# In Silico Assay for Assessing Phospholipidosis Potential of Small Druglike Molecules: Training, Validation, and Refinement Using Several Data Sets

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ABSTRACT: Phospholipidosis (PLD) is a lysosomal storage disorder induced by compounds, notably cationic amphiphilic drugs, which although reversible interferes with cellular phospholipids. The in silico method described utilizes the amphiphilic moment $\Delta\Delta G_{AM}$ (kJ/mol) together with basic pK_a values to assign PLD inducing potential to a compound. The new model was accurate and sensitive (85% and 82%, respectively) when compared to other data sets. Therefore, the parallel in vitro assay for PLD was discontinued. The data reinforce our view that the amphiphilic moment is far more informative for determining a compound's potential to induce PLD than the combined use of basic pK_a and ClogP values.
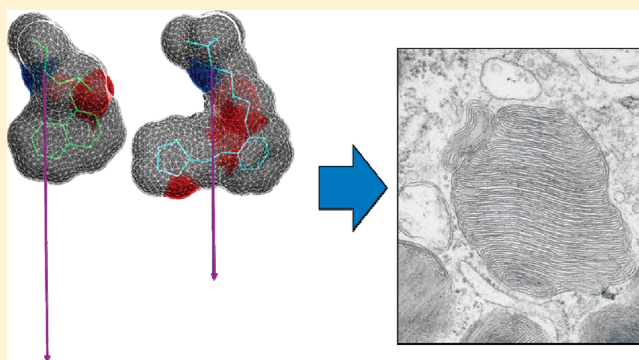
INTRODUCTION

From a commercial perspective, attrition late in drug development represents a serious problem to the pharmaceutical industry,[1] and early recognition of characteristics such as drug induced phospholipidosis (PLD), which may cause a compound to fail at later stages of development, may yield appreciable cost savings.[2] As part of the critical path initiative by the FDA,[3] PLD was targeted as a potential bottleneck in drug development. As a result, there are several ongoing projects under this initiative that concern the assessment of PLD potential early in drug development.

With respect to patient safety, there is a need to recognize early in development the potential of a compound to cause PLD and any associated toxicity before exposing a large patient population to investigative compounds.

This paper describes a new accurate and sensitive in silico method for predicting the likelihood of a compound to induce PLD.

Phospholipidosis. PLD is associated with the excessive accumulation of phospholipids in cells, usually within the lysosomes. PLD is characterized by the presence of inclusion bodies that are mainly concentric lamellar which are thought to arise as an adaptive cellular response and can be viewed using electron microscopy. Multiple tissue types may be affected including lymphocytes, lung, and liver.

Drug Induced Phospholipidosis. Drug induced PLD is commonly induced by the repeated administration of cationic amphiphilic drugs (CADs). CADs are a class of drugs that contain both a hydrophobic region (usually an aromatic ring/system) and a hydrophilic side chain (positively charged at physiological pH).[4] Drug induced PLD is thought to be an adaptive response to the administration of CADs whereby the drugs are sequestered into the inclusion bodies, thus possibly reducing cellular toxicity. The accumulation of phospholipids can occur via several mechanisms including the inhibition of lysosomal phospholipases, most likely through binding of CADs to phospholipids,[5,6] the direct inhibition of lysosomal phospholipases,[7] and the regulation of phospholipid synthesis.[8] Mitochondrial dysfunction has been identified as a toxic consequence of drug induced PLD.[9,10] PLD is reversible and generally disappears upon withdrawal of the CAD. The long-term toxicological effects of drug induced PLD are unknown, although it does share many characteristics with lipid storage disorders.[11]

Studies of Drug-Induced PLD. Drug-induced PLD has been studied in a variety of in vivo and in vitro systems. In 1978, Lullmann et al.[6] reported that CADs form complexes with acidic phospholipids that are resistant to degradation by phospholipases, the net result being lysosomal accumulation of the complexes. Predictions of PLD based on molecular structures could be verified more easily in cell culture. In whole animals this was more difficult probably because of other factors such as drug metabolism. CADs that are basic and have pK_a values higher than 7−8 were preferentially concentrated in the lysosomes.[6] Lullmann-Rauch and von Wizendorff[12] used cultured bovine corneal fibroblasts to assess the potency of the immunomodulatory drug tilorone to cause lysosomal storage of glycosaminoglycans. Tilorone interacts both electrostatically and intramolecularly (via its planar tricyclic ring system) with

glycosaminoglycans to form nondegradable complexes that accumulate in the lysosomes.

Over 50 drugs with cationic amphiphilic structures have been reported to induce PLD.[11] The mechanisms underlying PLD induction by CADs are complex and probably involve more than one mechanism. Investigations into the toxicity arising from PLD in humans are limited by the lack of available clinical specimens. Taken together, it is likely that PLD in humans is under-reported. From a biomarker perspective, lysobisphosphatidic acid (LBPA) remains a useful candidate for drug-induced PLD and further studies into this and other promising candidate biomarkers are warranted. The regulatory concerns regarding PLD induction will only be overcome when the potential functional and toxicological effects of PLD are elucidated.[11]

Hostetler[13] suggested a three-stage mechanism for the induction of PLD in rats and cultured Madin−Darby canine kidney (MDCK) cells. The drug must be able to enter the target cells readily, be trafficked to the lysosomes, and inhibit the lysosomal phospholipases A and C either directly or via alteration to the lysosomal pH. It was hypothesized that this mechanism may be relevant to other cell types and systems, i.e., humans. The administration of either gentamicin or chlorphentermine to rats resulted in the inhibition of phospholipases and the accumulation of phospholipids leading to PLD.[14] Gentamicin is a cationic hydrophilic compound and has been shown to have a mechanistic link to nephrotoxicity via phospholipidosis.[15]

Phospholipases are located at the inner leaflet of the lysosomal membrane where a lipid anchor connects the lipase with the membrane. A disruption of the binding of this lipid anchor, e.g., by increasing the lateral membrane pressure, leads to disruption of the phospholipase activity.[16] It can be speculated that increasing the lateral membrane packing density by an excessive binding of cationic amphiphilic drugs would nonspecifically inhibit the phospholipase activity. This mechanism would account for the fact that not only cationic compounds but particularly cationic amphiphilic compounds are prone to induce phospholipidosis.

Models for Determining Phospholipidosis Potential. Cell based and in silico models have been developed for the early determination of a compound's PLD inducing ability.[17−19] In silico techniques represent the fastest and most cost-effective method. In 2000, Fischer et al.[20] described a method for calculating the amphiphilic properties of a small molecule/compound. The program, Calculated Free Energy of Amphiphilicity of Small Charged Amphiphiles (CAFCA), was used to quantify amphiphilicity in terms of the free energy of amphiphilicity ($\Delta\Delta G_{AM}$). This value is proportional to the distance between the hydrophilic and the hydrophobic residues. By use of this model, those compounds that induced PLD had a basic pK_a higher than 6.3 and $\Delta\Delta G_{AM} \leq -6$ kJ/mol. Ploemen et al.[21] devised a model that used two calculated physicochemical values, pK_a (basicity) and ClogP (partition coefficient of a compound between octanol/water), to determine whether a compound would induce PLD. ClogP and pK_a clustered differently for the CADs when compared to the chemical series of piperazines. Compounds were found to induce PLD if $ClogP^2 + pK_a^2 \geq 90$, provided that ClogP was ≥ 1 and $pK_a \geq 8$. The results were validated in an in vitro cell line model where each compound was assessed for its ability to form lamellar inclusion bodies. These were observed in cells treated with the CADs but not in those exposed to the piperazines. In summary, compounds with relatively high ClogP and pK_a values (i.e., the CADs) induced PLD.

In 2007 Pelletier et al.[22] defined the predictive capacity of the Ploemen model[21] and suggested a series of improvements that included improving the rules by which a compound was predicted as being positive or negative for the induction of PLD and correcting the limit of the pK_a threshold to almost the same limits as Fischer et al.[20] Compounds induced PLD if $ClogP^2 + pK_a^2 \geq 50$, provided that ClogP ≥ 2 and $pK_a \geq 6$. Further improvements were achieved by using a Bayesian model with additional descriptors including amphiphilic moment, number of basic and acidic centers, and FCFP_4 fingerprints. The final Pelletier model therefore has 92% sensitivity, 93% negative predictive value, and 83% concordance with in vivo data for the induction of PLD.

It would be unwise to rely solely on one approach for determining how a compound may behave with respect to its potential for inducing PLD. The major drawback of this approach occurs because of noninducers being classified as PLD inducers via in silico or cell based assays, i.e., false positives. With respect to drug induced PLD, in vitro results are not a guarantee of in vivo behavior. Another shortcoming of this approach is the large number of structural properties that have to be controlled in order to come up with concise suggestions on how to improve molecules having a high potential for PL. Therefore, the most suitable model should be selected not only by concordance, selectivity, and sensitivity but also by the number and interpretability of the required molecular parameters. The ideal solution would be a combined approach using a high-throughput in silico screening test, followed by the further investigation of a small number of test compounds using in vivo models.

History and Aims. The in silico model described here was generated in 1999 and blind validated against an in vivo data set comprising 32 compounds for which PLD inducing potential had already been published. The model was then used continuously alongside in-house in vitro data from projects up to the end of 2004. The phospholipidosis in vitro assay described within this paper was then abandoned as no additional value was generated by running this assay alongside the in silico model.

The aim of this work is (1) to further describe a new in silico model for the screening of compounds for their potential to induce phospholipidosis in vivo, (2) to provide details of experiences with the new predictive model over the past decade in addition to earlier publications,[20,23] and (3) to compare and validate the reliability of the new model via reference to in-house in vivo data and previously published data sets,[24] together with alternative in silico models.[21,22] The model should enable an easy molecular interpretation of the findings such that investigators could use this information to optimize their molecules.

■ MATERIALS AND METHODS

Measurement of the Free Energy of Amphiphilicity. In terms of chemical structures, amphiphilicity is defined as the spatial difference between a distinct hydrophilic and hydrophobic region in a molecule. After the amphiphiles are dissolved in water, the hydrophobic portion is segregated from water either by the partitioning of the hydrophobic part into the air−water interface or by self-aggregation (micelle formation). The difference between both processes, the partitioning of the compound in the air−water interface and the concentration at which micelles are formed, is very nicely correlated with the amphiphilicity of a molecule. Hence, the free energy of amphiphilicity, $\Delta\Delta G_{AM}$, is defined as the difference in free energy between the partitioning of a compound into the air−water

interface, ΔG_{AW}, and free energy of the critical micellar concentration, ΔG_{MIC}.

$$\Delta\Delta G_{AM} = \Delta G_{AW} - \Delta G_{MIC} \qquad (1)$$

Calculation of the Amphiphilic Moment. The amphiphilic moment of a molecule $\vec{A}$ can be calculated by vector addition of all hydrophilic/hydrophobic residues in a molecule starting from the gravity of the charged parts in the molecule.

$$\vec{A} = \sum_i d_i \cdot \vec{\alpha}_i \qquad (2)$$

$\vec{\alpha}_i$ stands for the hydrophobic/hydrophilic contribution of an atom/fragment as described in Meylan and Howard (1995),[50] and d defines the distance between the center of gravity of the charged part of a molecule and the hydrophobic/hydrophilic moiety. For phospholipidosis the amphiphilicity is calculated for monoprotic bases. In the case of diprotic basic compounds, the midpoint between the charged parts of the molecule is taken as origin for vector addition.

Next, the amphiphilic moment was correlated with a homologous series of amphiphiles. This equation was used to transfer the amphiphilic moment into free energy of amphiphilicities $\Delta\Delta G_{AM}$.

The effect of various conformations was investigated, and it turned out that the most extended conformer with the highest amphiphilic moment shows the best correlation with the experimental determined $\Delta\Delta G_{AM}$ value.

CAFCA can be used to quantify the amphiphilic properties of molecules and also estimate their preferred conformation and orientation in biological membranes.[20]

In more detail, CORINA, version 3.46 (Molecular Networks GmbH, Erlangen, Germany), was used to generate a 3D conformer in a canonized way which was used as an input structure for the generation of multiple conformers within OMEGA, version 1.8.1 (OpenEye Scientific Software, Santa Fe, NM, U.S.). The resulting SDF file containing the multiple conformers was transferred into SMILES (simplified molecular input line entry specifications) together with the 3D coordinates of the individual atoms. This was done with the help of the Daylight toolkit, version 4.94 (Daylight Chemical Information Systems Inc., Santa Fe, NM, U.S.).

The assignment of lipophilicities to atoms/fragments was done on the level of SMILES. This was used to calculated the amphiphilic moment in a way similar to that used for calculating the dipole moment[20] but always starting from the most basic center in the molecule.

In Silico pK_a Calculation. In silico determination of the dissociation constant (pK_a) remains the fastest and cheapest way of determining pK_a values for a compound. MoKa[25] uses a novel approach for the in silico calculation of pK_a. The program was trained using a diverse set of more than 25 000 pK_a values and uses an algorithm based on descriptors derived from GRID molecular interaction fields. The predicted pK_a values generated by MoKa are accurate to 0.4 pK_a units for most compounds. As the program was developed using pK_a measured in aqueous media, the predicted pK_a values are water pK_a values. MoKa automatically warns of tautomeric forms that are unstable in the aqueous environment and also warns about possible covalent hydration events.

Phospholipidosis in Vitro Assay. Normal bovine corneal fibroblasts were isolated from corneal explants following collagenase treatment. Cells were cultured under standard tissue culture conditions (37 °C and 5% CO_2) in DMEM supplemented with 10% FCS, L-glutamine, and antibiotics. In the assay, cells from passage 2−8 were used and adjusted to a concentration of 1×10^6 cells/mL, and 2 mL of cell suspension was added to preprepared 35 mm culture dishes. Confluent cells were exposed to different concentrations of the test compounds for 72 h. Test compounds were dissolved in either DMSO or PBS. These were then diluted with culture medium so the test compound was at 20 μM and, if appropriate, DMSO was at 1%. Seven concentrations of test article were prepared by further dilution with culture medium such that 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, and 20 μM solutions of test compounds were prepared. Control dishes

(in duplicate) comprised culture medium only controls, imipramine (at 10 μM) as a positive control, and a 1% DMSO control. In each culture dish the medium was replaced with 3 mL of the respective test or control culture solution. Dishes were then incubated for 3 days under standard culture conditions.

After exposure the cells were fixed in 1% glutaraldehyde and stained with 1% tannic acid overnight. Phospholipids were identified qualitatively using light microscopy as punctuate, black intracytoplasmic grains, or inclusions.

■ RESULTS

Use of Amphiphilicity and Basic pK_a To Describe PLD. Amphiphilicity and basic pK_a can both be calculated in silico. Table 1 summarizes this data for a training set of 24 compounds, together with in vitro PLD data and ClogP values. Amphiphilicity is expressed as $\Delta\Delta G_{AM}$ according to Fischer,[20,23] and pK_a values were calculated using MoKa.[25] Of the 24 compounds, 12 had been shown to induce PLD using the bovine corneal fibroblast assay[26] and 12 had not induced PLD in vitro. It is interesting to note that the threshold values defined according to the training set of 24 compounds (in ref 23) were never changed even after the application of hundreds of compounds with measured in vitro PLD data.

Because of intellectual property rights, details of the original data set cannot be shared. Therefore, a new training set covering the same properties as the compounds of the original training set was defined (Table 1). This can be used to rebuild the published model[23] or used as training data for new approaches. Because of the simplicity of the approach that we developed and its dependency on other commercially available software (see Material and Methods), we are unable to share the code. However, we are open to answering any questions from programmers who need support to set up the program.

Figure 1 is a plot of in silico data and shows amphiphilicity versus basic pK_a. Those compounds with $\Delta\Delta G_{AM} \leq -6$ kJ/mol and basic $pK_a \geq 6.3$ but $pK_a \leq 11$ induced PLD. Compounds with $\Delta\Delta G_{AM} > -6$ kJ/mol did not induce PLD. A third set of compounds that had $\Delta\Delta G_{AM} \leq -6$ kJ/mol and basic $pK_a < 6.3$ or $pK_a > 11$ did not induce PLD either. The parameters for determining the induction of PLD from in silico data would appear easier to define than those for compounds that do not induce PLD.

Validation of in Silico Method with Published in Vivo Data. In a series of 32 compounds for which in vivo data were available from the literature, the capacity to induce PLD is illustrated in Table 2 as predicted via the in vitro assay and also using the in silico method described here. Calculated values for amphiphilicity, basic pK_a, and ClogP are also shown for the compounds. There is 91% agreement (29/32) between the in vivo data and in silico data for assigning PLD inducing potential to a compound. From the in vivo PLD positive compounds 93% (25/27) and 80% (4/5) in vivo PLD negative compounds are predicted correctly. In fact irrespective of which pair of PLD inducing potential data is analyzed there is an 88% (in vitro/in vivo) to 91% (in silico/in vivo) concordance between all the methods. Therefore, the in silico method is as good at predicting PLD inducing potential as either the in vivo or in vitro methods when looking at these 32 compounds.

Specific analysis of these 32 compound using three in silico methods, namely, the in silico method described here, together with those of Ploemen[21] and Pelletier,[22] reveals that the new method has a higher degree of accuracy and sensitivity when compared to the other two. However, specificity and precision levels with the new method do fall slightly from 100% with the

Table 1. Molecular Structure and Physicochemical Properties of a 24 Compound Training Set[a]
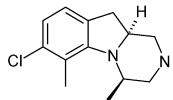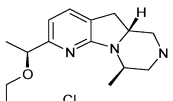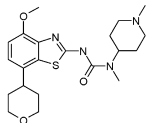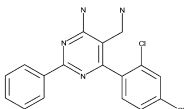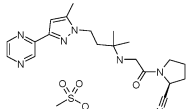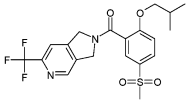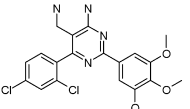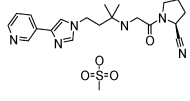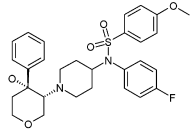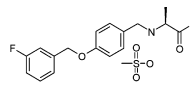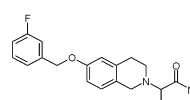
ID	Structure	in-vitro finding PLD	$\Delta\Delta G_{AM}$ [kJ/mol]	Calculated basic pKa	ClogP	Reference
1		positive	-6.44	8.89	3.46	31
2		negative	-4.03	8.22	2.11	31
3		positive	-8.95	8.5	2.38	52
4		positive	-7.45	7.47	3.66	30
5		negative	-5.12	7.24	0.95	53
6		negative	-6.12	4.46	2.59	55
7		positive	-8.21	7.47	3.11	51
8		negative	-5.24	7.22	1.07	53
9		negative	-6.76	5.98	3.95	54
10 (safinamide)		positive	-8.79	7.12	2.49	65
11		positive	-9.01	6.64	3.18	56

Table 1. continued
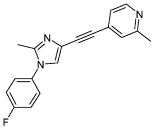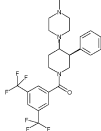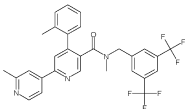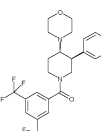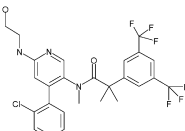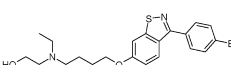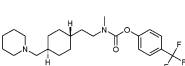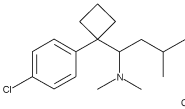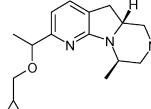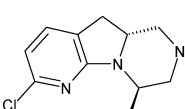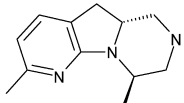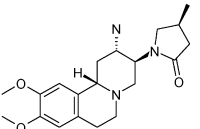
ID	Structure	in-vitro finding PLD	$\Delta\Delta G_{AM}$ [kJ/mol]	Calculated basic pKa	ClogP	Reference
12		negative	-7.13	5.44	3.15	57
13		positive	-16.00	8.5	3.13	58
14		negative	-17.30	4.52	6.74	59
15		positive	-10.34	7.11	4.77	58
16		negative	-14.83	4.98	6.39	60
17		positive	-17.19	9.44	5.85	61
18		positive	-11.47	9.82	6.41	62
19 (sibutramine)		positive	-7.07	9.67	5.59	64
20		positive	-6.05	8.22	2.56	31
21		negative	-8.73	11.45	1.65	63
22		negative	-4.34	8.22	2.29	31

Table 1. continued

ID	Structure	in-vitro finding PLD	$\Delta\Delta G_{AM}$ [kJ/mol]	Calculated basic pKa	ClogP	Reference
23		negative	-3.67	8.22	1.99	31
24		negative	-5.35	7.66	1.29	34

[a]Structural and physicochemical data for the training set of 24 compounds, together with invitro PLD data, calculated pK_a data (which are in good agreement with experimentally determined data, not shown), and ClogP values. Amphiphilicity is expressed as $\Delta\Delta G_{AM}$ according to Fischer,[20,23] and pK_a values were calculated using MoKa.[25]

Figure 1. Plot of amphiphilicity ($\Delta\Delta G_{AM}$) versus calculated basic pK_a for the training set of 24 compounds. The red area defines the region where a positive PLD response is expected, and the green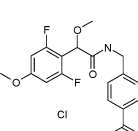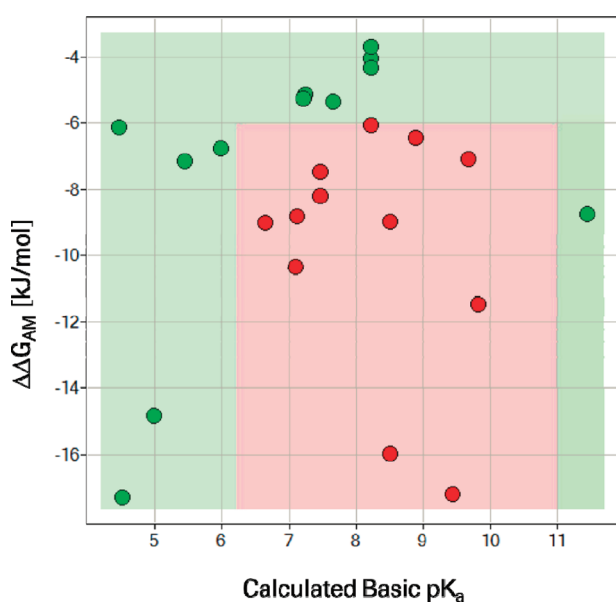 area defines where a negative response is expected according to the in silico tool. Red data points indicate a positive in vitro PLD finding and green a negative PLD finding. Compounds that do not induce phospholipidosis are represented by solid green circles (12 compounds) and those that do induce phospholipidosis by solid red circles (12 compounds). The inducers all have $\Delta\Delta G_{AM} \leq -6$ kJ/mol and p$K_a \geq 6.3$ and $K_a \leq 11$.

other two methods to 80% (4/5) and 96% (25/26), respectively.

Apart from two exceptions (chloramphenicol and trospectomycin) all the inducers of PLD determined during in vivo experiments have calculated $\Delta\Delta G_{AM} \leq -6$ kJ/mol and there are 2 out of the 3 compounds that show discordance between the in vivo and in silico results (the other being amikacin). Interestingly where discordance occurs between the three methods, the compound involved is in all cases an antibiotic.

Assessment of Compounds via In-House in Vitro and in Silico Methods. When compared to the in-house in vitro data for induction of PLD over a 6-year period, during which 422 compounds were assessed, the in silico data maintain a

concordance rate of ≥79% and a specificity rate of ≥89% when compared to the in vitro data (Figure 2). The sensitivity values observed were ≥60% throughout the six years. Overall, the in silico data show good concordance and specificity with respect to the in vitro data.

A direct comparison between the in silico model described here and the Ploemen[21] and modified Ploemen models[22] was performed for 422 compounds. The model presented here showed the highest accuracy (86%), was vastly more sensitive than the Ploemen model (80% vs 33%), and yet retained a specificity of 90%. Overall, the new model demonstrated the same level of precision as the Ploemen model.

Validation with FDA Data Set. From the original FDA data set[24] of 583 compounds only those that demonstrated either no phospholipidosis or strong phospholipidosis by electron microscopy were selected (provided that the molecular structure was available). A total of 91 compounds (56 positive and 35 negative) from the FDA PLD data set were analyzed by in silico methods for their potential to induce PLD. In terms of chemical structures this data set of 91 compounds is quite diverse, similar to the in-house in vivo data set shown in Table 2. The in silico method described here once again provides the highest levels of accuracy and sensitivity: 85% and 84%, respectively. The new method shows enhanced specificity and precision when compared to the modified Ploemen method. However, both specificity and precision are 97% using the Ploemen model, falling to 86% and 90%, respectively, for the new model.

Specific Physicochemical Properties and PLD. Aminoglycosides are an important class of antibiotics whose members include gentamicin, amikacin, and netilmicin. These antibiotics are highly charged, polycationic, hydrophilic drugs that are unable to cross cellular membranes to any great extent. Cationic hydrophilic drugs (e.g., gentamicin) are therefore taken up by endocytosis, and the resulting vacuoles fuse with lysosomes which lose their membrane integrity when aminoglycoside levels exceed the phospholipid content of the lysosomes. Aminoglycosides have low or negative ClogP values (Figure 3) and have been reported to cause phospholipidosis in certain cell types including renal proximal tubule cells. Gentamicin is the only compound where PLD induction has been linked to a clinical adverse event in humans, namely, nephrotoxicity.[27] Aminoglycosides have a distinct ClogP profile when compared to all the other compounds tested in vitro for their ability to cause PLD (Figure 3).

Table 2. Validation of the Predicted in Silico PL Classification with Published in Vivo Data for 32 Compounds[a]

name	in vivo finding	in vitro finding	PL classification (in silico)	$\Delta\Delta G_{AM}$ (kJ/mol)	calcd basic pK_a	ClogP	comment	ref
amiodarone	positive	positive	positive	−17.282	8.52	8.945		21
chloramphenicol	positive	negative	negative			1.283	neutral	28
chloroquine	positive	positive	positive	−8.598	10.28	5.06		26, 28
clozapine	positive	positive	positive	−8.051	7.76	3.714		22
cyclizine	positive	positive	positive	−6.814	8.02	3.798		38
chlorcyclizine	positive	positive	positive	−8.456	8.02	4.511		38
diazepam	negative	negative	negative	−5.143	3.05	2.961		39
erythromycin	positive	negative	positive	−7.275	8.7	1.611		39
fluoxetine	positive	positive	positive	−9.066	9.84	4.566		36, 40, 41
amikacin	negative	negative	positive	−34.051	9.33	−6.298	cationic hydrophilic	39
gentamicin	positive	negative	positive	−16.321	8.89	−2.395	cationic hydrophilic	39
haloperidol	positive	positive	positive	−7.748	8.37	3.849		39
hydroxyzine	positive	positive	positive	−9.935	7.29	3.995		42
imipramine	positive	positive	positive	−7.971	9.2	5.037		21, 28, 43
iprindole	positive	positive	positive	−10.3	8.98	5.66		44
maprotiline	positive	positive	positive	−7.958	10.19	4.524		39
5-ethyl-5-phenylbarbituric acid	negative	negative	negative			1.365	neutral	39
promazine	positive	positive	positive	−7.352	9.2	4.399		28
propranolol	positive	positive	positive	−7.675	9.27	2.753		21, 28
streptomycin	negative	negative	negative	−22.71	15.46	−4.263	cationic hydrophilic/fully charged	45, 46
tamoxifen	positive	positive	positive	−13.012	8.64	6.818		47
valproicacid	negative	negative	negative			2.76	acidic	39, 47
chlorphentermine	positive	positive	positive	−6.027	9.78	2.854		28
meclizine	positive	positive	positive	−8.354	7.21	6.732		38
perhexiline	positive	positive	positive	−8.746	10.45	7.153		47
chlorpromazine	positive	positive	positive	−7.411	8.98	4.036		21, 28
quinacrine	positive	positive	positive	−10.984	10.28	6.723		26, 39
tilorone	positive	positive	positive	−7.442	8.95	3.696		48
piperamide	positive	positive	positive	−6.743	9.83	1.716		51
citalopram	positive	positive	positive	−6.926	9.46	3.132		39
trospectomycin	positive	negative	negative	−5.353	9.58	−1.291	cationic hydrophilic	49
clomipramine	positive	positive	positive	−9.296	9.2	5.921		39, 47

[a]A comparison of PLD findings between previously published in vivo data for 32 compounds and the results obtained from the in house in vitro assay and the predictive in silico method described in this paper. Where available, $\Delta\Delta G_{AM}$, calculated basic pK_a, and ClogP data are included for each compound.
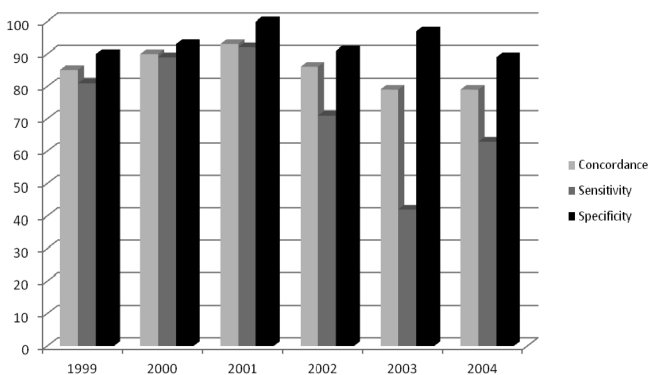
Figure 2. Performance of the in silico phospholipidosis model over a six-year period. The in silico method was applied to a library of 422 compounds that were assessed for phospholipidosis inducing potential over a 6-year period from 1999 to 2004 using the in vitro assay. Overall the in silico data show good concordance and specificity with the data obtained using the in vitro assay. Sensitivity was ≥60% across the study period.

With respect to the in-house in vitro data (422 compounds tested), acidic molecules that are negatively charged to more than 75% [acidic pK_a < 6.93] are not able to induce PLD.
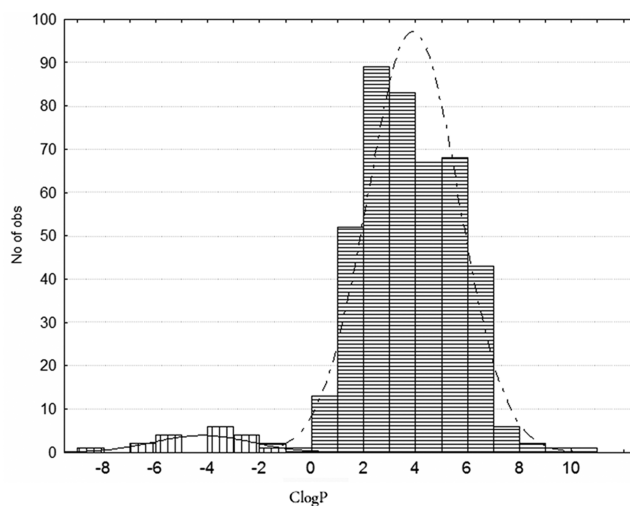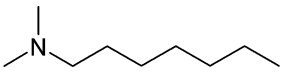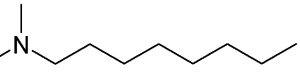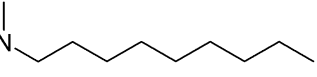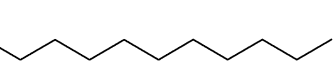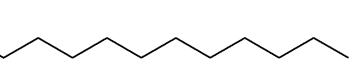
Figure 3. ClogP distribution of in vitro PLD data for aminoglycosides and all other compounds tested. The data for all the compounds tested in the in vitro PLD assay (bars with horizontal lines) show a normal distribution and are distinct when compared to the data for the smaller number of aminoglycosides (bars with vertical lines) which were tested.

Table 3. Calculated Amphiphilicity, $\Delta\Delta G_{AM}$, Lipophilicity, and Predicted PLD Potential for a Series of *N,N*-Dimethylacylamines of Various Chain Lengths

ID	Structure	NumberC-atoms [a]	Calc. $\Delta\Delta G_{AM}$ [kJ/mol]	Calc. $\Delta\Delta G_{AM}$ [kJ/mol]	Pred. PLD [b]
25		7	-5.35	3.19	Neg.
26		8	-6.30	3.72	Pos.
27		9	-7.43	4.25	Pos.
28		10	-8.66	4.78	Pos.
29		11	-10.0	5.31	Pos.
30		12	-11.5	5.84	Pos.
31		11	-6.40	2.31	Pos.
32		11	-6.00	2.31	Pos.
33		11	-5.15	2.31	Neg.

[a]Number of carbon atoms in the acyl chain. [b]Predicted phospholipidosis based on calculated amphiphilicity and calculated basic pK_a. Calculated basic pK_a for all compounds is 10.0.

The net charge carried by a compound at physiological pH is important when defining the likelihood of PLD induction. Data from the in silico model shown in Table 2 reconfirm the findings from the in vivo and in vitro methods that the acidic molecules tested do not induce PLD.

Molecules that exist as zwitterions (defined from the in-house data set as having acidic $pK_a < 6.93$ and basic $pK_a > 7.87$) at physiological pH do not induce PLD. Finally, compounds with limited permeability such as streptomycin, a cationic hydrophilic drug that is fully charged, or compound **21** in Table 1 do not induce phospholipidosis. In such cases the majority of the compound remains in the extracellular environment and is thus unable to bind to membrane phospholipids or interfere with intracellular processes.

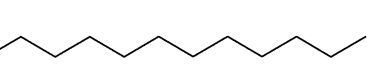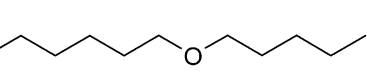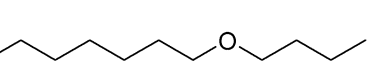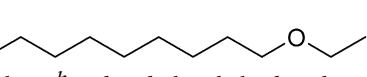**Structural Illustration of the Free Energy of Amphiphilicity.** The calibration of the calculated amphiphilic moment, $\vec{A}$, with experimental determined free energies of amphiphilicities, $\Delta\Delta G_{AM}$, as it is defined in eq 1, is required to identify the conformer that shows the best correlation with measured amphiphilicities. Beside that we compare the correctness of the calculated amphiphilicities from time to time with measured $\Delta\Delta G_{AM}$ if possible. Therefore, we still use

this rather "unvisualised" measure, $\Delta\Delta G_{AM}$, to describe the amphiphilic properties of molecules. Tables 3 and 4 should help to illustrate the concept of amphiphilicity based on a homologous series of hypothetical structures.

Table 3 shows how $\Delta\Delta G_{AM}$ for the *N,N*-dimethylacylamines nonlinearly increases with longer chains in comparison to the linear increase of the calculated lipophilicity. **26** has a $\Delta\Delta G_{AM}$ of -6.30 kJ/mol, which is pretty close to the threshold value of -6 kJ/mol that separates PLD positive from PLD negative compounds. The properties of highly amphiphilic compounds like **30** with $\Delta\Delta G_{AM} = -11.5$ kJ/mol can be clearly reduced by replacing a carbon with an oxygen in the acyl chain (**31**–**33**). Compounds **31**–**33** illustrate that $\Delta\Delta G_{AM}$ can be reduced by moving the oxygen to a more remote position to the charged nitrogen while the ClogP remains constant. **31** is predicted to be PLD positive, while **33** with the exact the same lipophilicity is predicted to be PLD negative. The predicted basic pK_a is 10.0 for all compounds in Table 3.

The concept of "isolipophilic" molecules with modified $\Delta\Delta G_{AM}$ is illustrated in Table 4. When the gem-methyl moiety is moved from C6 in the acyl chain (**33**) to C2 (**35**), $\Delta\Delta G_{AM}$ increases from -6.79 to -5.66 kJ/mol, which is above the

Table 4. Calculated Amphiphilicity, $\Delta\Delta G_{AM}$, Lipophilicity, and Predicted PLD Potential for a Series of Differently Branched *N,N*-Dimethylacylamines

ID	Structure	Number C-atoms [a]	Calc. $\Delta\Delta G_{AM}$ [kJ/mol]	ClogP	Pred. PLD [b]
27		9	-7.43	4.25	Pos.
33		9	-6.79	3.99	Pos.
34		9	-6.27	3.99	Pos.
35		9	-5.66	3.99	Neg.
25		7	-5.35	3.19	Neg.

[a]Number of carbon atoms in the acyl chain. [b]Predicted phospholipidosis based on calculated amphiphilicity and calculated basic pK_a. Calculated basic pK_a for all compounds is 10.0.

threshold value of −6 kJ/mol. The predicted basic pK_a is 10.0 for all molecules in Table 4.

Case Studies. *Addition of an Allyl Group (C₃H₅).* Compounds **2** and **20** shown in Table 1 only differ from one another because of the addition of an allyl group (C_3H_5) (see Figure 4B). The calculated basic pK_a remains unaltered, and the ClogP increases to 2.56 from 2.11. The greatest change is to the amphiphilicity of the molecules, which rises to a $\Delta\Delta G_{AM}$ of −6.05 kJ/mol from −4.03 kJ/mol because the slightly more lipophilic allyl group was introduced at a position that is far away from the basic center of the molecule (Figure 4B). This alone appears sufficient to induce PLD in the in vitro assay and results in a positive PLD prediction from the in silico model. As shown in the charts (Figure 4B) the concentration at which the compound induces PLD is reduced to 7.5 μM from 20 μM (Figure 4A). For either molecule the in silico prediction is in total agreement with the in vitro observations.

Replacing a Methylaryl (1) by a Pyridine (22). The removal of a methylaryl group from the compound **1** increases the polar nature of the molecule (Figure 5A). The basic pK_a is only slightly affected by this change. The ClogP decreases to 2.29, and the $\Delta\Delta G_{AM}$ rises above the −6 kJ/mol threshold. The reason is the same as described in the former example: the lipophilic change was made at a position that is far away from the basic center. The upper panels of Figure 5 represent data from the in vitro assay for induction of PLD. It can clearly be seen that although the compound on the left only slightly induces PLD at 20 μM, the compound on the right induces PLD at all the concentrations tested (2.5−20 μM). Once again the in silico method is in agreement with the observed in vitro studies on PLD induction.

Propranolol Compared to Bufetolol. Figure 6 shows 3D representations of **25** (bufetolol) (left side) and **26** propranolol (right side). Propranolol is considered to be a direct inhibitor of phospholipase A, and within a cell this may lead to an

accumulation of phospholipids.[8] Propranolol has been observed to induce PLD in several studies,[21,28] although this depends on the context in which PLD was being assessed, with low potential during in vivo studies but pronounced potency within in vitro assays. However, the $\Delta\Delta G_{AM}$ measurement is once again lower than the threshold of −6 kJ/mol, and the in silico method, which utilizes amphiphilicity measurements together with pK_a and ClogP data, identifies propranolol as an inducer of PLD. Bufetolol, although having similar values for both pK_a and ClogP when compared to propranolol, has a $\Delta\Delta G_{AM}$ of −5.75 kJ/mol and is not identified as an inducer of PLD by either in vitro[21] or in silico methods.

■ DISCUSSION

Lipophilicity and Amphiphilicity. Lipophilicity is a physicochemical property representing the affinity of a molecule to a lipophilic environment, and it is usually measured as a partition coefficient in biphasic systems (e.g., 1-octanol/water). Amphiphilic molecules possess an organic cation or anion with a larger hydrocarbon chain. Within the molecule there are distinct hydrophilic (polar) and hydrophobic (nonpolar) regions. Amphiphilicity can be measured by surface tension experiments as described in Fischer et al.[29] The program used to calculate the amphiphilic moment (CAFCA) was calibrated[20] and validated with experimentally determined $\Delta\Delta G_{AM}$ values. Although calculated lipophilic fragment values, similar to those used for the ClogP calculation, are used for the amphiphilicity calculation, $\Delta\Delta G_{AM}$ and ClogP are not necessarily correlated. Compounds may possess similar pK_a and ClogP values but have very different amphiphilic values ($\Delta\Delta G_{AM}$) and thus behave differently with respect to their induction of PLD. This can be seen from the examples in Figures 4−6. Over the past 10 years this approach has been successively used to optimize compounds against phospholipidosis.[30−34] The results presented here suggest that methods
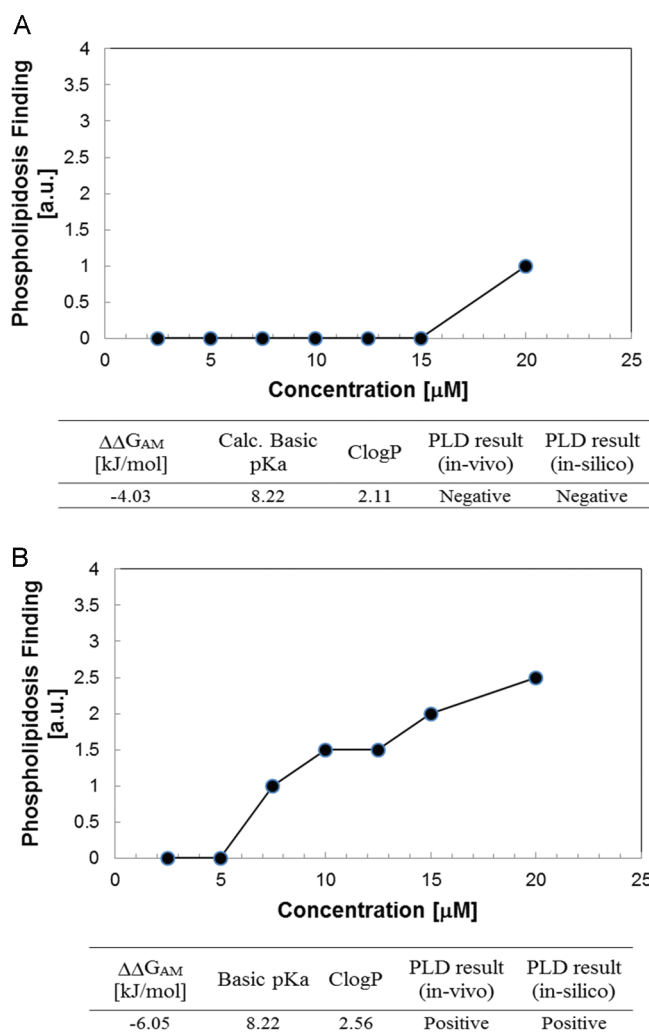
A

$\Delta\Delta G_{AM}$ [kJ/mol]	Calc. Basic pKa	ClogP	PLD result (in-vivo)	PLD result (in-silico)
-4.03	8.22	2.11	Negative	Negative

B

$\Delta\Delta G_{AM}$ [kJ/mol]	Basic pKa	ClogP	PLD result (in-vivo)	PLD result (in-silico)
-6.05	8.22	2.56	Positive	Positive

Figure 4. PLD can be reduced via removing an allyl group from a compound ((A) compound **2**; (B) compound **20**). The compounds differ only by the addition of an allyl group at a site distant from the basic center of the compound. This has the effect of reducing the concentration of the compound needed to induce PLD (as assessed by the in vitro assay) from 20 μM for compound **2** to 7.5 μM for compound **20**. The lower panels illustrate physicochemical data for the two compounds alongside PLD results from the in vitro and in silico methods. Although the basic pK_a remains unchanged, $\Delta\Delta G_{AM}$ is clearly decreased and ClogP only slightly increased in compound **20**. For both compounds the in silico prediction is in total agreement with the invitro observations.

A

$\Delta\Delta G_{AM}$ [kJ/mol]	Basic pKa	ClogP	PLD result (in-vivo)	PLD result (in-silico)
-4.34	8.22	2.29	Negative	Negative

B

$\Delta\Delta G_{AM}$ [kJ/mol]	Basic pKa	ClogP	PLD result (in-vivo)	PLD result (in-silico)
-6.44	8.89	3.46	Positive	Positive

Figure 5. PLD can be reduced via replacing a methylaryl with a pyridine group ((A) compound **1**; (B) compound **22**). Upper panel, left to right, shows structural representations of compounds **22** and **1** from Table 1. The compounds differ by a pyridine instead of a methylaryl moiety. This has the effect of increasing the concentration of the compound needed to induce PLD (as assessed by the invitro assay) from 2.5 μM for compound **1** to 20 μM for compound **22**. The lower panels illustrate physicochemical data: both compounds have similar basic pK_a values, while ClogP is increased and $\Delta\Delta G_{AM}$ decreased below the −6 kJ/mol threshold in compound **1**. The in silico predictions match the in-vitro observations.

that utilize amphiphilic measurements are more likely to accurately differentiate an inducer from a noninducer of PLD.

Physicochemical Properties and PLD. Phospholipases are attached to the lysosomal membrane via a lipid anchor, and disruption of this, for example, by increasing the lateral membrane pressure, leads to disruption of phospholipase activity.[16] Therefore, it can be speculated that increasing the lateral membrane packing density by an excessive binding of cationic amphiphilic drugs would nonspecifically inhibit the phospholipase activity. This mechanism would account for the fact that not only cationic compounds but particularly cationic amphiphilic compounds are prone to induce phospholipidosis. Therefore, we propose that amphiphilicity which describes the "similarity" of a molecule to the properties of the hydrophilic/lipophilic interface region should be a more predictive
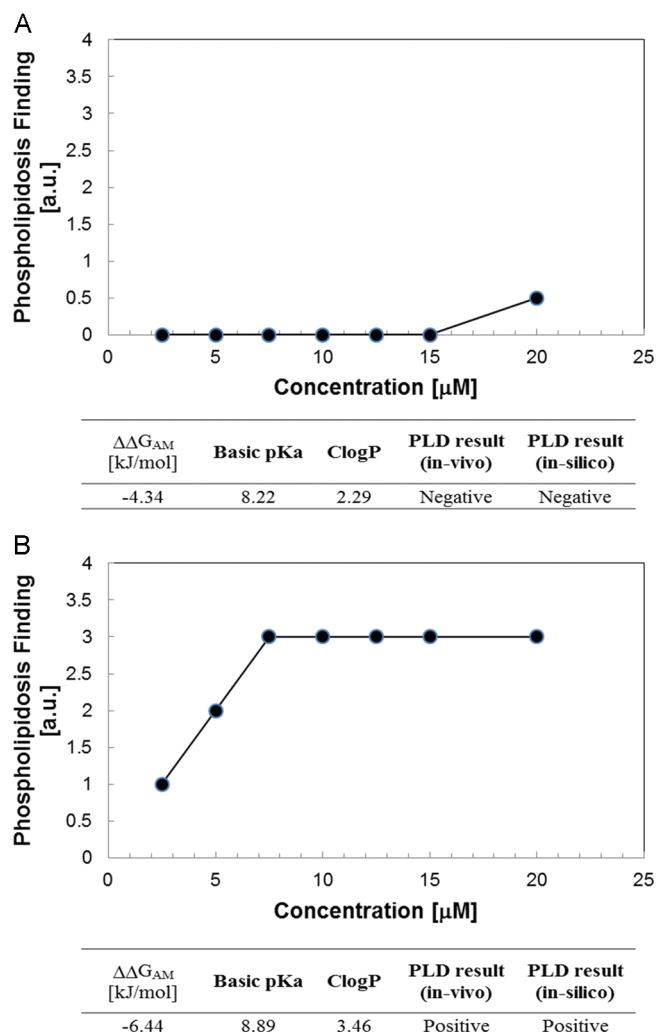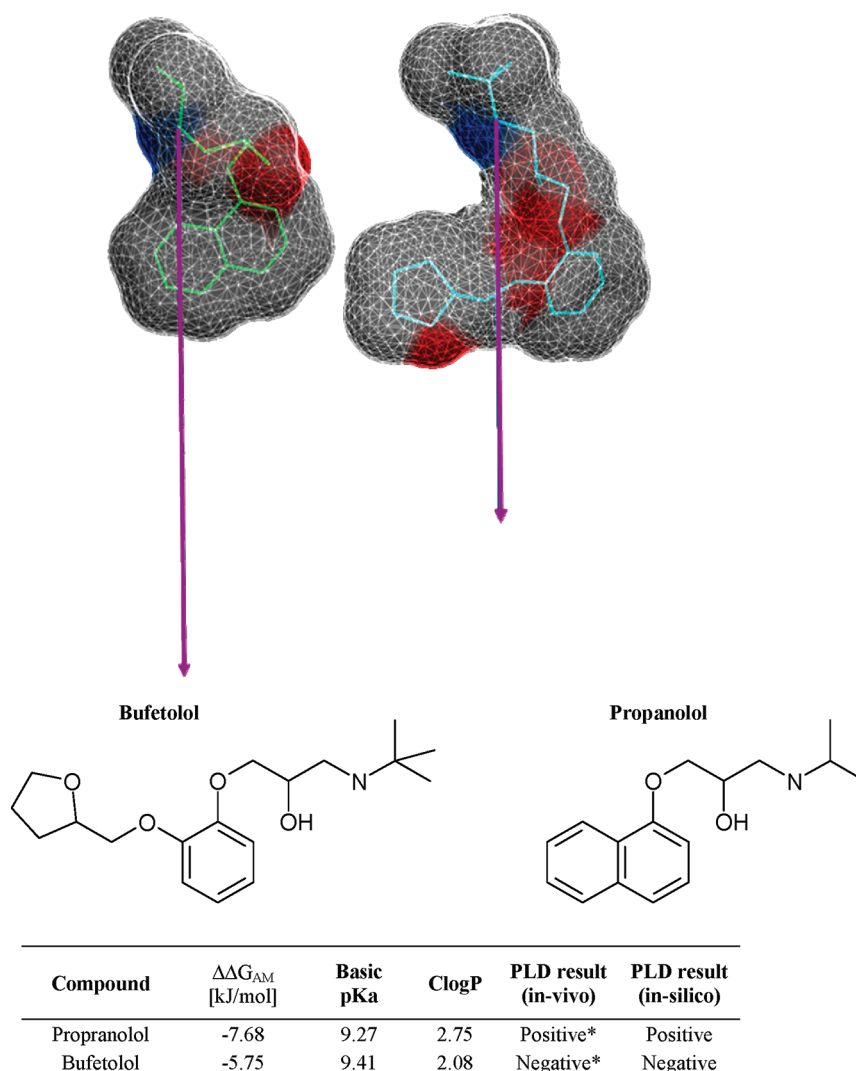
descriptor for PLD when compared to ClogP. This is supported by the finding that for a very large and diverse data set like the in-house PLD database or the FDA data set, concordance, sensitivity, and specificity are not completely different. The key difference between lipophilicity and amphiphilicity comes into play when molecules with similar structures are compared (Figures 4−6). For lead optimization the structural modifications are limited. Large changes in the chemical structure most often lead to inactive molecules. However, small changes in the overall structure leading to large improvements in amphiphilicity are much more successful in identifying molecules with similar target activities but a clearly lower risk of inducing PLD.[26,29−31,31−34]

CADs are well established as inducers of PLD. These compounds possess key physicochemical properties that can be directly related to their potential to cause PLD. These

Bufetolol **Propanolol**

Compound	$\Delta\Delta G_{AM}$ [kJ/mol]	Basic pKa	ClogP	PLD result (in-vivo)	PLD result (in-silico)
Propranolol	-7.68	9.27	2.75	Positive*	Positive
Bufetolol	-5.75	9.41	2.08	Negative*	Negative

* According to Figure 2 in Ploemen *et al* 2003 (21)

Figure 6. Physicochemical comparison of propranolol and bufetolol. Left hand panel shows 3D representations of **25** bufetolol and **26** propranolol, respectively. The purple arrows indicate the amphiphilic moments for each molecule. Propranolol has a larger amphiphilic moment ($\Delta\Delta G_{AM}$, kJ/mol) than bufetolol (-7.68 vs -5.75 kJ/mol, respectively). As shown in the tables, the two molecules have similar basic pK_a values, and ClogP is slightly higher for propranolol than bufetolol. The in vitro PL data for these two molecules was from Ploemen et al.,[21] and the in silico PLD prediction is in total agreement with the published data.

properties include their amphiphilic nature, relating to the distance between the hydrophilic and hydrophobic regions, and a hydrophilic side chain that is positively charged at physiological pH. In silico methods have been developed that rely upon these physicochemical properties to assign PLD-inducing potential to compounds.[21,22] Ploemen et al. simply utilize pK_a and ClogP to define a compound's PLD-inducing potential. This method was later refined to include other physicochemical properties such as amphiphilic moment and the number of acidic and basic centers within the compound.[22] However, a reliance upon physicochemical properties may lead to an overprediction of compounds likely to cause PLD, since other prerequisites like tissue exposure are not taken into account.[35] The model described here takes into account additional parameters, for example, the overall molecular charge that may in turn affect the exposure of tissues to the compound. Compound **21** (Table 1) is one example where, because of a high basic pK_a, the compound does not enter the systemic circulation and does not induce PLD even though it has a

strong amphiphilic moment ($\Delta\Delta G_{AM}$ = -8.7 kJ/mol). Therefore, new improved in silico methods that incorporate additional parameters should provide more sensitive and accurate predictions of PLD-inducing potential. Additionally, any new method should, like the in silico method described here, allow the easy imputation of structural changes to molecules in a rational manner and is thus unlike the complex approach required to input such changes into Bayesian modeling/methods.

Strategic Consequences for Early Detection of PLD Inducers. The adoption of the in silico method for predicting the likelihood that a compound will induce PLD has several benefits. Statistically the new in silico method is as good as the in vitro method for determining PLD-inducing potential. Additionally, the in silico method is faster and more economical to perform than the in vitro assay. The in silico method provides an accurate and sensitive high-throughput method for assessing compound libraries for PLD inducers. The overall result is that compounds identified as likely to induce PLD can

be removed early during their development, thus avoiding later compound attrition during lead optimization with the associated cost implications.

However, CADs have many advantageous properties including good solubility, lipophilicity, and good penetration into brain tissues. Hence, the vast majority of central nervous system compounds are CADs. Therefore, during the early drug discovery phase only the CADs that exhibit "extreme" amphiphilic properties ($\Delta\Delta G_{AM} \leq -12$ kJ/mol) are removed. Over the past decade, following the removal of early compounds that have "extreme" amphiphilic properties, there have been no issues with PLD in GLP toxicology studies within our organization.

Because of the variable nature of PLD induction with the same compound, i.e., across different tissue types or strains of the same species, it will still be necessary to perform in vivo studies in the foreseeable future. However, early detection of PLD inducers should reduce the number of animals required for these investigations. The excellent performance of the in silico method over a number of years (Figure 2) led to the discontinuation of the in vitro PLD assay.

Current drug labels include information relating to PLD induction for in vitro animal studies without providing any indication as to the consequences of these findings for human subjects. However, one intriguing case was reported involving a patient on fluoxetine hydrochloride (Prozac) who developed lung deficiency; pathological examinations revealed evidence consistent with hypersensitivity pneumonitis and lamellar bodies indicative of PLD.[36] On the other hand, at the Advisory Committee for Pharmaceutical Science and Clinical Pharmacology meeting on phospholipidosis (April 14, 2010), a very good correlation was shown between the toxicity-lowest observed effect level (LOEL) and the PL LOEL (http://www.fda.gov/downloads/AdvisoryCommittees/Committees-MeetingMaterials/Drugs/AdvisoryCommitteeforPharmaceuticalScienceandClinicalPharmacology/UCM210798.pdf). Since PLD is a lipid storage disorder, lamellar inclusion bodies may be observed without any signs of toxicity. The appearance of necrotic cells in proximity to those containing lamellar inclusion bodies may suggest, but does not prove, a mechanistic link between PLD and toxicity.

PLD-inducing activity is related to the dose and the duration of treatment and is normally reversible following cessation of treatment. Lower doses are associated with a reduction in risk, and for those compounds that induce PLD at high concentrations, the doses required may never be reached in the clinical setting. Compounds with a high volume of distribution at steady state are associated with a higher risk of inducing PLD. Additionally, if a compound is rapidly cleared to a metabolite that is not PLD positive, then there is a reduced risk associated with the parent molecule. The duration of treatment is another factor to consider; chronic drug administration requires a full consideration of PLD risk, whereas for short-term treatments this is less important (see above). Compounds have therefore been considered on a case-by-case basis, taking into account areas such as risk/benefit, the indication, and the disease burden. Other courses of action include termination of promising candidate drugs due to the observation of PLD in certain organs.

Further studies are needed to determine whether drug-induced PLD affects other cellular processes in addition to phospholipid metabolism and mitochondrial function.[5−10] The long-term biological consequences of drug-induced PLD remain unknown and merit further investigations.[37] Therefore, the discovery of new methods, such as the in silico method described here, for determining PLD-inducing potential remains an important area within the fields of drug safety and development.

Abbreviations Used

CAD, cationic amphiphilic drug; CAFCA, program entitled Calculated Free Energy of Amphiphilicity of Small Charged Amphiphile; ClogP, calculated octanol−water partition coefficient; CORINA, program for the fast and efficient generation of high-quality three-dimensional molecular models; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethylsulfoxide; FCS, fatal calf serum; GRID, computational procedure for determining energetically favorable binding sites on molecules of known structure; LBPA, lysobisphosphatidic acid; LOEL, lowest observed effect level; MDCK, Madin−Darby canine kidney; MoKa, program for the calculation of pK_a values; OMEGA, program that generates multiconformer structure databases; PBS, phosphate buffered saline; pK_a, ionization constant; PLD, phospholipidosis; SMILES, simplified molecular input line entry specification

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