent a significant step in understanding and managing this form of drug toxicity.

Our group identified an enzyme with 1-O-acyl-ceramide synthase activity and subsequently characterized a purified enzyme as lysosomal phospholipase A2 (LPLA2), now designated PLA2G15 (17–19). LPLA2 has an acidic pH optimum and colocalizes with lysosomes and late endosomes. Loss of function of LPLA2 in mice results in alveolar macrophage foam cell formation and surfactant accumulation, a phenotype similar to that observed with amiodarone-associated phospholipidosis (20). In subsequent work we reported that amiodarone is a potent inhibitor of LPLA2, but does so by inhibition of electrostatic charge interactions between the hydrolase and anionic phospholipids (21). This mechanism of action was further substantiated by our determination of the crystal structure of LPLA2 and the identification of critical residues in the lipid membrane-binding domain (22).

Based on these studies we considered whether the inhibition of LPLA2 by cationic amphiphilic compounds is a more general mechanism for DIP. We assayed two libraries of small molecules for their ability to inhibit LPLA2 and correlate this inhibition with physical properties of these compounds used by others as basis for predictive models of phospholipidosis. The first library consisted of drugs known to cause phospholipidosis in either in vitro or in vivo studies based on published reports. The second library consisted of compounds for which DIP has not been previously reported. We observed that inhibition of LPLA2 strongly correlates with drugs reported to cause phospholipidosis and have identified drugs that have not previously known to cause phospholipidosis, not all of which are cationic amphiphiles.

**MATERIALS AND METHODS**

**Materials**

1,2-dioleoyl-palmitoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-di-O-octadecenyl-sn-glycero-3-phosphocholine (DODPC), and brain porcine sulfatide ammonium salt were purchased from Avanti Polar Lipids (Birmingham, AL). p-Nitro-phenyl butyrate (pNPB) was from Sigma (St. Louis, MO). Purified recombinant mouse LPLA2 was produced by Proteos Inc. (Kalamazoo, MI) as previously reported (22). High-performance thin layer chromatography (HPTLC) silica gel plates (10 x 20 cm) were from Merck KGaA (Darmstadt, Germany). All cationic amphiphilic drugs and controls used in this study were obtained from Sigma-Aldrich (St. Louis, MO) with the following exceptions ay-9944 and suramin from Calbiochem (San Diego, CA), and clenbuterol and yohimbine were from Cayman Chemicals (Ann Arbor, MI).

**Transacylase activity of LPLA2**

The LPLA2 activity assay is based on the following principles (23). LPLA2 is uniquely characterized as having an acidic pH optimum and as a transacylase recognizing short-chain lipophilic alcohols as acceptors. Based on these properties, short-chain 1-O-acyl-ceramides are unique products of this reaction. Because LPLA2 binds preferentially to negatively charged liposomes, sulfatide was included in the liposomes but is not itself a substrate and does not function as a cofactor for lysosomal hydrolases. The transacylase reaction is based on the unique property of LPLA2 to transfer an acyl group from the sn2 or sn1 position of a glycerophospholipid to N-acetyl-sphingosine (NAS) forming 1-O-acyl-N-acetyl-sphingosine (1-O-acyl-NAS) (18, 22, 24). 1-O-acyl-NAS is not known to be a product of any other enzyme. The reaction mixture included 50 mM sodium citrate buffer (pH 4.5), 10 µg/ml bovine serum albumin, and liposomes consisting of 38 µM N-acetyl-sphingosine, 127 µM DOPC, 12.7 µM sulfatide, and test compound in a total volume of 0.5 µl. The test compounds were dissolved in DMSO. The final DMSO concentration in the reaction mixture was 0.125%. The reaction was initiated by the addition of recombinant LPLA2 protein (30 ng) and carried out at 37°C for 10 min. The reaction was terminated by the addition of 3 ml chloroform/methanol (2/1, v/v), followed by 0.3 ml of 9% (w/v) NaCl. After centrifugation for 7 min at 1800 × g, the resulting lower layer was transferred to new tube and dried under stream of nitrogen gas. The dried lipid was dissolved in 40 µl of chloroform/methanol (2/1, v/v) and applied to HPTLC plates. HPTLC plates were run in chloroform/acetic acid (9/1, v/v). The plates were dried and soaked in 8% (w/v) CuSO4.5H2O, 6.8% (v/v) H3PO4, and 32% (v/v) methanol and then charred for 15 min in an oven at 150°C. Scanned plates were analyzed by NIH ImageJ 1.65i (National Institutes of Health).

**LPLA2 esterase assay**

pNPB was used to directly measure the activity of LPLA2. pNPB is a water-soluble substrate that can directly access the catalytic site in the absence of liposomes (25). A reaction mixture of pNPB (0.2 mM) and cationic amphiphilic compounds at varying concentrations in sodium citrate buffer (pH 4.5) was prepared and prewarmed to 37°C for 5 min in a total volume of 500 µl. The reaction was initiated by the addition of recombinant LPLA2 (5 µg). At predetermined times, 120 µl of the reaction mixture was transferred to a tube.
containing 120 μl of 0.2 M NaHCO₃ and kept on ice. The cold reaction product was subsequently warmed to 37°C, and the absorbance of the reaction product, 4-nitrophenoxide, was measured at 400 nm with a Beckman Du-640 spectrophotometer.

**Liposome LPLA₂ cosedimentation assay**

Liposomes consisting of DOPC and sulfatide (10:1 M ratio, 127 μM total lipid) were incubated with 5 μg of LPLA₂ in 500 μl 50 mM sodium citrate, at pH 4.5 for 30 min on ice. The reaction mixture was then centrifuged for 1 h at 150,000 g at 4°C. The resulting precipitate was rinsed with cold 50 mM sodium citrate pH 4.5 and dissolved with 40 μl of SDS-PAGE sample buffer. The sample was separated by using 10% SDS-PAGE. After electrophoresis, LPLA₂ was detected with Coomassie brilliant blue. Band quantification was performed with ImageJ software (25).

**LPLA₂ thermal stability measurement**

A thermal stability assay was employed to determine the melting point (T_m) of LPLA₂ (26). An incubation mixture consisting of 2.5 μl of 8x SYPRO Orange, 1 μg of LPLA₂ in 50 mM Na citrate at pH 4.5, and ddH₂O in a final volume of 20 μl was added to wells of a 48-well thin-wall PCR plate. The plates were sealed with Optical-Quality Sealing Tape (Bio-Rad) and heated in a Real-Time PCR Detection System Life Technology (Thermo Fisher, Ann Arbor, MI) from 20 to 90°C in steps of 0.5°C. T_m values were calculated as the inflection point of the melting curve using the instrument software.

**Screening phospholipidosis assay**

The assay was modified from one reported previously (27). MDCK cells were seeded in 100 μl culture medium at cell density 3,000 cells per well in 96-well black-walled clear bottom Greiner micro plates (Sigma-Aldrich) and were allowed to adhere overnight. Cell culture medium was replaced with phospholipidosis staining solution (1:1,000 dilution) of LipidTOX Red Phospholipidosis detection reagent (Invitrogen), and simultaneously with different concentrations of fosinopril or amiodarone in total volume of 100 μl. Compounds were prepared as stock solutions at 200-fold higher concentration than the desired top concentration (solvent concentration maintained at 0.5%). Compound treatment was performed for 24 h with 5% CO₂ at 37°C. Then the culture medium was removed and cells were fixed with 100 μl fixation solution consisting of 4% formaldehyde in phosphate buffered saline (PBS). After washing, cells were incubated with 1 drop of NucBlue Live (NBL) for 20 min. Cells were washed three times with PBS, and fluorescence image acquisition was performed using the Molecular Devices (San Jose, CA) spectrophotometer. Cell nuclei fluorescence was detected using a 410–480 nm emission filter, red phospholipidosis detection was performed using 549–615 nm emission filter.

**Image acquisition and processing**

Ninety six-well plates were visualized under a Leica DM IRB microscope and images acquired with an Olympus DP70 camera via Olympus DP Manager software. All images were identically adjusted in GNU Image Manipulation Program to improve background and overall image clarity postacquisition.

**LipidTOX red particle quantification**

Images were quantified utilizing ImageJ as follows. Images were initially processed with the Subtract Background feature with a rolling ball radius of 50 pixels. Following conversion to 8 bit, images were subjected to Auto Local Threshold processing using the Bernsen algorithm with a radius of 15. Particles were subsequently quantified and analyzed utilizing the Analyze Particle feature. A total of six 10x fields (2 per triplicate) were quantified with an average of over 4,000 cells per field.

**Statistical analysis**

Data from at least three independent experiments were analyzed with a paired t test in GraphPad Prism 7 and expressed as mean ± SD. The differences between control and treated samples were considered statistically significant at P < 0.05.

**RESULTS**

A library of 163 compounds was assembled and assayed for inhibition of LPLA₂. One hundred and nine compounds were identified via literature review as causing phospholipidosis based on either in vitro or in vivo assays (Table 1). In the latter case, the animal species employed is indicated. These compounds were chosen represent a wide spectrum of therapeutic indications, having a range of pKa and ClogP that fell within and outside of values commonly associated with DIP and in which the lysosomal pathology is observed across a range of organs. Most, but not all, of the compounds are cationic amphiphiles, and several are central nervous system penetrant. A second set of 54 compounds was assayed representing drugs for which no reports of phospholipidosis were found but which were representative of a similar spectrum of chemical properties (Table 2). Included in this set were metabolites chosen as negative controls (glucose, leucine, and uridine). The primary clinical indications listed in these tables are consistent with a wide range of cellular targets for these compounds.

Two primary physical properties of a drug have been used to predict whether a compound may be lysosomotropic. These are the ClogP and pKa (basic). ClogP, a measure of partitioning between octanol and water, predictive of transport independent distribution across cell membranes. pKa (basic) is a determinant of the protonation of an amine at lysosomal pH. ClogP and pKa (basic) were employed by Ploemen and colleagues to generate an in silico model that is predictive of phospholipidosis (Table 3) (115). In a subsequent paper, a modification was proposed to improve the positive and negative predictive value of the model (1). In contrast, the assay used for the measurement of LPLA₂ activity is cell-free and thus not dependent on the ability of a particular compound to enter a target cell and distribute into late endosomes or lysosomes. A comparison between the physical properties and
| Generic name | UPAC Designation | CAS Number | Indication                                                                 | ClogP | pKa (basic) | Ploemen Value | Pred Ploemen | Pred modified Ploemen | LPLA2 IC50 (μM) | In Vitro PLD | In Vivo PLD | Refs          |
|--------------|------------------|------------|----------------------------------------------------------------------------|-------|------------|---------------|--------------|------------------------|----------------|--------------|--------------|---------------|
| Alprenolol   | 1-(o-allylphenoxy)-3- (isopropylamino)-2-propanol | 13707-88-5 | Antihypertensive, antiarrhythmic, sympatholytic agent | 3.1  | 9.67       | 103           | +            | +                      | 172.7          | Yes          | (28, 29)     |
| Alverine     | ethyl bis (3-phenylpropyl)amine | 150-59-4 | Antidiarrheal                                                              | 5.73  | 10.44      | 142           | +            | +                      | 40.01          | Yes          | (28, 30)     |
| Ambroxol     | 2-amino-3,5-dibromo-N((trans-4-hydroxy cyclohexyl) benzylamine | 28828-92-4 | Mucolytic                                                                 | 3.72  | 9.01       | 95            | +            | +                      | 59             | Yes          | (28, 31)     |
| Amiodarone   | [2-[(2-butyl-1-benzoturan-3-carbonyl)-2-diodophenoxy]ethyl] diethyl amine | 1951-25-3 | Antiarrhythmic                                                             | 7.57  | 8.47       | 130           | +            | +                      | 8.3            | Yes          | H,R (16, 25, 27, 28, 32–38) |
| Amitriptyline| dimethyl[tricyclo[9.4.0.0³,8]pentadeca-1(15),3,5,7,11,13-hexaen-3,8-yldiene]propyl]piperazine | 50-48-6 | Antidepressant                                                             | 5.1   | 9.76       | 121           | +            | +                      | 14.7           | Yes R        | (11, 16, 27, 28, 35, 36, 39, 40) |
| Amorolfine   | 2R,6S-2,6-dimethyl-4-[(2-[4-(2-methylbutan-2-yl) phenyl] methyl] propyl]morpholine | 78613-35-1 | Antifungal                                                                 | 5.62  | 8.49       | 104           | +            | +                      | 41.47          | Yes          | (28, 30)     |
| Anastrozole  | 2,2'-[5-(IH-1,2,4-Triazol-1-methyl)-1,3-phenylenebis(2-methylpropiononitrile)] | 120511-73-1 | Chemotherapy                                                               | 2.31  | 2          | 9.3           | -            | -                      | 4.82           | Yes          | (41, 42)     |
| Astemizole   | 1-((4-fluorobenzy1)-2-(1-(1-methyl-2-(3-[trifluoromethyl]phenyl)ethyl)amino benzimidazol | 68844-77-9 | Antihistamine                                                             | 5.92  | 8.75       | 112           | +            | +                      | 8.19           | Yes (28, 33, 40) |
| ay-9944      | trans1,4-bis(2-chlorobenzylaminomethyl)cyclohexane | 366-93-8 | Hypocholesteremic                                                         | 6.4   | 9.1        | 124           | -            | +                      | 116            | Yes R,Ra,M   | (16, 43–46) |
| Benzbromarone| 2,6-dibromo-4-(2-ethyl-1-benzoturan-3-carbonyl)phenol | 3562-84-3 | Xanthine oxidase inhibitor                                                | 5.52  | -3.8       | 30.5          | -            | -                      | 3.8            | Yes          | (28, 32)     |
| Benfluorex   | N(1-methyl-2-[3-(trifluoromethyl)-phenyl]ethyl)amino ethanol benzoate ester | 23642-66-2 | Anorectic and hypolipidemic                                                | 4.26  | 9.14       | 102           | +            | +                      | 19.9           | Yes (35, 47, 48) |
| Bepridil     | hydrochloride 1-isobutoxoy-2-pyrrolidino-3(N-benzyllalino)propane hydrochloride | 74764-40-2 | Calcium channel blocker                                                   | 5.33  | 9.16       | 112           | +            | +                      | 7.17           | Yes          | (28, 36)     |
| Betaxolol    | 1-{4-[2-(cyclopropylmethoxy) ethyl]phenoxyl}-3-{[prop-2-yl] amino]propan-2-ol | 63659-18-7 | Beta blocker                                                              | 2.81  | 9.07       | 101           | +            | +                      | 0              | Yes          | (28, 40)     |
| Bromhexine   | 2-amino-3,5-dibromo-N-(cyclohexyl)-N-methylbenzylamine hydrochloride | 611-75-6 | Mucolytic                                                                 | 4.08  | 9.32       | 104           | +            | +                      | 30.78          | Yes          | (28, 31, 49, 50) |
| Buclizine    | 1-[4-chlorophenyl](phenylmethy1)-4-((4-(1,1-dimethylethyl)phenyl)methyl)piperazine | 82-95-1 | Antihistamine                                                             | 6.16  | 8.04       | 103           | +            | +                      | 9.13           | Yes          | (12, 28)     |
| Generic name | UPAC Designation | CAS Number | Indication | ClogP | pKa (basic) | Pred Ploemen Value | Pred modified Ploemen | LPLA2 IC50 (μM) | In Vivo PLD | In Vivo PLD | Refs |
|--------------|-----------------|------------|------------|-------|-------------|-------------------|----------------------|-------------------|--------------|-------------|------|
| Bromocriptine | (4R,7R)-10-bromo-N-{[(S,R,7S)-2-hydroxy-7-(2-methylpropyl)-5,8-dioxo-4-[propan-2-yl]-3-oxa-6,9-diazatricyclo[7.3.0.0^2,6]dodecan-4-yl]6-methyl-6,11diazatetracyclo[7.10^2,7]0^12,16]hexadeca-1(16),29,14,11-pentaene-4-carboxamide | 25614-03-3 | Dopamine promoter | 3.2 | 6.71 | 55 | - | + | 125 | Yes (28, 51) |
| Chloroquine | 7-chloro-N-[5-(diethylamino)pentan-2-yl]quinolin-4-amine | 54-05-7 | Immunosuppressive and anti-parasitic | 4.63 | 10.32 | 128 | + | + | 655 | Yes H, R, D, M (28, 34, 36–38, 40, 52–55) |
| Chlorpheniramine | [3-(4-chlorophenyl)-3-(pyridin-2-yl)propyl] dimethylamine | 132-22-9 | Antihistamine | 3.38 | 9.47 | 101 | + | + | 147 | Yes (28, 35, 56) |
| Chlorprothixene | 3-(9Z)-2-chloro-9H-thioxanthen-9-ylidenecarbonyl)dimethylamine | 11-59-7 | Antipsychotic | 5.18 | 9.76 | 122 | + | + | 7.78 | Yes (28) |
| Chlorpromazine | [3-(2-chloro-10H-phenothiazin-10-yl)propyl] dimethylamine | 50-53-3 | Antipsychotic | 5.41 | 9.3 | 116 | + | + | 9.01 | Yes R, D (16, 27, 28, 34, 36, 37, 39, 40) |
| Cinnarizine | 1-(diphenyl methyl)-4-(3-phenylprop-2-en-1-yl)piperazine | 298-57-7 | Antihistamine | 5.77 | 8.44 | 105 | + | + | 40.6 | Yes (28, 57) |
| Citalopram | 1-{[(3-dimethylamino)propyl]propyl}dimethylamine | 59729-33-8 | Antidepressant | 3.76 | 9.78 | 110 | + | + | 19.5 | Yes Yes (12, 27, 28, 34–37, 40) |
| Clemastine | (2R)2-[2-{(1R)-4-chlorophenyl)-1-phenylethoxy} ethyl-methylpyrrolidine | 14976-57-9 | Antihistamine | 5.29 | 9.55 | 119 | + | + | 13.21 | Yes (28) |
| Clenbuterol | 4-amino-3,5-dichloro-6-{[(1L-dimethylamino)benzene}-ethanol, monohydrochloride | 21898-19-1 | Muscle relaxer, decongestant, bronchodilator | 2.94 | 9.63 | 101 | + | + | 7298 | Yes (28) |
| Clindamycin | (2S,4R)-N-{2-chloro-1-[(2R,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(methylsulfanyl)oxan-2-yl]propyl}1-methyl-4-propylpyrrolidine-2-carboxamide | 18323-44-9 | Antibiotic | 2.16 | 7.55 | 617 | - | + | ND | Yes Yes (12, 28, 36, 40) |
| Clofazimine | N,N-diisopropyl-3,5-dihydro-3-(isopropylamino)phenazin-2-amine | 2050-63-9 | Anti-mycobacterial, anti-inflammatory properties | 7.66 | 9.29 | 145 | + | + | 10.8 | Yes (28, 58, 59) |
| Clozapine | (3′-4′-chloro-2′-azatricyclo[9.4.0.0^3,8]pentaedeca-1′(1′),3′,5′,7′,12′,14-hexaenal-2′-yl)propyl)dimethylamine | 303-49-1 | Antipsychotic | 5.04 | 9.2 | 104 | + | + | 17 | Yes Yes (16, 28, 34–37, 39) |
| Cloperastin | 1-[2-{4-chlorophenyl} (phenyl)methoxy] ethyl piperidine | 3703-76-2 | Antihistamine | 5.11 | 8.82 | 104 | + | + | 40.9 | Yes (28, 30) |
| Clozapine | 8-Chloro-N-[1-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e]1,4-diazepine | 34233-69-7 | Antipsychotic | 3.23 | 7.35 | 64.5 | - | + | 10.8 | Yes Yes (16, 28, 34, 39, 40) |

(continued)
| Generic name | CAS Number | Indication | ClogP (basic) | Pred Ploemen Value | Pred modified Ploemen | LPLA2 IC50 (μM) | Refs |
|--------------|------------|------------|--------------|--------------------|----------------------|------------------|------|
| Dibenzosuberane | 10,11-dihydro-5H-dibenzo-a,d-cycloheptene | 833-48-7 | Protein inhibitor | 4.7 | 10 | 122 | + | 346 | Yes (28) |
| Diphenhydramine | 2-hydroxyethyl[dimethylammoniumchloride] | 147-24-0 | Antihistamine | 3.27 | 8.87 | 89.4 | - | 270 | Yes (28) |
| Doxepin | dimethyl[3H]-dibenz[a,d]cycloheptene | 1668-19-5 | Psychotropic | 4.29 | 9.76 | 114 | + | 301 | Yes (28, 37) |
| Drofenine | Anticholinergic 5α-reductase inhibitor | 5.3 | 921 | 113 | + | 7.29 | Yes (35, 63) |
| Dutasteride | Ethyl(dimethylammonium)(dimethylammoniumchloride) | 548-66-3 | Anticholinergic 5α-reductase inhibitor | 6.8 | 2.17 | 50.9 | + | 1048 | Yes (28) |
| Erythromycin | Bacteriostatic antibiotic | 2.37 | 8.38 | 75.9 | + | 117 | Yes R, D (28, 34, 36, 39, 65) |
| Etomidate | Ethyl (1(R)-1-phenyethyl) benzamide | 33125-97-2 | Anesthetic | 3 | 4.54 | 29.6 | - | - | 1155 | Yes (28, 40) |

(continued)
| Generic name | UPAC Designation | CAS Number | Indication | ClogP | pK<sub>a</sub> (basic) | LPLA<sub>2</sub> IC<sub>50</sub> (μM) | In Vitro PLD | In Vivo PLD | Refs |
|--------------|-----------------|------------|------------|-------|-------------------|----------------------|--------------|-------------|------|
| Fenofibrate  | 2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid isopropyl ester | 49562-28-9 | Cholesterol lowering | 5.3 | -4.9 | 28.1 | - | - | (28, 66) |
| Fexofenadine | 2-(4-[1-hydroxy-4-[1-(hydroxydiphenylmethyl)piperidin-1-yl]butyl] phenyl)-2-methylpropanoic acid | 83799-24-0 | Antihistamine | 5.02 | 9.01 | 106 | + | + | 179 | Yes | (28, 67) |
| Fipexide     | 1-[2-[4-chlorophenoxy]acetyl]-4-(3,4-methylenedioxybenzyl)piperazine | 34161-24-5 | Attention deficit | 2.95 | 6.09 | 45.7 | - | - | 17.19 | Yes | (28) |
| Flunarizine  | 1-[bis(4-fluorophenyl)methyl]-4-[[2E]-3-phenylprop-2-en-1-yl]piperazine | 52468-60-7 | Calcium entry blocker | 5.3 | 7.6 | 85.9 | - | + | 7.49 | Yes | (28, 68) |
| Fluroxetine  | methyl[[3-phenyl-3-[trifluoromethyl]phenoxy]propyl] amine | 54910-89-3 | Antidepressant | 4.5 | 9.8 | 112 | + | + | 13.5 | Yes | H,R,M | (16, 27, 28, 34–37, 39, 40, 69, 70) |
| Flufenamic acid | 2-[3-[(3 trifluoromethyl)phenyl]phenyl]-2,5-dihydroxybenzoic acid sodium salt | 530-78-9 | Analgesic, anti-inflammatory, antipyretic | 5.25 | -2.1 | 32 | - | - | 177.9 | Yes | (28, 71) |
| Gentisic acid | 2,5-dihydroxybenzoic acid sodium salt | 4955-90-2 | Antihistaminic | -1.1 | -5.9 | 34.8 | - | - | 99.4 | Yes | (28, 72) |
| Hydroxyzine  | 2-(4-[4-[4-(4-chlorophenyl) (phenyl)methyl] piperazin-1-yl] ethoxy)ethan-1-ol | 68-88-2 | Antihistaminic | 3.43 | 7.82 | 729 | - | + | 63 | R | (34, 36, 55, 73) |
| Imipramine   | 3-[2-azatricyclo[9.4.0.0]<sup>3,8</sup>]pentadeca-1(15),3,5,7,11,13-hexaen-2-yl]ethanol-1-yl | 50-49-7 | Antidepressant | 4.8 | 9.2 | 108 | + | + | 27.6 | Yes | R | (16, 28, 34–37, 40, 74, 75) |
| Indoramin    | N-[1-[2-(1H-indol-3-yl)ethyl]piperidin-4-yl] benzamide | 26844-12-2 | Antidrenergic | 4.02 | 9.59 | 108 | + | + | 123.9 | Yes | R | (28, 36, 40) |
| Ketoconazole | 1-[4-[3-[(2,4-dichlorophenyl)-2-(H-imidazole-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-2,2-Dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate | 65277-42-1 | Antifungal | 4.35 | 6.75 | 64.5 | - | + | 39 | Yes | M | (16, 27, 28, 34, 36, 37, 40) |
| Ketotifen    | 4-(1-methylpiperidin-4-ylidene)-4H-benzo[4,5]cyclohepta[1,2-6]thiophen-4-yl-dione | 34580-14-8 | Antihistaminic | 2.2 | 7.15 | 56 | - | + | 19.68 | Yes | (16, 28, 36, 76) |
| Lercanidipine | 3-[1-[3,3-diphenylpropyl](methyl)amino]-2-methylpropan-2-yl| 100427-26-7 | Calcium channel blocker | 6.4 | 9.36 | 129 | + | + | 37.25 | Yes | (28, 77) |
| Lofepramine  | 2-[3-azatricyclo[9.4.0.0]<sup>3,8</sup>]pentadeca-1(15),3,5,7,11,13-hexaen-2-yl]propyl (methyl) amino]-1-[4-chlorophenyl] ethan-1-one | 23047-25-8 | Antidepressant | 6.11 | 6.53 | 80 | - | + | 13.25 | Yes | (28) |
| Loperamide   | 4-[4-[1-chlorophenyl]-4-hydroxypiperidin-1-yl]-N,N-dimethyl-2,2'-diphenylbutanamide | 53179-11-6 | Antidiarrheal | 4.44 | 9.41 | 108 | + | + | 149.12 | Yes | (28) |

(continued)
| Generic name | CAS Number | Indication | ClogP | Pred pKa (basic) | Pred Ploemen | Pred modified Ploemen | LPLA2 IC50 (μM) | In Vivo PLD | Refs |
|--------------|------------|------------|-------|-----------------|--------------|-----------------------|-----------------|-------------|------|
| Loratadine   | 79794-75-5 | Antihistamine | 4.8  | 4.33            | 41.8         | 8.94                  | Yes             | (16, 28, 36, 37, 40, 78) |
| Mannitol     | 69-65-8    | Osmotic diuretic | -2.7 | 123             | 150          | 160                  | Yes             | (28, 38, 79) |
| Maprotiline  | 10262-69-8 | Antidepressant | 4.82 | 10.54           | 134          | 12                    | R              | (34–36, 79, 80) |
| Mebeverine   | 2743-45-9  | Anti-diarrheal | 4.6  | 10.31           | 127          | 417                  | Yes             | (28) |
| Memantine    | 41100-52-1 | NMDA receptor antagonist | 3.32 | 10.7           | 125          | 35.7                  | Yes             | (35, 37, 81, 82) |
| Methapyrilene| 91-80-5    | Antihistamine | 2.87 | 8.85            | 86.6         | ND                    | Yes             | (28) |
| Mianserin    | 24219-97-4 | Antidepressant | 3.52 | 6.9            | 60.3         | 29.98                 | Yes             | (28, 33, 36) |
| Mifepristone | 84371-65-3 | Progesterone blocker | 5.3  | 4.89           | 52           | -                     | 27.69           | Yes (28) |
| Mirtazapine  | 85650-52-8 | Antidepressant | 2.9  | 6.67            | 529          | -                     | 8.7             | Yes (28, 83, 84) |
| Mitotane     | 53-19-0    | Chemotherapy | 6    | 36              | -            | 132.1                 | Yes             | (28, 85) |
| Naphazoline  | 835-31-4   | Sympathomimetic | 3.44 | 10.19           | 115          | 6.98                  | Yes             | (35) |
| Oxalazine citrate | 959-14-8 | Anti-psychotic | 2.7  | 8.96            | 87.6         | -                     | 369             | Yes (28, 86) |
| Oxybutynin   | 1508-65-2  | Anticholinergic | 4.36 | 8.77           | 95.9         | +                     | 26.39           | Yes (28) |
| Pantoprazole | 102626-70-7 | Proton pump inhibitor | 2.11 | 3.55           | 17.1         | -                     | 6.23            | Yes (28, 35, 87) |
| Paroxetine   | 61869-08-7 | Antidepressant | 3.1  | 9.77            | 105          | +                     | 5.12             | R (28, 36, 37, 69) |

(continues)
| Generic name | UPAC Designation | CAS Number | Indication | ClogP | pKα (basic) | Prediction | Pred Modified | LPLA2 IC50 (μM) | In Vitro PLD | In Vivo PLD | Refs |
|--------------|-----------------|------------|------------|-------|------------|------------|-------------|-----------------|-------------|-------------|------|
| Penfluridol  | 1-[4,4-bis(4-fluorophenyl)butyl]-4-[4-chloro-3-[trifluoromethyl]phenyl]piperidin-4-ol | 26864-56-2 | Antipsychotic | 6.09 | 8.96 | 117 | - | + | 9.09 | | | (28, 36, 63) |
| Perhexiline  | 2-(2,2-dicyclohexylethyl) piperidine | 6621-47-2 | Coronary vasodilator | 6.2 | 10.58 | 150 | + | + | 11.72 | Yes H.R.M | | (16, 28, 34–36, 40, 88–96) |
| Perphenazine | 2-[4-[3-(2-chloro-10H-phenothiazin-10-yl)propyl]piperazin-1-yl]ethanol | 58-39-9 | Antipsychotic | 4.2 | 8.21 | 85 | - | + | 3.92 | Yes | | (28) |
| Phenacetin   | N-[4-(ethoxyphenyl) acetamide | 6621-47-2 | Non-steroidal | 1.58 | -4.2 | 2.5 | - | - | 2140 | Yes R | | (6, 28, 40, 79, 91) |
| Pimozide     | 1-[1-[4,4-bis(4-fluorophenyl)butyl]piperidin-4-yl]2,3-dihydro-1H-1,3-benzodiazol-2-one | 2062278-4 | Antipsychotic | 6.36 | 8.38 | 111 | + | + | 10.6 | Yes | | (28, 92) |
| Pirenperone  | 3-[2-[4-(1-fluorobenzoyl)piperidin-1-yl]ethyl]-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one | 75444-65-4 | Antipsychotic | 2.6 | 8.02 | 71.1 | - | + | 25.2 | Yes | | (28, 93) |
| Pranlukast   | N-[4-oxo-2-[2H-1,2,3,4-tetrazol-5-yl]-4H-chromen-8-yl]-4-(4-phenylbutoxy)benzamide | 103177-37-3 | Antiasthmatic | 4.82 | -1.7 | 23.2 | - | - | ND | Yes | | (28, 30) |
| Pridinol     | 1,1-diphenyl-3-[piperidin-1-yl]propan-1-ol | 511-45-5 | Muscle relaxant | 3.69 | 9.34 | 101 | + | + | 285.4 | Yes | | (28, 94) |
| Profenamine  | diethyl[1-(10H-phenothiazin-10-yl)propan-2-yl]amine | 522-00-9 | Antidyskinetic | 5.75 | 9.6 | 125 | + | + | 8.7 | Yes | | (28, 95) |
| Progesterone | (1S,3aS,3bS,9aR,9bS,11aS)-1-acetyl-9a,11a-dimethyl-1H,2H,3H,3aH,3bH,4H,5H,7H,8H,9H, 9aH,10H,11H,11aH-cyclopenta[a]phenanthren-7-one | 57-83-0 | Hormone | 3.58 | -4.8 | 128 | - | - | 10.52 | Yes | | (28, 96) |
| Promazine    | dimethyl[3-(10H-phenothiazin-10-yl)propan-2-yl]amine | 58-40-2 | Antipsychotic | 4.55 | 9.2 | 105 | + | + | 39 | Yes R | | (28, 34, 35, 40) |
| Promethazine | dimethyl[1-(10H-phenothiazin-10-yl)propan-2-yl]amine | 60-87-7 | Antihistamine | 4.81 | 9.05 | 105 | + | + | 40.3 | Yes | Yes | (28, 37, 40) |
| Propafenone  | 1-[2-[2-hydroxy-3-(propylamino)propoxy]phenyl]phenyl-3-phenylprop-1-one | 54063-53-5 | Antiarrhythmic | 3.1 | 9.63 | 102 | + | + | 48.22 | Yes | | (28, 97) |
| Proparacaine | 2-(diethylaminomethyl)3-amino-4-propoxybenzoate | 499-67-2 | Antiepileptic | 2.5 | 8.56 | 79.5 | - | + | 2469 | Yes | | (28, 60) |
| Propanol     | 1-(naphthalen-1-yl)-3-[propylamino]propan-2-ol | 525-66-6 | Antihypertensive | 3.48 | 9.67 | 106 | + | + | 49.9 | Yes | | (1, 28, 34–37, 40) |
| Pyrilamine   | N-[2-(dimethyl amino)ethyl]-N-[4-methoxyphenyl]methylpyridin-2-amine | 91-84-9 | Antihistamine | 3.27 | 8.76 | 87.4 | - | + | 21.4 | Yes | | (28, 98) |
| Quinacrine   | 6-chloro-9-(4-diethylamino-1-methylbutylamino)2-methoxyacridine dihydrochloride | 6905-05-6 | Antimalarial and antibiotic | 5.5 | 10.33 | 137 | + | + | 30.13 | Yes R | | (28, 34–37, 40, 99) |
| Quinidine    | (R)-[1S,2S,4S,5R]-5-cyclopropyl-2,3-azabicyclo[2.2.2]octan-2-yl]-6-methoxyquinolin-4-yl)methanol | 130-95-0 | Antimarial | 3.34 | 9.05 | 93.7 | + | + | 164 | Yes | | (36, 40) |

(continued)
| Generic name          | UPAC Designation | CAS Number  | Indication            | ClogP | pKa (basic) | Pred Ploemen Value | Pred modified Ploemen | PLA2 IC50 (μM) | In Vivo PLD | In Vivo PLD | Refs                  |
|-----------------------|------------------|-------------|-----------------------|-------|-------------|--------------------|------------------------|----------------|-------------|--------------|-----------------------|
| Repaglinide           | 2-ethoxy-4[2-(3-methyl-1[2-(1-piperidinyl)phenyl]-butyl)amino]o-2-oxoethyl]benzoic acid | 135062-02-1 | Antihyperglycemic     | 5.9   | 4.28        | 53.1               | -                      | 86.49         | Yes         |             | (28, 29)              |
| Retinol               | (2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexan-1-en-1-yl)nona-2,4,6,8-tetraen-1-ol | 68-26-8 | Vitamin A             | 5.68  | -2.2        | 37.1               | -                      | 52.52         | Yes         |             | (28, 100)             |
| Ropinirole            | 4-[2(2-dipropylamino)ethyl]-2,3-dihydro-4H-indol-2-one | 91374-21-9 | Dopamine agonist      | 3.06  | 10.17       | 113                | +                      | ND            | Yes         |             | (28, 101)             |
| Sertraline            | (1S,4S)-4-[3,4-dichlorophenyl]-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine | 79617-96-2 | Antidepressant        | 5.15  | 9.85        | 123                | +                      | 8.6           | Yes         | Yes         | (16, 27, 28, 34-37, 40) |
| Spiperone             | 8-[5-(p-fluor benzoyl)propyl]-1-phenyl-1,3,8-triazaspiro[4.5]decane-4-one | 749-02-0 | Antipsychotic         | 3.03  | 8.89        | 88.2               | -                      | 519.2         | Yes         |             | (28, 37)              |
| Sulindac              | 2-[2(RS)-5-fluoro-1-[4-methanesulfinylphenyl]methylidene]-2-methyl-1H-inden-3-yl]acetic acid | 38194-50-2 | Nonsteroidal          | 3.42  | -6.7        | 11.7               | -                      | -             | 10.93       | Yes         | (28, 36, 102)          |
| Sulpiride             | N-[1-ethylpyrrolidin-2-yl]methyl]-2-methoxy-5-sulfoamoylbenzamide | 15676-16-1 | Antidepressant, antipsychotic | 1.2   | 8.4         | 71.8               | -                      | ND            | Yes         |             | (103)                 |
| Suramin               | S-[4-methyl-3-[3-[3-[[2-methyl-5-[(4,6,8-trisulphonaphthalen-1-yl)carbamoyl]phenyl]carbamoyl]phenyl]carbamoyl]amino]benzamido]benzamido]naphthalene-1,5,5-trisulfonic acid | 129-46-4 | Antineoplastic        | 5.58  | -6          | 31.1               | -                      | 35.55         | Yes R       |             | (28, 104, 105)         |
| Tacrine               | 1,2,3,4-tetrahydroacridin-9-amine | 321-64-2 | Cholinesterase inhibitor | 2.71  | 8.95        | 87.5               | -                      | +             | 63.5        | Yes         | (28)                   |
| Tamoxifen             | 2-[4-[4-[4-[2,4-diphenylbut-1-en-1-yl]phenoxoy]ethyl]dimethylamine | 10540-29-1 | Antiestrogenic        | 5.93  | 8.76        | 112                | +                      | 7.7           | Yes R       |             | (16, 27, 28, 34-36, 39) |
| Tetracaine            | 2-(dimethylamino)ethyl 4- (butylamino)benzoate | 94-24-6 | Anaesthetic           | 3.54  | 8.42        | 83.4               | -                      | +             | 333         | Yes         | (28)                   |
| Thioridazine          | 10-[2-(1-methylpiperidin-2-yl)ethyl]-2-(methylsulfanyl)-10H-phenothiazine | 50-52-2 | Antipsychotic         | 5.9   | 8.93        | 115                | +                      | +             | 38          | Yes R       | (16, 28, 36, 40, 106)  |
| Tobramycin            | O-[3-amino-3-deoxy-α-D-glucopyranosyl(1→6)]O-[2,6-diamino-2,3,6-trideoxy-α-D-ribohexitopyranosyl(1→4)]O-[2-deoxy-D-streptamine | 32986-56-4 | Antibiotic            | -5.8  | 9.8         | 130                | +                      | +             | 33.03       | Yes R,H     | (28, 36, 40, 107-109) |
| Trimipramine          | 3-(2-azatricyclo[9.4.0.08,15]pentadeca-1(15),3,5,7,11,13-hexaen-2-yl)-2-methylpropyl]dimethylamine | 739-71-9 | Antidepressant        | 4.67  | 9.42        | 111                | +                      | +             | 267.9       | Yes         | (7, 28, 40)            |
| Triparanol            | 1-(4-(2-diethylaminoethoxy)phenyl)-1-p-tolyl-2-(4-chlorophenyl)ethanol | 78-41-1 | Cholesterol synthesis inhibitor | 6.2   | 13.44       | 219                | +                      | +             | 78          | Yes R, M, Ha | (28, 33, 35, 73, 110-113) |

(continued)
TABLE 1. Continued

| Generic name | UPAC Designation | CAS Number | Indication | ClogP | pKa (basic) | Ploemen Value | Pred Ploemen | Pred modified Ploemen | Pred | LPLA2 IC50 (μM) | In Vitro PLD | In Vivo PLD | Refs |
|--------------|------------------|------------|------------|-------|-------------|---------------|--------------|------------------------|------|---------------|-------------|-------------|------|
| Vinblastine  | methyl (1R,9R,10S,11R,12R,13R)-11-(acetyloxy)-12-ethyl-4-[[13S,15R,17S]-17-ethyl-17-hydroxy-13-(methoxycarbonyl)-1,1-\[diazatetracyclo[13.3.1.0^{1.0}]\_nonadeca-4(12),5(10),6,8-tetraen-13-yl]-10-hydroxy-6-methoxy-8-methyl-8,16-diazapentacyclo[10,6,10\_1.0\_1.0\_1.0\_3]nonadeca-2,4,6,13-tetraene-10-carboxylate | 865-21-4 | Chemotherapeutic | 3.7   | 8.86 | 92.2 | + | + | 2123 | Yes | (28, 97) |
| Warfarin     | 4-hydroxy-3-(3-oxo-1-phenylbutyl)-2H-chromen-2-one | 81-81-2 | Anticoagulant | 2.41  | -6.6 | 49.4 | - | - | 94 | Yes | (28, 114) |
| Yohimbine    | methyl (1S,15R,18S,19R,20S)-18-hydroxy-3,13-diazapentacyclo[11.8.0.0^{2,10.0}_{4,9.0}_{15,20}]henicosan-2(10),4,6,8-tetraene-19-carboxylate | 146-48-5 | Alpha adrenergic antagonist | 2.73  | 7.65 | 66 | - | + | 200 | Yes | (28) |
| Zafirlukast  | cyclopentyl N-[3-[(2-methoxy-4-[(2-methylbenzenesulfonyl)carbamoyl]phenyl)methyl]-1-methyl-1H-indol-5-yl]carbamate | 107753-78-6 | Leukotriene receptor antagonist | 5.4   | -1.1 | 29.2 | - | - | 3.1 | Yes | (35) |

The generic name, International Union of Pure and Applied Chemistry (IUPAC) designation, chemical abstracts registry (CAS) number, and clinical indication are provided. Chemical properties, including ClogP and pKa (basic) were obtained from the Pubchem and chEMBL databases of bioactive molecules and used to calculate the Ploemen value for each compound. By convention, negative pKa values were assigned a value of 0 for calculation of the Ploemen number and would be predicted negative based on pKa values of less than 8 or 6 for the Ploemen and modified Ploemen models respectively (Table 3). The species for in vivo data are designated as human (H), dog (D), rat (R), mouse (M), and hamster (Ha). LPLA2 IC50 denotes the concentration at which 50% of the LPLA2 dependent 1-O-acyl N-acetylsphingosine synthase activity is observed.
| Generic Name | UPAC Designation | CAS Number | Indication | ClogP | pKa (basic) | Ploemen Value | Pred Ploemen | Pred Mod Ploemen | LPLA2 IC50 (μM) |
|--------------|------------------|------------|------------|-------|-------------|---------------|--------------|-----------------|-----------------|
| Amisulpride  | 4-amino-N-[(1-ethyl-2-pyrrolidinyl)methyl]-5-ethylsulfonyl)-2-methoxybenzamide | 71675-85-9 | antipsychotic | 1.5 | 7.05 | 52 | - | - | 1915 |
| Allopurinol  | 3-(4H-1,2,4-triazol-4-yl)1H-pyrazole-4-carboxamide | 315-30-0 | xanthine oxidase inhibitor | -0.55 | 2.57 | 6.9 | - | - | ND |
| Atovaquone   | 2-hydroxy-3-[(1r,4r)-4-(4-chlorophenyl)cyclohexyl] -1,4-dihydropyridinethalone-1,4-dione | 95233-18-4 | anti-pneumocystis, anti-malarial | 1.59 | 8.16 | 69.1 | - | - | ND |
| Atropine     | (1R,3R,5S)-8-methyl-8-azabicyclo[3.2.1]octan-3-yl 3-hydroxy-2-phenylpropanoate | 51-55-8 | antimuscarinic | 1.83 | 9.39 | 91.5 | - | - | 1874 |
| Azaperone    | 1-(4-fluorophenyl)-4-(pyridin-2-yl)piperazin-1-yl butan-1-one | 1649-18-9 | tranquilizer | 2.73 | 7.16 | 58.7 | - | + | ND |
| Benztropine  | (1R,3R,5S)-3-(diphenylmethoxy)-8-methyl-8-azabicyclo[3.2.1]octane | 86-13-5 | antitremor | 4.27 | 9.54 | 109 | - | 58.97 | ND |
| 18 Beta-glycyrrhetinic acid | 3β-hydroxy-11-oxo-18β,20-β-olean-12-en-29-oic acid | 471-53-4 | anti-inflammatory, antioxidant | 3.7 | 16 | 270 | + | + | 2432 |
| Butenafine   | [(4-tert-butylphenyl)methyl](methyl)(naphthalen-1-yl)methylamine | 101828-21-1 | antifungal | 5.85 | 9.23 | 119 | + | + | 41.2 |
| Carbamazepine | 2-azatricyclo[9.4.0.0^3,8]pentadeca-1(15),3,5,7,9,11,13-heptaene-2-carboxamide | 298-46-4 | anticonvulsant | 2.77 | -3.8 | 7.7 | - | - | 105.6 |
| Clomifene    | 2-[4-(2-chloro-1,2-diphenylethenyl) phenoxy]ethyl diethy lamine | 911-45-5 | estrogen agonist | 7.2 | 9.31 | 139 | + | + | 12.86 |
| Clonidine    | N-[2-(2-chlorophenol)-4,5-dihydro-1H-imidazole-2-amine | 4205-90-7 | antihypertensive | 1.59 | 8.16 | 69.1 | - | - | ND |
| Clorocromen  | 8-chloro-3-(2-diethylaminoethyl)-7-ethoxycarbonylmethoxy-4-methylcoumarin hydrochloride | 74697-28-2 | platelet aggregation inhibitor | 3.97 | 9.1 | 98.6 | - | + | 285 |
| Conessin     | (3S)-N,N-dimethyl-3-en-2-amine | 546-06-5 | antihistamine | 4.9 | 5 | 49 | - | - | 31.28 |
| Desloratadine | 13-chloro-2-(piperidin-4-ylidene)4-azatricyclo[9.4.0.0^3,8]pentadeca-1(1),3(8),4,6,12,14-hexa | 100643-71-8 | antihistamine | 3.48 | 9.73 | 107 | + | + | 836 |
| D(+)-glucose | (2R,3S,4R,5R)-2,3,4,5,6-pentahydroxyhexanal | 50-99-7 | monosaccharide | -2.4 | -3 | 14.8 | - | - | ND |
| Diclofenac   | (2R,2S)-dichloroethylene|benzene acid sodium salt | 15307-79-6 | nonsteroidal anti inflammatory | 4.51 | -2.1 | 20.3 | - | - | 7.2 |
| 5,7-Dichloro-8-hydroxy-2-methylquinolone | 5,7-Dichloro-2-methyl-8-quinolinol | 72-80-0 | antioxidant | 3.61 | 4 | 29 | - | - | 1179 |
| Dilazep      | 3-[4-[3,4,5-trimethoxybenzoyloxy]propyl]-1,4-diazepan-1-yl) propyl 3,4,5-trimethoxybenzoate | 35898-87-4 | adenosine uptake inhibitor | 3.21 | 9.54 | 101 | + | + | 59.76 |
| 17-Dimethylxanthine | 1,7-Dimethyl-1H-purine-2,6-dione | 611-59-6 | stimulant | -0.5 | 13.5 | 183 | - | - | ND |
| Disopyramide | 4-[bis(propan-2-yl)amino]-2-phenyl-2-(pyridin-2-yl)butanamide | 3737-09-5 | antiarrhythmic | 2.58 | 10.31 | 115 | + | + | ND |
| Fenspiride   | 8-(2-phenylethyl)-1-oxa-3,8-diazaspiro[4.5]decan-2-one | 50533-08-7 | anti-inflammatory | 1.81 | 9.37 | 91.1 | - | - | ND |
| Fosinopril   | (2S,4S)-4-cyclohexyl-1-[2[(R)-[4]]-2-methyl-1-[propanoloxopyroxopy]4-phenylbutyl] | 98048-97-6 | Angiotensin-converting enzyme inhibitor | 5.62 | -4.4 | 31.6 | - | - | 0.181 |
| Fosinoprilat | (2S,4S)-4-cyclohexyl-1-[2{(R)-[4]}-2-methyl-1-[propanoloxopyroxopy]4-phenylbutyl] | 95399-71-6 | active fosinopril metabolite | 3.7 | -4.7 | 35.8 | - | - | 5.764 |
| Fulvestrant  | (1S,3aS,3bR,4R,4bR,11aS)-11a-methyl-4-[9(4,4,5,5,5-pentafluoropentanesulfinyl)nonyl]-1H,2H,3H,3aH,3bH,4H,5H,9bH,10H,11H,11aH-cyclopenta[a]phenanthrene-1,7-diol | 129453-61-8 | chemotherapeutic | 6.54 | -0.88 | 42.8 | - | - | 61.85 |

(continued)
| Generic Name | UPAC Designation | CAS Number | Indication | ClogP | pKa (basic) | Pred Ploemen Value | Pred Ploemen | Pred Mod Ploemen | LPLA2 IC50 (μM) |
|--------------|------------------|------------|------------|-------|------------|---------------------|--------------|------------------|-----------------|
| Fusidic acid | (Sα,Sα,5α,5α,8α,9β,11α,13α,14,16β,17Z)-6-acetoxy-3,11-dihydroxy-4,8,14-trimethyl-18-norcholesta-17,24-dien-21-11-oic acid | 6990-06-3 | antibiotic | 4.97 | -0.2 | 24.7 | - | - | 231 |
| Gabapentin  | 2-[1-amino methyl] cyclohexylacetic acid | 60142-96-3 | antiepileptic | -1.9 | 9.91 | 102 | - | - | 37.37 |
| Harmine     | 7-methoxy-1-methyl-9H-pyridin-(3,4-)bindole | 442-51-3 | central nervous system stimulant | 2.61 | 6.46 | 48.5 | - | - | 633 |
| Hydralazine | 1-hydraylnylphthalazine | 86-54-4 | antihypertensive | 1 | 6.4 | 41 | - | - | 100 |
| Hydrocortisone | (1β,11β,17,21)-trihydroxyprogren-4-ene-3,20-dione | 50-23-7 | glucocorticoid | 1.61 | -28 | 10.4 | - | - | 402 |
| Hydroxytyramine | 2-(3,4-dihydroxyphenyl)acetic acid | 28094-15-7 | neurotoxin | 0.26 | 9.85 | 97.1 | - | - | 88.02 |
| hydrochloride | 3,4-dihydroxyphenylalanine | 62-31-7 | catecholamine neurotransmitter | -0.91 | 9.27 | 86.9 | - | - | 56.08 |
| Imiquimod  | 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine | 99011-02-6 | topical immunomodulatory | 2.83 | 5.01 | 33.1 | + | + | 160 |
| Isoxsuprine | 4-(L8,2R)-1-hydroxy-2-[[2R]-1-phenoxypropan-2-yl] amino propyl]benzol | 395-28-8 | vasodilator | 2.06 | 9 | 85.2 | - | + | UND |
| Lidocaine   | 2-diethylamino-6-[2,6-dimethylphenyl]acetamide | 137-58-6 | antiarrhythmic | 1.8 | 7.75 | 63.3 | - | - | ND |
| L-leucine   | (2S)-2-amino-4-methylpentanoic acid | 61-90-5 | amino acid | -1.52 | 9.52 | 92.9 | - | - | ND |
| Mebhydrolin | 5-benzyl-2-methyl-1H,2H,3H,4H,5H-pyrido[4,3-b]-indole | 524-81-2 | antihistamine | 3.5 | 6.7 | 57.1 | - | + | 146 |
| Meclofenamic acid | 2-[2-[2-dichloro-3-methylphenyl]amino]benzoic acid | 644-62-2 | nonsteroidal | 5.11 | -36 | 39.1 | - | - | ND |
| Melatonin   | N-acetyl-5-methoxyspyrmine | 73-31-4 | antagonistodrome | 1.42 | -16 | 4.6 | + | + | 38.03 |
| Mibefradil  | 1S,2S)-2-(2-[(3-(1H-1,3-benzodiazol-2-yl)propyl](methyl)amino]ethyl)-6-fluoro-1-(propan-2-yl)-1,2,3,4-tetrahydronaphthalen-2-yl 2-methoxyacetate | 116644-53-2 | calcium channel blocker | 5.34 | 9.82 | 124 | + | + | 12.92 |
| Naproxen    | (2S)-2-(6-methoxyphenethyl)-2-yl propanoic acid | 22204-53-1 | nonsteroidal | 3.18 | -48 | 33.2 | - | - | 1102 |
| Orphenadrine | (2S)-2-(6-phenoxynaphthalen-2-yl) methoxypropionate | 83-98-7 | muscle relaxant | 3.77 | 8.87 | 92.9 | + | + | 259.3 |
| Phenytoin   | 5,5-diphenyl-2,4-imidazolidinedione, 5,5-Diphenylhydantoin sodium salt | 630-93-3 | anticonvulsant | 2.47 | -9 | 61 | - | - | 60.05 |
| Pipamperone | 1-[4-[4-fluorophenyl]-4-oxoallyl]-[1,4]-bipiperidine]-4-carboxamide | 1893-33-0 | antipsychotic | 2.32 | 8.96 | 85.7 | - | + | 250 |
| PP 06424439 | (3R)-1-[2-[1-(4-chloro-1H-pyrazol-1-yl)cyclopropyl]-3H-imidazo[4,5-b]pyridin-5-yl]-3-piperidinyl]-1-pyrolidinyl-methanone | 1469284-79-4 | triglyceride and cholesterol lowering | 2.7 | 4.1 | 24.1 | - | - | 8217 |
| Prochlorperazine | 2-chloro-10-[3-[4-methylpiperazin-1-yl]propyl]-10H-phenothiazine | 58-38-8 | antipsychotic | 4.88 | 8.39 | 94.2 | + | + | 42.41 |
| Procyclidine | 1-cyclohexyl-1-phenyl-3-[pyrrolidin-1-yl]propan-1-ol | 77-37-2 | anticholinergic | 4.7 | 13.84 | 214 | + | + | 1470 |
| Ritalinser | 6-(2-[4-[bis(4-fluorophenyl)methylidene]piperidin-1-yl]ethyl)-7-methyl-5H-[1,3]isobenzofurane[3,2-a]pyridin 5-one | 87051-43-2 | serotonin receptor agonist | 5.02 | 8 | 89.2 | - | + | 13.39 |
| Rolipram  | 4-[3-cyclopentoxy]-4-methoxyphenylpyrroolidin-2-one | 61413-54-5 | antidepressant | 2.19 | -1.9 | 9.9 | - | - | 300 |
| SB222200 | (S)-3-methyl-2-phenyl-N-(1-phenylpropyl)-4-quinolinecarboxamide | 174635-69-9 | antihistamine | 2.17 | 9.57 | 98.2 | + | + | 14.94 |
| S-methyl-isothiourea | methyl carbamimidothioate | 867-44-7 | iNOS inhibitor | 1.47 | 9.83 | 98.8 | - | - | ND |
| Suloctidil | (1R,2S)-2-octylamino]-1-[4-(propan-2-ylsulfanyl)phenyl]propan-1-ol | 54767-75-8 | vasodilator | 5.54 | 9.76 | 126 | + | + | 6.82 |
| Trifluoperazine | 10-[3-[4-methylpiperazin-1-yl]propyl]-2-(trifluoromethyl)-10H-phenothiazine | 117-89-5 | antipsychotic, antiemic | 4.87 | 8.39 | 94.1 | + | + | 687 |

(continued)
inhibitory effects on LPLA2 of the compounds tested was therefore assessed as independent variables.

Under these assay conditions, 112 compounds from the entire library of known phospholipid transfer and control compounds inhibited LPLA2 acyl transferase activity at IC50 values less than 250 μM. Twenty-eight compounds inhibited with IC50s greater than 250 μM, and 19 compounds showed no inhibition (Fig. 1). Surprisingly, 35 compounds not previously reported to cause phospholipidosis inhibited LPLA2 with 22 compounds inhibiting at IC50s of 250 μM or less (black circles). The measured IC50s were continuous between 3.8 μM and 2 mM. The compounds assayed did not segregate based on therapeutic use or whether phospholipidosis had been reported as a result of in vitro (red circles), in vivo (blue circles) or both in vitro and in vivo studies (yellow circles). The most potent inhibitor among this group was fosinopril, an angiotensin-converting enzyme inhibitor not previously reported to cause phospholipidosis.

The library of compounds previously reported to cause phospholipidosis and assayed for LPLA2 inhibition were plotted based on their calculated ClogP and pKa basic values (Fig. 2). Following the convention employed in the Ploemen study, negative pKa values were assigned a value of 0 for purposes of calculating a Ploemen value. Eight compounds inhibited LPLA2 at concentrations greater than 500 μM. Three compounds (proparacaine, vinblastine, and clenbuterol) inhibited LPLA2 at millimolar concentrations, and two compounds, spiperone and chloroquine, inhibited LPLA2 with IC50s slightly greater than 500 μM. Four compounds (betaxolol, methapyrilene, ropinirole, and sulpiride) had no inhibitory activity against LPLA2 and could thus be considered to be true false-negatives for predicting phospholipidosis.

Importantly, 23 compounds in the library reported to cause phospholipidosis did not meet either the Ploemen or modified Ploemen criteria. Of these 23 compounds, 17 inhibited LPLA2, 9 of these compounds having IC50 values less than 50 μM. Thus in this limited library of compounds previously reported to cause phospholipidosis, LPLA2 inhibition was observed for almost three-quarters of the drugs that would have been considered false-negatives by the Ploemen or modified Ploemen criteria.

The second library consisting of compounds not reported to cause phospholipidosis was similarly graphed (Fig. 3). Thirty of 55 compounds inhibited LPLA2 at IC50 values less than 250 μM, and 15 of these compounds inhibited LPLA2 at less than 50 μM. Only half or 15 of the 30 inhibitors would have been identified by the modified Ploemen criteria. An in vitro assay using the LipidTOX red detection reagent was used to determine whether exposure of cells to these compounds was associated with lysosomal phospholipid accumulation (Table 4). This assay was validated using fosinopril, the
most potent inhibitor of LPLA2 activity (supplemental Figs. S1 and S2). In addition, none of the 163 compounds shifted the melting temperature of LPLA2 more than 2°C when assayed for thermal stability, consistent with a lack of direct binding of any compound to the phospholipase (supplemental Table 1). In contrast the fluorophosphonate inhibitors, isopropyl dodec-11-enyl fluorophophonate and methyl arachidonyl fluorophophonate, covalently bind to the catalytic serine of LPLA2 and increase the melting temperature by 10 and 12°C, respectively (22).

Fosinopril is an angiotensin-converting enzyme inhibitor not previously known to cause phospholipidosis. Fosinopril inhibited LPLA2 activity at an IC50 of 180 nM, considerably lower than that observed for any other compound (Fig. 4A and B). The basis for LPLA2 inhibition by this compound was therefore studied in greater detail. We had previously shown that the inhibition of electrostatic binding of liposomes to LPLA2 could be measured by loss of cosedimentation. Fosinopril partially inhibited the cosedimentation of liposomes and recombinant LPLA2 when centrifugation was performed at 150,000 g (Fig. 4C). As was previously reported with amiodarone (25), no inhibition of the soluble esterase activity of LPLA2 was observed in the presence of fosinopril. The transacylase activity of LPLA2 toward p-NPB as substrate was first confirmed by the formation of 1-O-butanoyl-N-acetylsphingosine when present in the fully constituted LPLA2 assay (Fig. 4D). The formation of 1-O-butanoyl-N-acetylsphingosine was also observed when LPLA2 activity was assayed only in the presence of LPLA2, N-acetylsphingosine, and p-NPB as a monodispersion (Fig. 4E) consistent with accessibility of the substrate and acceptor within the catalytic domain of LPLA2. However, in the presence of 250 nM fosinopril, no inhibition of 1-O-butanoyl-N-acetylsphingosine formation was observed. This is consistent with the absence of direct inhibition of LPLA2 by this drug.

Compared with untreated controls, 325 nM fosinopril significantly increased the number of LipidTOX Red particles as assessed by fluorescence microscopy (supplemental Fig. S1). Quantification of particles revealed an approximate 20-fold increase in particle number in fosinopril-treated cells compared with controls (supplemental Fig. S2), which was similar to that observed with amiodarone at the same concentration. Concomitant increases in percent area and mean fluorescence intensity (MFI) were also documented.

Fosinopril is a prodrug for the active metabolite fosinoprilat. The measured IC50 value for fosinoprilat (5.8 μM) was more than 50 times greater than that observed for fosinopril (0.18 μM). However, fosinoprilat also generated a positive signal in the LipidTOX assay (Table 4). Thus metabolism of fosinopril to its active metabolite cannot explain the previously reported absence of phospholipidosis in this case.

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**Table 3. The Ploemen and modified Ploemen criteria for prediction of phospholipidosis**

|                      | Ploemen Model                                                                 | Modified Ploemen Model                               |
|----------------------|------------------------------------------------------------------------------|------------------------------------------------------|
| Predicted positive   | If (pKa-basic)² + (ClogP)² ≥ 90, provided that pKa ≥ 8 and ClogP ≥ 1          | If (pKa-basic)² + (ClogP)² ≥ 50, provided that pKa ≥ 6 and ClogP ≥ 2 |
| Predicted negative   | When result < 90 or pKa < 8 or ClogP < 1                                      | When result < 50 or pKa < 6 or ClogP < 2             |

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**Fig. 1.** The range of LPLA2 inhibition by all compounds studied. The measured IC50 for inhibition of LPLA2 in the cell-free assay are plotted. Compounds from Table 1 in which phospholipidosis has been reported are denoted by in vitro studies (red circles), in vivo studies (blue circles), or both in vitro and in vivo studies (yellow circles). Compounds studied in which no prior reports of phospholipidosis are denoted by black circles.
DISCUSSION

There are three important findings in this study. First, inhibition of LPLA2, as measured by a decrease in 1-O-acylcarnitine formation in a cell-free assay, is observed in the presence of most of 110 drugs studied previously reported to cause phospholipidosis. None of the drugs assayed shifted the melting temperature of LPLA2 consistent with the absence of the direct binding of any compound to LPLA2. Thus the inhibition of LPLA2 activity by these compounds occurs within a concentration range and likely by a mechanism similar to amiodarone, namely interference with the electrostatic charge interaction between cationic residues in the lipid-binding domain of LPLA2 and anionic phospholipid head groups. This mechanism, however, would not explain the inhibition of LPLA2 by compounds that cause phospholipidosis but lack a functional group that would be protonated at lysosomal pH including fosinopril, mitotane, and mannitol.

Second, the LPLA2 inhibition assay identified several CADs known to cause phospholipidosis but that are not predicted to do so by use of in silico models based on the pKa and ClogP of CADs. The measurement of LPLA2 inhibition as a stand-alone assay for prediction of DIP is associated with a greater sensitivity and accuracy than models based on ClogP and pKa alone but slightly less than when these models are combined with an in vitro assay (28). Specifically, LPLA2 inhibition with an observed IC$_{50}$ < 500 μM is 86% accurate in predicting phospholipidosis compared with the 58 and 79% accuracies of the Ploemen and modified Ploemen models, respectively. The accuracy in predicting phospholipidosis is greater than 90% for any observed inhibition of LPLA2 (Table 5). Individual drugs that cause phospholipidosis may do so synergistically (116), and such drugs may achieve concentrations within the lysosome that are up to 50,000-fold greater than that measured extracellularly (117). It is therefore possible that compounds that inhibit LPLA2 may do so at concentrations significantly greater than those associated with their therapeutic activity.

Third, LPLA2 inhibition may identify chemical entities currently approved by regulatory agencies that cause phospholipidosis but not previously identified as such. This is exemplified in the current study by the potent inhibition of LPLA2 by fosinopril and its active metabolite fosinoprilat and by validation of their phospholipidotic potential in the LipidTOX Red phospholipidosis assay. The IC$_{50}$ value for fosinopril is significantly lower than that of the other drugs assayed in this study. Fosinopril is unique among the larger class of ACE inhibitors in that it contains a phosphinic-acid-containing ester that serves as the binding group as opposed to the more common carboxyl or sulfhydryl
functions that characterize other ACE inhibitors (118). It is also among the most lipophilic of this class of drugs. Like amiodarone, the mechanism of inhibition by fosinopril appears to occur by interference of binding between LPLA₂ and liposomes as supported by observed inhibition of cosedimentation of LPLA₂ and liposomes in the presence of fosinopril. However, fosinopril is an amide and as a weak base not protonated at lysosomal pH unlike cationic amphiphilic drugs. Thus a different mechanism of inhibition that is distinct from amiodarone is likely in this case.

While there is general agreement that phospholipidosis results from the lysosomal accumulation of CADs, there is less agreement regarding the cause. LPLA₂ is a good candidate for a cellular target by drugs that cause phospholipidosis. Although LPLA₂ was first characterized as a phospholipase with transacylase activity toward short-chain ceramide acceptors (17), it was later recognized to be a phospholipase A₂ with an acidic pH optimum (18). The further characterization of the enzymatic activity revealed broad substrate specificity to several glycerophospholipids including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylglycerol. Subsequent work characterized LPLA₂ as having both PLA₁ and PLA₂ activity (119).

The earliest observed phenotype of a transgenic mouse knocked out for LPLA₂ was the presence of alveolar macrophages with a foam cell appearance. Lipid analyses of both the macrophages and bronchoalveolar lavage fluid demonstrated increased levels of glycerophospholipids that were substrates for LPLA₂ (20). The pulmonary toxicity associated with amiodarone is consistent with several of these functions. In its classic form, amiodarone toxicity is manifest as the accumulation of lipid-laden alveolar macrophages. The ultrastructure of these foam cells is characterized by the presence of lamellar bodies within lysosomes. Because the knockout mouse phenotype bore a strong resemblance to that seen with pulmonary amiodarone toxicity, the possible inhibition of LPLA₂ by amiodarone was further studied (21). We observed at that time that amiodarone was not a direct inhibitor of LPLA₂, but appeared to block the electrostatic interaction between liposomes and enzyme. This mechanism was further supported by the loss of activity in the presence of buffers of higher ionic content.

More recently, we determined a structure of LPLA₂ by X-ray diffraction (22). The presence of the catalytic triad and the disulfide bond previously characterized was confirmed (120). Two tracks could accommodate the phospholipid head groups of a broad range of substrates. In the current model, sn-1 and sn-2 fatty acyl groups of these phospholipids can be oriented in track A within the catalytic domain and be recognized as the scissile fatty acyl group. This model is supported by the formation of 1-O-acyl-ceramides that are products of either sn-1 or sn-2 acyl groups on phospholipid substrates (121). The structural studies identified a distinct lipid binding domain and four cationic residues within the domain that are required for liposome binding. The observation that each of these residues was necessary for LPLA₂ activity lent further support for the

### TABLE 4. Screening phospholipidosis assay

| Drug                | Ratio (0.32 μM) | Ratio (15 μM) |
|---------------------|-----------------|---------------|
| Allopurinol         | 0.13            | 0.37          |
| Amisulpride         | 0.08            | 0.08          |
| Atropine            | 0.04            | 0.16          |
| Azaperone           | 0.16            | 0.43          |
| Benztropine         | 0.13            | 0.68          |
| Benzbramorone       | 0.0             | 0.5           |
| 18 Beta-glycyrrhetic acid | 0.36  | 0.4         |
| Butenafine          | 0.16            | 0.31          |
| Carbamazepine       | 0.12            | 0.18          |
| Clofazimine         | 0.11            | 0.15          |
| Clonidine           | 0.18            | 0.25          |
| Clomifene           | 0.09            | 0.38          |
| Cloricromen         | 0.1             | 0.1           |
| Conesin             | 0.01            | 1.08          |
| Corticosterone      | 0.12            | 0.28          |
| Desloratadine       | 0.14            | 1.13          |
| Diclofenac          | 0.22            | 0.49          |
| Meclofenamic acid   | 0.18            | 0.52          |
| Melatonin           | 0.17            | 0.29          |
| Hydrocortisone      | 0.12            | 0.15          |
| 6-Hydroxydopamine   | 0.2             | 0.93          |
| 3-Hydroxytyramine hydrochloride | 0.14  | 0.91         |
| Hydralazine         | 0.1             | 0.6           |
| Imiquimod           | 0.07            | 0.07          |
| Isoxsuprine         | 0.16            | 1.22          |
| Lidocaine           | 0.16            | 0.45          |
| Mehydroxydine       | 0.12            | 2.54          |
| Meclofenamic acid   | 0.18            | 0.52          |
| S-methylsulfoxide   | 0.15            | 0.38          |
| Melatonin           | 0.15            | 0.28          |
| Mibefradil          | 0.02            | 1.21          |
| Naproxen            | 0.07            | 0.1           |
| Orphenadrine        | 0.17            | 0.29          |
| Paroxetine          | 0.03            | 1.11          |
| Penfluridol         | 0.01            | 0.35          |
| Phenyoctin          | 0.23            | 0.38          |
| Pipperazine         | 0.17            | 0.37          |
| pp 0624439          | 0.15            | 0.31          |
| Prochlorperazine    | 0.16            | 0.81          |
| Prucyclidine        | 0.16            | 1.14          |
| Quinine             | 0.12            | 0.34          |
| Ritanserin          | 0.07            | 0.19          |
| Rolipram            | 0.11            | 0.19          |
| S922290             | 0.03            | 0.34          |
| Sulocitid           | 0.14            | 0.66          |
| Trifluoperazine     | 0.24            | 0.81          |
| Xylometazoline      | 0.14            | 0.16          |
| Amiodarone          | 0.32            | 1.64          |

Each compound was assayed at either 32 or 15 μM. The fluorescence ratio denotes LipidTOX Red Phospholipidosis detection (549–615 nm emission) to NucBlue Live detection (410–480 nm emission).
Fig. 4. Inhibition of LPLA₂ by fosinopril. A: Thin layer chromatography from the LPLA₂ assay in the presence of fosinopril. The reaction products include free fatty acid and 1-O-acyl-N-acetyl-ceramide (1-O-acyl-NAS). B: LPLA₂ activity in the presence of fosinopril as a percent of the control assay run in the absence of fosinopril. C: Cosedimentation of liposomes and LPLA₂ in the presence or absence of fosinopril. Liposomes consisting of DOPC/ sulfatide (10:1, molar ratio, 127 μM total) were incubated with 5 μg of LPLA₂ and different concentrations of fosinopril in 500 μl of 50 mM sodium citrate pH 4.5 for 30 min on ice. The reaction mixture was then centrifuged for 1 h at 150,000 g at 4°C. The resulting precipitate was rinsed with cold 50 mM sodium citrate pH 4.5 and dissolved with 40 μl of SDS-PAGE sample buffer. The sample was separated by using 10% SDS-PAGE. Following electrophoresis, LPLA₂ was detected with Simply Blue. Band quantification was performed with the Image J software I1.651j8. D: LPLA₂ transacylase activity against comparing DOPC to p-NPB as substrates. Liposomes containing DOPC-sulfatide (10:1 M ratio) were incubated with recombinant LPLA₂ (30 ng/ml) with or without p-NPB (200 μM) in the presence or absence of 10 μM NAS at 37 degrees C in 500 μl Na-citrate buffer (50 mM, pH 4.5). E: LPLA₂ transacylation activity toward p-NPB comparing liposomes to a monodispersed substrate. Fosinopril (250 nM) was present in lanes 5 and 6. The reactions as detailed in panels E and F were terminated by the addition of 3 ml chloroform/methanol (2/1, v/v), followed by 0.3 ml of 9% (w/v) NaCl. After centrifugation for 7 min at 1,800 g, the resulting lower layer was transferred to new tube and dried under stream of nitrogen gas. The dried lipid was dissolved in 40 μl of chloroform/methanol (2/1, v/v) and applied to HPTLC plates. HPTLC plates were run in chloroform/acetic acid (9/1, v/v). The plates were dried and soaked in 8% (w/v) CuSO₄₅H₂O, 6.8% (v/v) H₃PO₄, and 32% (v/v) methanol and then charred for 15 min in an oven at 150°C. Scanned plates were analyzed by NIH ImageJ 1.65i8 (National Institutes of Health).
proposed mechanism of inhibition by amiodarone. Acid sphingomyelinase has also been identified as another target for DIP. A role for acid sphingomyelinase is also supported by the possibility that the substrate recognition of this phospholipase C may extend to phospholipids and beyond sphingomyelin. However, while the phospholipase C activity of acid sphingomyelinase may extend to phosphatidylcholine as well as sphingomyelin, the comparative activity is an order of magnitude greater for sphingomyelin (122, 123).

Although the inhibition of both LPLA2 and lysosomal acid sphingomyelinase by drugs that cause phospholipidosis has been proposed to occur by inhibition of electrostatic interactions between the respective phospholipases and anionic lipids, this model would not explain the phospholipidosis observed by compounds that are not basic drugs. This is exemplified in the present study by mitotane and mannitol, which lack amines and thus have no assignable pKa.

DIP has been an active focus of regulatory agencies including the FDA for more than 20 years. In 2004, the FDA announced that it formed an initiative named the Phospholipidosis Working Group under the auspices of the Center for Drug Evaluation and Research (124). The overarching goal of this group was to establish regulatory guidance for drugs that were observed to cause phospholipidosis. Significant research was fostered by this initiative leading to new in silico and in vitro tests, studies on the relation

| Table 5. LPLA2 assay sensitivity, specificity, and accuracy in comparison to those based on the Ploemen criteria |
|---------------------------------------------------------------|
| **Sensitivity/Specificity** | **PPV/NPV** | **False Positives** | **False Negatives** | **Accuracy** |
|-----------------------------|-------------|---------------------|---------------------|-------------|
| Ploemen model               | 55/87       | 96.3/24             | atrapine, 18 beta-glycyrhetinic acid, S-methylisothioiurea | azaperone, benzbromarone, bromocriptine, carbamazepine, clozapine, conessin, corticosterone, cyclosporin, diethylstilbestrol, 5,7-dichloro-8-hydroxy-2-methyl quinolone, diphenhydramine, dutasteride, erythromycin, etomate, fenofibrate, fipexide, flunarizine, flufenamic acid, fosinopril, fosinoprilat, fulvestrant, fusidic acid, gabacon, gentamic acid, hydralazine, hydroxyurea, 6-hydroxydopamine, 3-hydroxytyramine, isoxsuprine, ketoconazole, ketotifen, losapramine, loratadine, mannitol, mebhydrolin, melatonin, mianserin, mirtazapine, mitotane, oxalate, oxalate, citrate, pantoprazole, pentfluoridol, perphenazine, phenacetin, phenytoin, pipamperone, pirenperone, pranlukast, progesterone, proparacaine, pyrillamine, repaglinide, ritanserin, sipiperone, sulindac, sulpiride, suramin, tacrine, tetracaine, warfarin, yohimbine, zafirlukast | 58.2 |
| Modified Ploemen model     | 76.4/95.7   | 99.1/40             | 18 beta-glycyrhetinic acid | benzbromarone, carbamazepine, conessin, corticosterone, cyclosporin, diethylstilbestrol, 5,7-dichloro-8-hydroxy-2-methyl quinolone, etomate, fenofibrate, fipexide, flufenamic acid, fosinopril, fosinopril at, fulvestrant, fusidic acid, gabacon, gentamic acid, hydralazine, 6-hydroxydopamine, 3-hydroxytyramine, loratadine, mannitol, melatonin, mitotane, pantoprazole, phenacetin, phenytoin, pranlukast, progesterone, sulindac, sulpiride, suramin, warfarin, zafirlukast | 79.1 |
| LPLA2 (IC50 ≤ 500 μM)     | 87.0/78.2   | 96/50               | carbamazepine, imiquimod, ritanserin, rolipram, ropinirole | azaperone, betaxolol, chloroquine (655), clenbuterol (7298), clindamycin, 5,7 dichloro-8-hydroxy-2-methyl quinolone (1179), disopyramide, dutasteride (1048), etomate (1155), isoxsuprine, lidocaine, methylnprilene, phenacetin (2140), pranlukast, procyclidine (1470), proparacaine (2469), sulpiride, vinblastine (2123) | 85.9 |
| LPLA2 (IC50 any concentration) | 92.8/78.2 | 96.2/64             | carbamazepine, imiquimod, ritanserin, rolipram, ropinirole | azaperone, betaxolol, clindamycin, disopyramide, isoxsuprine, lidocaine, methylnprilene, pranlukast, sulpiride | 90.7 |

The table compares the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the Ploemen, modified Ploemen, and LPLA2 assays. Compounds associated with false-positive and false-negative results are listed. The numbers in parentheses denote the μM IC50 for LPLA2 inhibition.
between CAD and DIP, efforts to understand potential connections between phospholipidosis and other toxicities such as QT prolongation and protein trafficking defects, and biomarker development including bis(monoacylglycerol) phosphate. However, these efforts did not provide a consensus as to a common mechanism or cellular target for CADs that cause phospholipidosis.

The identification here that LPLA2 inhibition is a primary basis for DIP provides further insight into the toxicological significance of DIP. While over 50 inherited monogenic lysosomal disorders have been identified, no clinical phenotype has yet to be described for an inherited loss of LPLA2 activity. However, a variety of potentially important biological roles for LPLA2 have been reported suggesting that long-term LPLA2 inhibition may be a pathological significance. These include a role for LPLA2 in surfactant degradation (20, 125), catabolism of oxidized phospholipids (126), oculomacular degeneration (127), host response to tuberculosis (128), and lipid antigen presentation through CD1d (129). While LPLA2 is expressed ubiquitously, the high activity of the phospholipase A2 in macrophages and other antigen presenting cells is consistent with an important role in host defense and antigen processing. Whether or not prolonged exposure to CADs that inhibit LPLA2 confers increased risk to loss of these functions will require further evaluation. Importantly, the recognition that LPLA2 is a primary target for DIP should aid in discerning drug-specific toxicities that are independent of LPLA2 inhibition and the result of other off-target effects.

Finally, the recognition that LPLA2 is the primary target for DIP raises the possibility that variants in the LPLA2 gene may account for differences in susceptibility to drugs that cause phospholipidosis within the population. Numerous sequence and splice variants have been identified for LPLA2, several of which are in the open reading frame of the LPLA2 gene and would predictably change the activity of the lipase either by resulting in the loss of catalytic activity or by conformational changes affecting the lipid-binding domain. Amiodarone, a highly effective antiarrhythmic, would be an obvious agent to study as DIP often limits its use. Future questions of interest might focus on establishing whether intrinsic differences in LPLA2 activity due to these variants account for susceptibility to amiodarone toxicity and whether structure activity studies of amiodarone might identify analogues that eliminate LPLA2 inhibition while maintaining antiarrhythmic activity.

Data availability

All data are contained within the article and supplemental data.

Supplemental data

This article contains supplemental data.

Author contributions

V. H.-G., T. T., J. M. S., A. L., and A. A. investigation; V. H.-G. and A. A. methodology; V. H.-G. and J. A. S. writing-original draft; A. A. and J. J. G. T. writing-review and editing; J. J. G. T. and J. A. S. funding acquisition; J. A. S. conceptualization; J. A. S. project administration; J. A. S. supervision.

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Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article. Recombinant LPLA2 and anti-LPLA2 monoclonal antibodies are licensed to Echelon Biosciences by the University of Michigan.

Abbreviations

CAD, cationic amphiphilic drug; DIP, drug-induced phospholipidosis; DODPC, 1,2-di(n-docosanyl)-sn-glycerol-3-phosphocholine; DOPC, 1,2-dioleoyl-sn-glycerol-3-phosphocholine; HPTLC, high-performance thin layer chromatography; LPLA2, lysosomal phospholipase A2; NAS, N-acetyl-sphingosine; pNPB, p-nitro-phenyl butyrate.

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