Assessment of Drug−Lipid Complex Formation by a High-Throughput Langmuir-Balance and Correlation to Phospholipidosis

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Phospholipidosis, the accumulation of phospholipids in cells, is a relatively frequent side effect of cationic amphiphilic drugs. In response to the industry need, several methods have been recently published for the prediction of the phospholipidosis-inducing potential of drug candidates. We describe here a high-throughput physicochemical approach, which is based on the measurement of drug−phospholipid complex formation observed by their effect on the critical micelle concentration (CMC) of a short-chain acidic phospholipid. The relative change due to the drug, CMC_{DL}/CMC_L provides a direct measure of the energy of the drug-phospholipid association, irrespective of the nature of the interaction. Comparison of results for 53 drugs to human data, animal testing, cell culture assays, and other screening methods reveals very good correlation to their phospholipidosis-inducing potential. The method is well suited for screening already in early phases of drug discovery.

1. Introduction

Phospholipidosis (PLD)[a] is a lipid storage disorder characterized by the accumulation of phospholipids within cells and has been found to be induced by several drugs.[1,2] While it remains uncertain whether there is an association between PLD and any adverse effects such drugs may have, the frequent occurrence of PLD with cell toxicity[3,4] has caused concern and initiated an interest in predicting the PLD-inducing potency of drugs.[5–12] Of particular interest is a large group of compounds, coined as cationic amphiphilic drugs (CADs). The therapeutic indications of CADs are diverse and include β-blockers, the antiarrhythmic drug amiodarone,[13,14] antibiotics,[15] antidepressants,[16] as well as compounds currently in clinical use for Alzheimer's and Parkinson's disease.[17–20] Characteristically, CADs contain a hydrophobic part consisting of an aromatic ring and a hydrophilic group with one or more nitrogen groups bearing a net positive charge at physiological pH.[21,22]

The positive charge in combination with the amphiphilic character allows CADs to partition by electrostatic and hydrophobic interactions into cellular membranes, in particular those containing anionic lipids. To this end, the mechanistic basis of PLD induction has been suggested to be complex formation with acidic phospholipids, resulting in the inhibition of lysosomal phospholipid degradation by acidic phospholipases.[1,2] Importantly, amphiphilicity of compounds correlates to several ADME/tox characteristics, such as the fraction absorbed from the intestinal lumen and the ability to cross the blood−brain barrier (BBB).[5,20,23] The latter is of major importance for drugs targeted at the central nervous system (CNS). Accordingly, in the absence of specific transporters this limits the freedom to modify the physicochemical properties of potential drug candidates, as they have to be relatively hydrophobic and small in order to be able to penetrate through BBB.[5,20,23] Simply avoiding the use of amphiphilic compounds is thus not possible.

The observed close connection of the physicochemical properties of a drug to the tendency to cause PLD[20] suggests that it should be possible to find *in vitro* physicochemical methods to estimate the PLD inducing potency of compounds. The techniques used to predict the induction of PLD by drugs range from computational approaches such as estimating log *P* with either pK_a[7,11] or compound net charge at lysosomal pH,[10] to more laborious cell-based assays based on the observation of the accumulation of a fluorescent phospholipid derivative,[9,11] the appearance of multilamellar morphological structures by electron microscopy (EM),[8,9,24] or changes in the levels of mRNA coding for enzymes involved in phospholipid metabolism.[8,11] Since the metabolism of a drug may also affect its PLD-inducing potential, the best yet also the most expensive and labor-intensive approach is testing in animals.[4,25] None of the above methods is suitable for compound screening in the early phases of drug discovery. The cell-based assays are inherently complex, have a low throughput, and yield false negatives. Given the complex physicochemical nature of the membrane environment and drug−lipid interactions, it is obvious that predictions also from computational analyses should be taken with caution.

Here we describe a physicochemical screen that measures complex formation by drugs and an acidic phospholipid (1,2-dioctanoyl-*sn*-glycero-3-phospho-L-serine, diC8PS), observed by quantitating their effect on the critical micelle concentration (CMC) of this lipid. The CMC values were obtained from surface tension values for samples contained in 96-well plates and recorded with a high-throughput multichannel tensiometer. In addition to the data on drug−phospholipid interactions, this method yields direct quantitative information on the hydrophobicity/amphiphilicity of compounds, providing their air/water interfacial partition coefficient.[6] When used in connection to an automated liquid handling system, this approach can be

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[a] Abbreviations: ADME, adsorption, distribution, metabolism, excretion; BBB, blood−brain barrier; CAD, cationic amphiphilic drugs; CMC, critical micelle concentration; CMC_{DL}, CMC for drug−phospholipid complex; CMC_L, CMC for diC8PS; CNS, central nervous system; coCMC, CMC_{DL}/CMC_L; DF, dilution factor; diC8PS, 1,2-dioctanoyl-*sn*-glycero-3-phospho-L-serine; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; EM, electron microscopy; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NPC, Niemann-Pick disease type C; PLD, phospholipidosis; SAPs, sphingosine activator proteins; $\Delta CMC_{max0.5}$, half-maximal change in coCMC.

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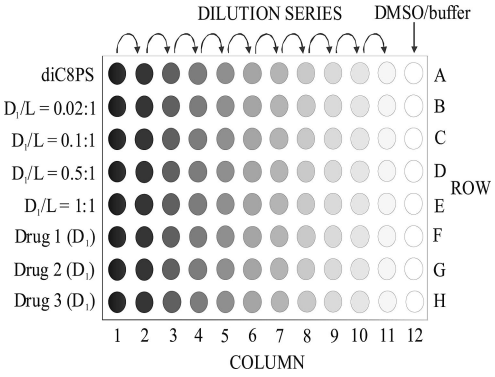

Figure 1. Preparation of dilution series for diC8PS, drugs as such (compounds **1**−**3**), and for drug:diC8PS mixtures at different molar ratios. Samples were applied into a disposable 96-well plate, as described in the text. The last column is filled with DMSO/buffer (or the solvent used for the drug, as indicated) as a reference for surface tension. After dilution, a 50 μL aliquot from each well was transferred onto the measurement plate.

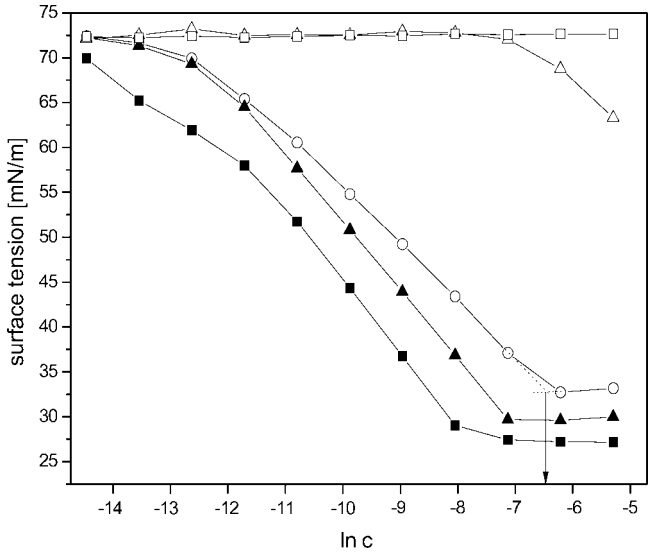

Figure 2. Surface tension π vs natural logarithm of concentration ln c for diC8PS (○), imipramine (△), and gentamicin (■), as well as imipramine/diC8PS (▲) and gentamicin/diC8PS (both at D/L = 0.1:1, □). The values for CMC were obtained from the intercept of the descending and subsequent horizontal parts of the isotherm, as illustrated for diC8PS and marked with an arrow.

estimated to have a throughput of nine drugs analyzed (in duplicate) per hour. The total consumption of a compound is typically less than 1 mg.

2. Results

By definition, surface active compounds accumulate into the air/water interface, forming a monolayer, this surface excess depending on the concentration of the surfactant in the subphase and its hydrophobicity (Figure 2). Increasing concentrations of the acidic phospholipid diC8PS in the subphase progressively lower γ, revealing the partitioning of this lipid to the surface (Figure 2). A discontinuity is seen in the isotherm between 3.3 μM and 5 mM diC8PS, and the depicted intercept of the slopes of the descending and constant parts of the curve yields the value of CMC = 1.6 mM. Also illustrated is the isotherm for gentamicin, which, as expected from its chemical structure, is highly water soluble and does not have any surface activity (Figure 2). For comparison, the isotherm for a somewhat

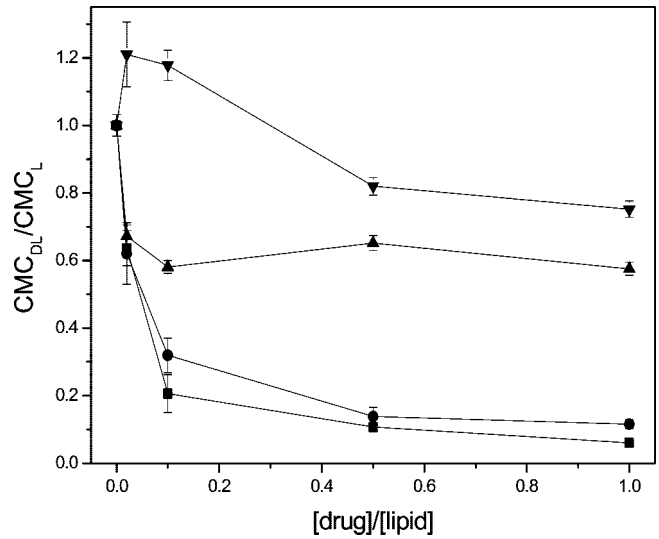

Figure 3. CMC_{DL}/CMC_L vs D/L data recorded at D/L varied as 0.02, 0.1, 0.5, and 1.0 for amiodarone (□), promazine (•), clozapine (▲), and amantadine (▼).

hydrophobic drug imipramine is depicted, lowering of γ seen at >0.8 mM concentrations. The latter type of isotherm was common for the drugs investigated in this study.

Affinity of a drug to the anionic diC8PS is readily anticipated to influence the CMC measured for a drug−phospholipid complex. Accordingly, both imipramine and gentamicin (both at molar drug/diC8PS molar ratio of 0.1:1) significantly decrease the CMC, to 285 and 95 μM phospholipid, respectively (Figure 2). Importantly, for these two drugs the driving forces for complex formation are qualitatively different. For gentamicin, it is merely electrostatic attraction of its five positive charges to the anionic headgroups of diC8PS, which upon complex formation reduces the repulsion between the negatively charged lipid phosphates, lowering CMC. For imipramine, both the hydrophobicity of the compound and its single positive charge are contributing, yet both drugs promote the nucleation of micelles.

Subsequently, it was of interest to study the impact of drug/phospholipid molar ratio D/L. More specifically, CMCs were measured at D/L of 0.02, 0.1, 0.5, and 1. The values for CMC_{DL}/CMC_L were then calculated, where CMC_{DL} is the CMC for drug/lipid complex and CMC_L is the CMC of the phospholipid as such. We then constructed the CMC_{DL}/CMC_L vs D/L curves for each drug with the use of the CMC_{DL}/CMC_L correcting for the impact of the different solvents. These data are illustrated for amiodarone, promazine, clozapine, and amantadine, which have very different potencies to induce PLD (Figure 3). Amiodarone, which is one of the most potent PLD inducing compounds, reveals a steep decrease of CMC_{DL}/CMC_L with increasing D/L. Similar behavior was evident for gentamicin, another efficient inducer of PLD (data not shown). Promazine, which has been found to induce PLD in animals,[26] exhibits a behavior similar to that of amiodarone with a rather low CMC_{DL}/CMC_L value. The decrement in CMC_{DL}/CMC_L for clozapine at D/L = 1.0 is significantly smaller than for amiodarone and promazine. This drug has been observed to induce PLD only in cells[26] and is not considered to induce PLD *in vivo*. A very small decrease in CMC_{DL}/CMC_L is evident for amantadine, which has been reported to induce PLD in animals, yet only in high doses.[27]

To illustrate the correlation between drug−phospholipid complex formation and PLD-induction potency, these data are shown as 50% of the maximum change of CMC $R_{½}$ vs minimum

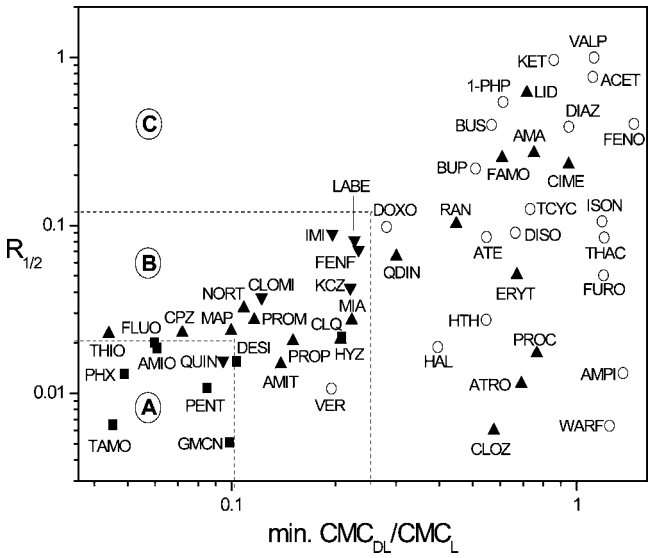

Figure 4. Drug:lipid (D/L) ratio causing half-maximal effect $R_{½}$ on CMC vs minimum of CMC_{DL}/CMC_L ratio recorded at D/L = 1:1.

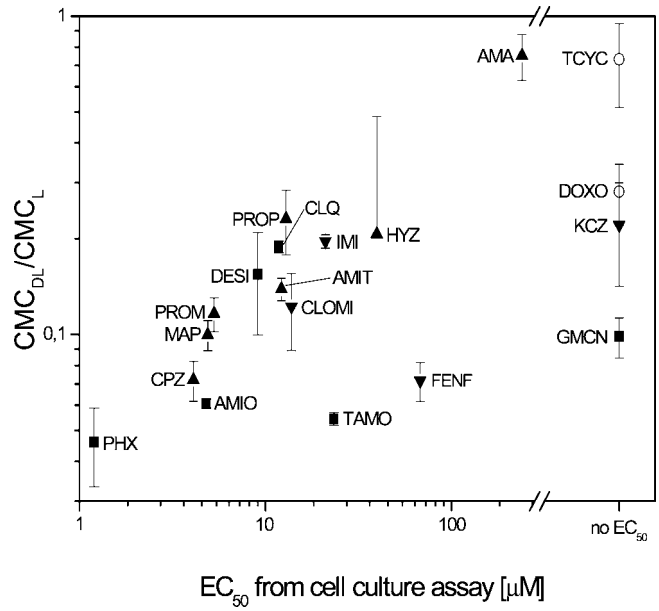

Figure 5. Minimum in CMC_{DL}/CMC_L (D/L = 1:1) vs EC_{50} values from the cell culture assay described by Morelli et al.[17] A numerical value for the induction of PLD in the latter test was not obtained for DOXO, TCYC, KCZ, and GMCN. Symbols used refer to the classification in Table 1.

in CMC_{DL}/CMC_L (observed at D/L = 1:1, Figure 4), measured for drugs causing PLD in humans, animals, cultured cells, and compounds for which PLD has not been reported. The division of drugs into three categories in these data is readily evident, illustrated here as classes A, B, and C, respectively, the most potent drugs in inducing PLD in humans (group A) being well separated from the other compounds. These data also reveal that to estimate the potency of a compound to induce PLD it is sufficient to use the value for CMC_{DL}/CMC_L measured at D/L = 1:1.

3. Discussion

The formation of micelles by diC8PS is driven by the hydrophobicity of its acyl chains, while there is the penalty as Gibbs free energy increase due to the repulsion of the negatively charged headgroups, in addition to the decrease in entropy caused by the aggregation of the phospholipid molecules. When positively charged drugs (such as gentamicin) are present, the negative charges of diC8PS are screened, thus promoting micelle formation. Drugs can also incorporate their hydrophobic part to the micelle core thus promoting the formation of the micelles at lower phospholipid concentrations. The intercalation of the drug into the micelle is constrained by the shape of its hydrophobic part as well as the spatial distribution of its charges and polar moieties. Highly hydrophobic compounds such as amiodarone, tamoxifen, and fluoxetine with charged amino groups readily assemble into mixed micelles with diC8PS and decrease the observed CMC, while more bulky, branched compounds or compounds with an amino group in the middle of a hydrophobic structure, such as amantadine, lidocaine, and haloperidol appear to have less affinity toward the phospholipid micelles.

Notably, the coCMC values acquired from the Gibbs absorption isotherms appear to reflect very well the degree of risk for PLD. In brief, the lower the coCMC, the higher the risk for PLD (Figure 4). It also appears that a single drug/lipid ratio is sufficient, as $R_{½}$ provides little additional information. Drugs with no PLD reported (e.g., atenolol, ranitidine, and bupivacaine) had little effect on CMC_{DL}.

We then compared our data with results from other assays predicting PLD. We plotted our CMC data against the EC_{50} for drug concentration inducing accumulation of a fluorescent lipid marker in cell cultures[9] (Figure 5). The correlation between the two methods is good. However, no data could be obtained in this cell-based assay for four compounds. One of these is gentamicin, representing a false negative in the data of Morelli et al.[9] Instead, our CMC assay correctly classifies it as a potent inducer of PLD. Gentamicin is fully water soluble and nonamphiphilic and thus structurally differs from CADs. It is the best known member of the aminoglycosides known to induce PLD in humans.[28] Unlike for the cell-based assay, the PLD risk for fenfluramine predicted by coCMC is high, in agreement with its impact in cells.[29] Significant PLD risk for ketoconazole, a potent PLD-inducing compound,[9] was correctly predicted by our method, whereas no numerical value was obtained for this drug in the cell based assay of Morelli et al.[8] A problem with assays monitoring fluorescent lipid accumulation into cells may be a limited entry of some drugs into cells. Gentamicin for instance is poorly soluble in nonpolar solvents, and thus may not be able to permeate the plasma membrane. The same problem yet to a lesser extent may be present for also less hydrophilic compounds. As both the fluorescently labeled phospholipids and the drugs accumulate into lysosomes, and as many of the drugs are efficient quenchers of fluorescent probes[30] also quenching may slightly distort the fluorescence data.

We next compared our coCMC data with the results of Tomizawa et al.[10] The correlation of the values for coCMC to their pathology score is very good (Figure 6A), with inverse relationship between coCMC and PLD risk. The perhaps most notable exception is disopyramide, which our method predicts not to induce PLD, whereas Tomizawa et al. assigned it as a high PLD risk compound. However, we have not come across independent data demonstrating PLD for disopyramide either in humans or experimental animals. Likewise, in contrast to Tomizawa's score, Sawada et al. obtained zero pathology score for disopyramide. The computational predictions of Tomizawa et al. divided compounds into either PLD+ or PLD−. Almost all compounds found to induce PLD in humans, animal, and cultured cells were correctly predicted by Tomizawa et al. as

Table 1. List of the Compounds Studied Including Name, Abbreviation, Pld-Induction Potency, and Reference, if Available[a]

name	abbreviation	PLD-induction	ref
amiodarone	AMIO	I	2, 34, 61
chloroquine	CLQ	I	7, 9, 61
desipramine	DESI	I	9
fluoxetine	FLUO	I	7, 19
gentamicin	GMCN	I	2, 9, 19
pentamidine isethionate	PENT	I	33, 10 (8)
perhexiline	PHX	I	7, 59
clomipramine	CLOMI	II	9, 61
fenfluramine	FENF	II	9, 59
imipramine	IMI	II	20, 40, 61
ketoconazole	KCZ	II	9
labetalol	LABE	II	10, 60
quinacrine dichloride (mepacrine)	QUIN	II	9, 59
tamoxifen	TAMO	II	9, 19
verapamil	VER	II	29
amantadine	AMA	III	9
amitriptyline	AMIT	III	9, 61
atropine	ATRO	III	10
cimetidine	CIME	III	10
clozapine	CLOZ	III	27 (7)
chlorpromazine	CPZ	III	2, 59, 63
erythromycin	ERYT	III	10
famotidine	FAMO	III	10
hydroxyzine	HYZ	III	9, 34
lidocaine	LIDO	III	56
maprotiline	MAP	III	9, 22, 62
mianserin	MIA	III	9
nortriptiline	NORT	III	9
procaine	PROC	III	56
promazine	PROM	III	9
propranolol	PROP	III	2, 9
quinidine	QDIN	III	56
ranitidine	RAN	III	29
thioridazine	THIO	III	9, 62
1-phenylpiperazine	1-PHP	IV	7
acetaminophen	ACET	IV	10, 38
ampicillin	AMPI	IV	10
atenolol	ATE	IV	30
bupivacaine	BUPI	IV	–
buspirone	BUS	IV	7
disopyramide	DISO	IV	9 (8)
doxorubicin	DOXO	IV	9
diazepam	DIAZ	IV	7
fenofibrate	FENO	IV	22
furosemide	FURO	IV	10
haloperidol	HAL	IV	7
hydroxythioridazinesa	HTH	IV	–
isoniazide	ISON	IV	10
ketoprofen	KET	IV	29
tetracycline	TCYC	IV	9
thioacetamide	THAC	IV	10
valproic acid	VALP	IV	22
warfarine	WARF	IV	10, 37

[a] In subsequent figures, the symbols for the compounds are PLD in humans (class I, □), in animals (class II, ▼), in cultured cells but not in animals (class III, ▲), and no PLD observed or reported (class IV, ○). For some compounds, discrepant data on PLD have been published (reference in parentheses).

PLD+ (Figure 6B), and correlation between their computational data and our coCMC data is good. However, compared to the analysis by Tomizawa et al. our method provides more scale with respect to the PLD risk, with the high risk compounds having low coCMC, and compounds with no PLD risk characterized by high coCMC (Figure 6B).

We also compared our data with the cell-based toxicogenomic assay by Sawada et al.[8] The correlation between the PLD pathology score used by Sawada et al. and PLD risk following the classification in Table 1 is poor (Figure 7A). Likewise, the values for coCMC do not correlate with the mRNA prediction score (Figure 7B). Notably, Sawada et al. assign only a moderate

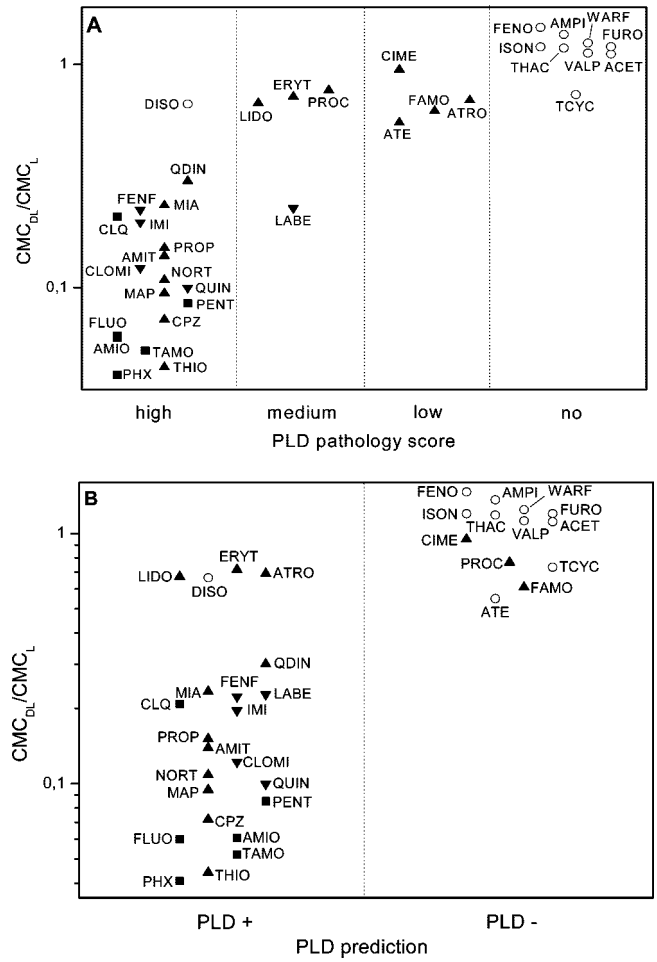

Figure 6. Panel A: Correlation between (CMC_{DL}/CMC_L, D/L = 1:1) and PLD risk assessed by a cell culture assay by Tomizawa et al. Panel B: (CMC_{DL}/CMC_L, D/L = 1:1) vs PLD risk predicted by the physicochemical method described by Tomizawa et al.[10] The symbols used refer to the classification in Table 1.

PLD risk to amiodarone and perhexiline, both of which are known to cause PLD in humans.[13,31] In fact, following their classification these compounds would have smaller risk than imipramine and clozapine, which are generally considered weak PLD inducers.[22,32] Also the pathology score of Sawada et al. for amiodarone deviates from published data.[7,33] Low PLD risk is predicted by the microarray assay for quinacrine and thioridazine, in contrast to PLD-induction by these drugs in animals and cells.[34,35] The gene expression microarrays may suffer from the high complexity of the assay and from the fact that the drugs may influence gene expression by mechanisms, which poorly reflect their potency to induce PLD. In addition, the primary problem is unlikely the expression of genes but in the functioning of the expressed proteins.

The PLD-inducing potency of drug *in vivo* depends not only on the inherent PLD-inducing potency of the compound, but also on the dose of the drug, its metabolism, and the duration of use.[17] This somewhat complicates the evaluation of different methods of PLD prediction, in particular as comprehensive long-term animal trials allowing quantitative comparison are not available. For example, amantadine is known to cause PLD in animals[27] but only at very high concentrations, around 100 times higher than amiodarone.[36] Pentamidine isethionate has been referred to have low pathological score in cell culture data of Sawada et al.[8] (at 8.3 μM drug), while high PLD risk is suggested by the physicochemical approach by Tomizawa et

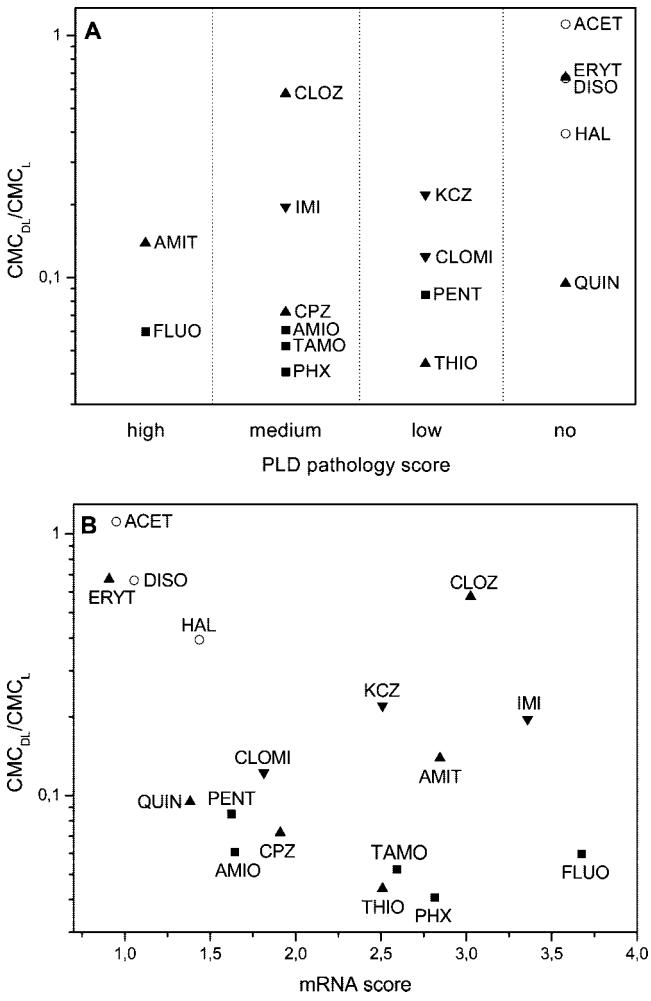

Figure 7. Panel A: (CMC_{DL}/CMC_L, D/L = 1:1) vs PLD pathology score used by Sawada et al. Panel B: (CMC_{DL}/CMC_L, D/L = 1:1) vs mRNA prediction score derived from the toxicogenomic assay by Sawada et al.[8] The symbols used refer to the classification in Table 1.

al.[10] As multilamellar structures form in rat liver lysosomes after the injection of low concentration of pentamidine,[37] the latter PLD-prediction method appears to be more reliable. Likewise, the coCMC derived for pentamidine is low, suggesting a high PLD risk. Notably, the recent study by Nioi et al.[11] demonstrates that part of the false PLD negatives predicted by Sawada et al.[8] are due to too low drug concentrations used in the latter study. It could be that similarly to gentamicin the highly charged and relatively hydrophilic pentamidine is problematic in cell cultures, not permeating into the cells. On the other hand, Sawada et al.[8] and Kasahara et al.[33] classified disopyramide as a low PLD pathology score compound, whereas Tomizawa et al.[10] obtained a high pathology score.

For proper functioning of cells both biosynthesis and catabolism have to cooperate to effectively control the levels of biomolecules, such as phospholipids. If any of these pathways are perturbed, pathological effects may result.[38,39] Drug-induced PLD has been suggested to arise from the inhibition of the phospholipases, responsible for the degradation of phospholipids, thus resulting in the accumulation of phospholipids in cells.[1,2,40,41] Our method assesses directly the formation of drug−lipid complexes, which has been suggested to underlie drug-induced PLD. Our results compare very well with literature data on PLD. This lends credence for the notion that drug−phospholipid complex formation indeed represents the direct causative factor for PLD.[2,42]

Not only lysosomal lipases but also lysosomal lipid transport proteins require anionic phospholipids in order to function.[38] Specific membrane lipid-binding proteins saponins are required for the lysosomal degradation of sphingolipids.[43−45] Mutations in these proteins in Niemann−Pick disease for instance have been suggested to elevate lysosomal levels of the natural cationic amphiphile sphingosine,[46] leading to the neutralization of anionic phospholipids that regulate the enzymes involved in lipid degradation,[38,39,43] thus leading to PLD. Accordingly, blocking of anionic phospholipids by cationic drugs may impair both degradation and lipid transport. In the light of the above, it is of interest that the coCMC for sphingosine with diC8PS (at 1:1 molar ratio) is 21% of CMC for diC8PS (data not shown), thus classifying sphingosine as a PLD inducer.

Techniques such as electron microscopy,[8] and fluorescence microscopy[22,47] have been employed in monitoring the PLD-inducing potencies of CADs in cells. These methods are very complex, time-consuming, and often require high amounts of drugs. Cell-based methods in general require careful maintenance of cell lines in order to avoid changes in response due to contamination, infection, or due to possible genetic changes in the cultures. These assays further require long incubation times. Complex changes in the metabolic pathways in cells upon exposure to drugs can lead to poor signal-to-noise ratio. The strength of cell-based assay is that the detection of PLD is not limited to any particular mechanism of PLD induction. For example, a drug may interact directly with a protein(s) to cause PLD or to elevate the levels of sphingosine as in Niemann-Pick disease.[39] Obviously, our method would be unable to predict PLD risk for drugs affecting protein function resulting in sphingosine accumulation, for instance. Mere physicochemical calculations of compound properties may also yield erraneous results, as the conditions *in vivo* differ from those used for isolated molecules. A limited number of parameters cannot fully cover the nature of phospholipid−drug interactions, let alone the complex nature of PLD induction. The main advantages of our method are good reproducibility, high throughput, low compound consumption, and, based on the data available so far, a very good correlation to PLD risk. Importantly, when discrepancies between the other prediction methods and our assay are evident, comparison with data in literature seems to be in favor of our technique.

4. Experimental Section

Preparation of the Drug Stock Solutions. All drugs were obtained from Sigma (Steinheim, Germany). Their purity was >95%, which is sufficiently high to allow the use of a Langmuir-balance, with negligible influence by possible contaminants. Dry compounds were carefully weighed and dissolved in dimethyl sulfoxide (DMSO, J. T. Baker, Deventer, The Netherlands) to yield a final concentration of 62.5 mM, of which 12.5 and 2.5 mM solutions were subsequently prepared. Due to limited solubility in DMSO, tamoxifen was dissolved in ethanol and gentamicin in water.

Compounds Studied. We characterized 53 drugs of differing pharmacological activity, selected according their availability and data reported in the literature on their PLD-inducing potency (Table 1). With regard to the latter the compounds were divided into four categories, i.e., drugs reported to induce PLD in humans (class I), animals (class II), in cultured cells (class III), and those for which no PLD has been demonstrated (class IV).

Preparation of the Lipid Solution. 1,2-Dioctanoyl-*sn*-glycero-3-phospho-L-serine (diC8PS, Na^+-salt, from Avanti Polar Lipids, Alabaster, AL) was dissolved in chloroform/methanol (5:1, by volume) to yield approximately 35 mM solution. Concentration of this stock solution was determined gravimetrically by a high precision electronic microbalance (Cahn 2000, Cahn Instruments, Cerritos, CA). An appropriate amount of diC8PS solution was then transferred into a carefully cleaned glass vial and the solvents evaporated under a gentle stream of nitrogen. The dry lipid film was dispersed in 20 mM HEPES, 0.1 mM EDTA, pH 7.4 to obtain a final concentration of 5.0 mM diC8PS, followed by vigorous mixing and incubation at 60 °C for 45 min in a thermostated water bath.

Sample Preparation. The principle of the method is based on measuring surface tension as a function of concentration of the drug, a short chain phospholipid (diC8PS), and their mixtures. When indicated, the latter were prepared at varying drug/lipid molar ratios (D/L), i.e. 1:1, 0.5:1, 0.1:1, and 0.02:1. Dilution series of drugs, diC8PS, and their mixtures were prepared in disposable 96-well plates (Greiner Bio-One, Kremsmünster, Austria) with the wells addressed as 8 rows (from A to H), and 12 columns (from 1 to 12, Figure 1). As the drugs were dissolved in DMSO, this solvent was (unless otherwise indicated) present at 4% final concentration in the first column of the dilution series in all samples analyzed, except at 8% for D/L = 1:1 (including lipid).

Row A was used for measuring the CMC of pure phospholipid (in the presence of solvent, usually DMSO). Into wells 1A−E was applied 175 μL of the 5.0 mM lipid stock solution, 175 μL of buffer was added to the wells 1F−H, while to the rest of the wells (2−12) in the rows A−H 105 μL of buffer was pipetted. To obtain D/L molar ratios 0.02, 0.1, and 0.5, respectively, 7 μl of 2.5, 12.5, and 62.5 mM drug stock solutions were added to wells 1B, 1C, and 1D. Likewise, to obtain D/L = 1:1, for drug 1 14 μL of 62.5 mM drug stock solution was added to 1E. The surface activity profiles of drugs (numbered here from 1 to 3) in the absence of phospholipid were measured by adding 7 μL of 62.5 mM drug stock solution into the wells 1F, 1G, and 1H.

Dilution series with 11 concentrations of the compound were prepared using a multichannel pipet with the concentration of lipid given as cx^{n-1}, where x is the dilution factor (DF), c is the concentration of diC8PS in column 1 (i.e., 5.0 mM), and n increases from 1 to 11, corresponding to columns 1−11. For DF = 0.4, an aliquot of 70 μL was transferred from one column to the consecutive one, up to column 11. Column 12 was filled with buffer to provide a reference value for surface tension.

After the dilution series in the 96-well plate were complete, 50 μL from each well was transferred into the corresponding well of the 96-well plate used for the measurement of the surface tension. Subsequently, the plate was covered with a lid and allowed to equilibrate for 15 min prior to the recording of surface tension.

Measurement of Surface Tension. Surface tension measurements were performed using an eight-channel tensiometer (Delta 8, Kibron Inc., Helsinki, Finland), with the samples applied into the wells of a dedicated 96-well plate (Kibron, Inc.) as described above. The instrument records the value of surface tension by determining in triplicate the maximum weight of the meniscus adhering to a 0.5 mm diameter du Nouy probe upon its withdrawal from the solution.[48] To minimize the impact of carry-over, the plates were measured starting with column 12 containing only the buffer and then continuing toward increasing drug and lipid concentrations. The probes were automatically cleaned between each 96-well plate by heating in the built-in electric oven of the tensiometer.

Analysis of the Langmuir Isotherms. Reflecting the lack of favorable interactions between the liquid (water) molecules for the molecules at the liquid–vapor interface, this interface has a high free energy evident as interfacial tension. Surface active molecules adsorb at the liquid−gas interface and reduce the surface tension γ, i.e., increase the surface pressure π defined as

$$\pi = \gamma^0 - \gamma \tag{1}$$

where γ° is the surface tension of the free interface and γ the value recorded in the presence of the surface active molecules. Gibbs adsorption isotherm connects the surface tension with the compound concentration in the subphase by

$$d\gamma = -d\pi = -\Gamma d\mu = -RT\Gamma d\ \ln c \tag{2}$$

where $R = 8.314\ J\cdot K^{-1}\cdot mol^{-1}$, T = temperature, Γ = surface excess of the compound in concentration per unit area, μ = chemical potential, and c = concentration of the surfactant.

Surface active compounds contain both hydrophilic and hydrophobic part(s). In a gas/water interface, the former resides in the aqueous phase, while the latter becomes accommodated in gas. This interfacial absorption of the amphiphile thus reduces both the free energy of the gas/water interface and is driven by the removal of the unfavorable contacts of the surfactant's hydrophobic parts with water. After sufficient concentration is reached, it also becomes favorable in terms of free energy to remove the hydrophobic parts from the contact with the water by the formation of micelles, aggregates in which the hydrophobic part(s) of the amphiphile are clustered in its core and hydrophilic parts form the surface. Upon reaching the critical micellar concentration (CMC) the free monomer concentration remains constant and $d\ln c = 0 => d\gamma = 0$. Any further addition of the amphiphile increases the number of micelles, and neither the concentration of free monomers nor the surface excess no longer increase, and the surface tension thus remains constant.

CMCs were determined by dedicated software (Delta-8 Manager) provided by the instrument manufacturer. After recording of the Gibbs adsorption isotherms for varying drug/lipid, the cubic interpolation function of Matlab (MathWorks, Natick, MA) was used to obtain drug/lipid ratio $R_{½}$ causing 50% of the maximum change in CMC, $\Delta CMC_{max0.5}$

$$\Delta CMC_{max0.5} = \frac{1}{2}(p + m) \tag{3}$$

where p is the CMC for diC8PS as such and m is the CMC measured in the presence of the drug.

To correct the coCMC for the impact of solvent, the values measured in the presence of the drug (CMC_{DL}) were divided by CMC_L, the CMC for the lipid (measured in the presence of the solvent). For the sake of clarity, the values for CMC_{DL}/ CMC_L are referred to as relative coCMC.

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Supporting Information Available: Characterization data for cationic amphiphilic drugs including name, structure, abbreviation, phospholipidosis-inducing potency, and reference, if available. This material is available free of charge via the Internet at http://pubs.acs.org.

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