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Modeling Phospholipidosis Induction: Reliability and Warnings

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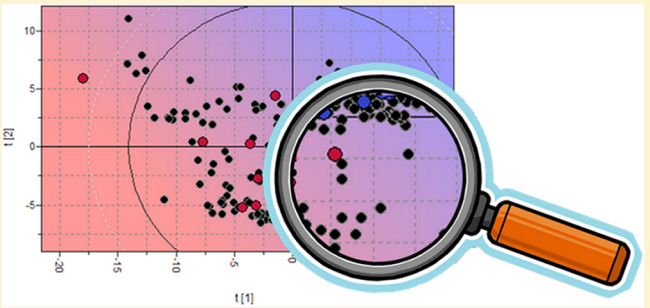
Supporting Information

ABSTRACT: Drug-induced phospholipidosis (PLD) is characterized by accumulation of phospholipids, the inducing drugs and lamellar inclusion bodies in the lysosomes of affected tissues. These side effects must be considered as early as possible during drug discovery, and, in fact, numerous in silico models designed to predict PLD have been published. However, the quality of any in silico model cannot be better than the quality of the experimental data set used to build it. The present paper reports an overview of the difficulties and errors encountered in the generation of databases used for the published PLD models. A new database of 466 compounds was constructed from seven literature sources, containing only publicly available compounds. A comparison of the PLD assignations in selected databases proved useful in revealing some inconsistencies and raised doubts about the previously assigned PLD+ and PLD− classifications for some chemicals. Finally, a Partial Least Squares Discriminant Analysis (PLS-DA) approach was also applied, revealing further anomalies and clearly showing that metabolism as well as data quality must be taken into account when generating accurate methods for predicting the likelihood that a compound will induce PLD. A new curated database of 331 compounds is proposed.



INTRODUCTION

Drug-induced phospholipidosis (PLD) is a phospholipid storage disorder characterized by the excessive accumulation of phospholipids and the inducing drug in the lysosomes of the affected tissues.[1−3] The hallmark feature of phospholipidosis is the formation of the characteristic lamellar bodies in cells, which are detected by transmission electron microscopy (TEM), and represents the most accepted indication of this condition. Numerous drugs possessing a cationic amphiphilic structure (cationic amphiphilic drugs, CADs) are PLD inducers in vivo and in vitro. The first report of CAD-induced PLD was provided by Nelson and Fitzhugh in 1948, when they observed the induction of foamy macrophages in rats due to treatment with chloroquine, even though at that time this phenomenon of PLD was unknown.[4] More than 50 marketed drugs having a cationic amphiphilic structure have been reported to induce phospholipidosis in vivo or in vitro, since then. These PLD inducers include antidepressant, antiarrhythmic, antipsychotic, and antihistaminic drugs.[2] Regardless of their pharmacologic category, CADs commonly share two structural features: a rigid hydrophobic moiety (mainly aromatic rings) and a polar "head group", which includes an amine group charged under physiological conditions. In addition to CADs, aminoglycoside, aminocyclitol, and macrolide antibiotics can also induce PLD, despite the lack of a hydrophobic moiety.[2,5]

PLD has been extensively described in numerous review articles in the last two decades,[1−3,5−7] but an unresolved issue is whether phospholipidosis is a toxicological condition or an adaptive response to xenobiotics. In 2004, the FDA formed the Phospholipidosis Working Group to address this question.[3] A result was that many pharmaceutical companies now apply PLD screening assays on their drug candidates, in order to detect the risk of PLD induction as soon as possible. This need to assess the phospholipidosis issue early in drug discovery programs shifted the interest of scientists from animal testing and in vitro assays toward in silico models. The use of in silico models to predict the PLD risk may represent an efficient high-throughput and cost-effective method. A major advantage of the in silico method, with respect to other high-throughput methods under investigation (e.g., the use of biomarkers),[8−10] is that it allows for outcome predictions prior to drug synthesis.

Numerous in silico models for PLD prediction have been developed, since 2004. For example, Ploemen et al.[11] described a simple model based on two physicochemical properties: the predicted pK_a of the most basic center in the molecule and the ClogP value. This simple method was apparently accurate enough to be useful as a fast screening tool to discriminate compounds suspected of being phospholipidosis inducers. Then, Tomizawa et al.[12] modified the Ploemen model replacing the pK_a with the net charge (NC) to improve the prediction capability in the presence of zwitterionic compounds. Later, Pelletier et al.[13] validated the Ploemen model using a rigorously selected list of positive and negative compounds (using literature and proprietary data) for evaluation, then proposed

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a "Modified Ploemen model" and a Bayesian Model to improve predictivity.

Hanumegowda et al.[14] took into account that PLD in vivo is related not only to the physicochemical properties of the compounds but also to their residence in tissues. They combined the volume of distribution (V_d) with the pK_a of the most basic group and the ClogP, which improved the ability to predict phospholipidosis in vivo. Recently, Fisher et al.[15] showed that methods based on the amphiphilic moment, rather than on the lipophilicity, can accurately differentiate between PLD inducers (PLD+) and PLD noninducers (PLD−). This finding is in agreement with the most accepted mechanism of phospholipidosis induction, which is that the charged amphiphilic drugs interact with the lysosomal membranes leading to the inhibition of the phospholipases activities. Although more complex models have also been reported,[16−20] concerns on the usability of a complex approach in a drug discovery phase have been raised.[15] Regardless of the fact that all the mentioned models report good prediction abilities, there are still many efforts to enhance the discrimination between PLD+ and PLD− compounds.

Despite the variety of in silico methods available, the lack of information about the mechanism of phospholipidosis induction and the creation of a reliable data set to be used to build the models represent the main obstacles to improve model usability and predictive value. Indeed, at least three hypotheses for the mechanism of PLD induction have been proposed.[1,7] So, further research is necessary to establish the biochemical mechanisms involved and apply them to PLD models. Unfortunately, the available data sets normally mix together PLD data from different species, because not much is known about drugs inducing phospholipidosis in humans. Also, the degree of the induced PLD and the affected tissues are not considered when compounds are PLD+ classified. In addition, data sets listing PLD− compounds usually include compounds that were not tested for PLD induction: thus, their PLD effects are actually unknown. Also, it is well-known that drug biotransformations can strongly affect the induction of phospholipidosis, but metabolism is not carefully considered when building in silico models for PLD prediction.

A number of data sets are publicly available,[13,14,16,17,20] which contain proprietary data whose structures are not disclosed.

In this paper seven databases containing nonproprietary compounds were compared, and a new data set of 466 unique structures was compiled. Comparing PLD data from the different literature sources led to the identification of several inconsistencies, which raised doubts about the previously assigned PLD+ and PLD− classifications. These inconsistencies are discussed, along with the importance of considering drug metabolism in the creation of the models.

MATERIALS AND METHODS

Data Sources. Data for drug induced PLD were collected by comparing seven recently published data sets. Their compositions and abbreviations are reported in Table 1.

The data set published by Kruhlak et al.[16] was not selected, since it represents a previous version of O2012. The database used in a more recent paper by Lowe and co-workers[18] is the same as published elsewhere. So, here we refer only to the original paper.[17] The O2012 database is the most complete data set available and includes structures from databases T2006, P2007, and V2008. However, in this study we report all the databases, because inconsistencies among them were noticed.

Table 1. Selected Databases for the Study of Publicly Available PLD Data

ref	authors, year	database abbrev	PLD+ public compds	PLD− public compds	unique[a] compds
20	Orogo et al., 2012	O2012	215	232	262
12	Tomizawa et al., 2006	T2006	35	17	2
13	Pelletier et al., 2007	P2007	55	60	0
21	Vitovic et al., 2008	V2008	34	18	0
17	Lowe et al., 2010	L2010	99	82	2
14	Hanumegowda, 2010	H2010	38/33[b]	42/9[b]	3
15	Fisher et al., 2012	F2012	27/23[b]	5/9[b]	2

[a]Compounds that are reported in that database only. [b]Numbers refer to in vitro and in vivo data, respectively. Although some databases do not contain new compounds, they report useful information.

These will be discussed in the Results and Discussion section. The database L2010 also includes data from P2007 and from the Kruhlak et al.[16] source. The database of H2010 contains literature data from T2006, P2007, V2008, while the database V2008 includes data from T2006. In the case of F2012, in vivo PLD findings are from various literature sources, while the in vitro PLD findings are from their in house tests.

The PLD findings reported in the selected databases were measured in a variety of species (including humans, rats, dogs, and mice), and PLD effects refer to a variety of target tissues. The strategies for data sets construction are different and are discussed in the Results and Discussion section.

Generation of the PLS Model. A Partial Least Squares Discriminant Analysis (PLS-DA) approach was applied to model the L2010 and the O2012 database using VolSurf+. VolSurf+ is an automatic procedure to convert information coded into the 3D GRID Molecular Interaction Fields into 128 physico-chemically relevant molecular descriptors.[22,23] The interaction of drug molecules with biological membranes is based on a three-dimensional recognition that is mediated by surface properties such as shape, van der Waals forces, electrostatics, hydrogen-bonding, and hydrophobicity.[24] Thus, the VolSurf+ descriptors refer to molecular size and shape, hydrophilic and hydrophobic properties, hydrogen bonding, amphiphilic moments, and critical packing parameters. Pharmacokinetic descriptors related to solubility, metabolic stability, and cell permeability are also generated. A complete list of the VolSurf+ descriptors has been published elsewhere.[24] It should be noted that VolSurf+ descriptors are independent of the alignment of molecules and relatively independent of conformational sampling and averaging. Numerous applications of VolSurf+ have been reported,[25−28] showing that the obtained models are fast to compute, easy to interpret, and well-suited for external predictions. In particular, the VolSurf+ approach was previously used to successfully predict the blood brain barrier (BBB) permeation of compounds from the 3D molecular structure of drug candidates.[25] In this work, each molecule of the data set was imported in its most abundant protonation state at pH 7.4, using MoKa[29,30] for pK_a prediction. A categorical score was assigned to the PLD+ and PLD− compounds (+1 and −1, respectively). Two significant latent variables emerged from the cross-validated PLS model. The statistical parameters for the models discussed are reported in Table 2 under the "Results and Discussion" section. The same

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Table 2. List of the Inconsistencies Detected When Comparing Seven Literature Sources

				V2008			H2010		F2012		O2012	
	name	T2006 PLD	P2007 PLD	class[a]	PLD	L2010 PLD	PLD in vivo	PLD in vitro	PLD in vivo	PLD in vitro	conf[b]	PLD
1	chloramphenicol					pos	neg		pos	neg[c]		
2	clarithromycin					neg					medium	pos
3	disopyramide	pos[d]		IV	neg		neg	pos[d]				
4	procaine	neg	neg	III	pos[e]	neg	neg				medium	pos[e]
5	cloforex					neg					high	pos
6	erythromycin	pos	pos	III	pos[e]	pos	pos	pos	pos	neg[c]	high	pos
7	gentamicin		pos	I	pos		pos		pos	neg[c]		
8	trospectomycin sulfate		pos			pos			pos	neg[c]	high	pos[d]
9	amikacin		pos			pos	pos		neg	neg[c]	high	pos
10	bupropion hydrochloride		neg			neg	neg				medium	pos
11	buspirone hydrochloride			IV	neg	neg					medium	pos
12	felbamate		neg			neg					high	pos
13	flutamide		neg			neg					medium	pos
14	haloperidol			IV	neg	pos	pos	pos[e]	pos	pos[c]	high	pos
15	sotalol hydrochloride					neg	neg	neg[e]			medium	pos
16	stavudine		neg			neg					high	pos
17	cimetidine	neg[d]		III	pos[d]	neg	neg	neg[d]			high	neg[d]
18	doxepin hydrochloride						pos	pos[e]			medium	neg
19	famotidine	neg[d]	neg	III	pos[d]	neg	neg	neg[e]				
20	atropine	pos[d]		III	pos[d]	pos	neg	pos[d]			medium	pos[d]
21	lidocaine	pos[d]		III	pos[e]	pos	neg	pos[d]			medium	pos[d]
22	mianserin	pos[e]		III	pos[e]	pos	neg	pos[e]			medium	pos[e]
23	propranolol	pos[d]		III	pos[e]	pos	neg	pos[d]	pos	pos[c]	high	pos[d]
24	sertraline					pos	neg	pos[d]			medium	pos

[a]Four classes of PLD induction as reported by Vitovic et al.:[21] I: in humans; II: in animals; III: in cultured cells; IV: compounds for which PLD was not experimentally demonstrated. Information from in vitro tests is reported when available. [b]Confidence levels of the data, as defined by Orogo et al.[20] [c]Bovine corneal fibroblasts. [d]Isolated rat hepatocytes. [e]Cultured cells.

data sets were also processed using different descriptors, and the obtained models are reported in the Supporting Information.

RESULTS AND DISCUSSION

Data Analysis. A careful investigation of the collections of publicly available PLD findings was performed, focusing attention on the most recent sources used for in silico or high-throughput studies. Thus, seven data sets were selected (see Table 1 for abbreviations), although the most recent databases include the majority of the previously published compounds. A detailed description of the links among the seven databases is reported in the Materials and Methods section. An initial database of 466 compounds was obtained, combining all the information available in the selected databases, which is also available in the Supporting Information. The common connections among the databases should provide identical information with regards to the ability of a given compound to induce PLD. However, comparisons of the PLD findings showed a number of inconsistencies, as reported in Table 2.

Data reported in Table 2 did not allow entries 1−4 (chloramphenicol, clarithromycin, disopyramide, procaine) to be categorized as PLD+ or PLD−, because the respective PLD findings were too variable among the different literature sources. So, entries 1−4 were removed from the database. Cloforex (5) is reported to be PLD+ in O2012 and PLD− in L2010. However, a more recent paper by Lowe and co-workers[18] raised doubts about the previous PLD− assignation, suggesting that cloforex should be labeled as PLD+. Thus, the PLD+ assignment proposed in O2012 was used in our database. The antibiotics erythromycin, gentamicin, and trospectomycin (entries 6−8) are cationic drugs that do not belong to the CAD family, because they lack the typical hydrophobic moiety. Still, these compounds induce phospholipisosis[2,13] and, in the case of the aminoglycosides (e.g., gentamicin), a causal relationship between PLD and nephrotoxicity has been suggested.[31] These compounds are likely to have different interactions with phospholipids, due to their peculiar structures and in vitro tests performed by Fisher et al.[15] on normal bovine corneal fibroblasts failed to detect PLD induction. Based on the literature data, entries 6−8 must be considered as PLD+. However, the differences in the internalization of these compounds in lysosomes and the peculiar physicochemical properties of aminoglycosides antibiotics and also macrolides and aminocyclitol compounds[2] suggest that a separate model for these antibiotics is needed.

Entries 9−19 represent those compounds for which only one of the selected databases is in disagreement with the other literature sources. For example, amikacin is reported as PLD+, while Fisher et al.[15] report the opposite finding both in vivo and in vitro. It appears that therapeutic doses of amikacin induce early lysosomal phospholipidosis in human kidney cortex, which is comparable to that observed in animals at low doses.[32,33] However, animal and human studies have shown that amikacin induces significantly less lysosomal overloading than the other aminoglycosides (e.g., gentamicin and tobramycin) with no loss of phospholipase A_1 activity. Thus, amikacin was labeled as PLD+, even though it may be a weak inducer of PLD.

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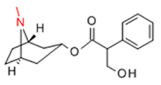
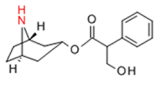
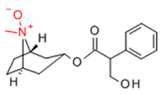
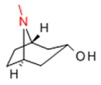
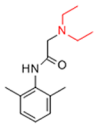
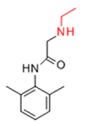
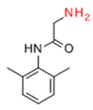
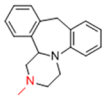
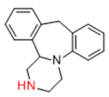
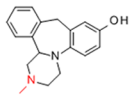
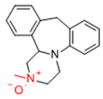
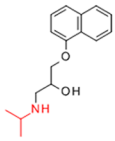
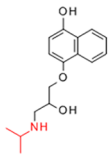
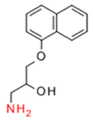
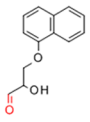
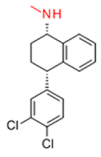
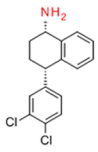
Table 3. Main Metabolites for Entries 20−24 in Table 2

Entry	Ref.	Parent	Metabolite A	Metabolite B	Metabolite C
Atropine	36				
Lidocaine	37				
Mianserin	38				
Propranolol	39				
Sertraline	40				

Reference O2012 contains seven entries (10−13, 15, 16, and 18) reported to have PLD effects which disagree with those reported in the other data sets. In particular, compounds 10−13, 15, and 16 are reported to be PLD− in older databases, meaning that at that time these compounds were not positively tested for PLD induction. Unfortunately, no references are provided by O2010 for these 6 compounds to prove their tested PLD+ effects, and, due to the limited number of structures, such compounds were labeled as "uncertain" and removed from the final version of the database. In the case of doxepin hydrochloride (entry 18), H2010 and O2012 differ in the PLD assignation, but no clear references for these findings were provided. Thus, doxepin was also removed.

In contrast, entries 14, 17, and 19 in V2008 database differ from the other data sets, but haloperidol (14) is included in class IV (PLD− compounds) by Vitovic and co-workers because the PLD induction was not experimentally demonstrated at the time of publication of the database, while cimetidine and famotidine are reported to induce PLD only in vitro (class III). More recent findings proved that haloperidol induces PLD, according to the more recent databases, while cimetidine and famotidine are recently proved to be PLD− and indeed are included among the experimentally proved PLD− compounds in L2010. Thus, haloperidol was labeled PLD+, while cimetidine and famotidine were labeled PLD−, based on the referenced information from the other data sets.

Finally, the last five entries are generally reported to be PLD +, except for the in vivo data reported in H2010. Differences between in vivo and in vitro assays might be explained by different dispositions of the compounds, since lipophilicity, basicity, or the net charge influence uptake and distribution. Hanumegowda et al.[14] included the volume of distribution (V_d) in their model to account for this, in order to provide a better prediction of PLD. However, the in vivo distribution might be also influenced by the occurrence of biotransformations. Unfortunately, the species used for in vivo tests in H2010 are not reported.[14] In addition, in vitro tests for entries 20−24 (see Table 2) were performed mostly in rat hepatocytes by Tomizawa et al.[12] A strong correlation among the databases was detected, since V2008, H2010, and O2012 report the T2006 data at most. The uncertain origin of the in vivo data and the high correlation of the in vitro results do not allow a detailed interpretation of the role that metabolism can play in PLD induction by these compounds. However, marked differences in metabolism studies between in vitro data using hepatocytes and in vivo data have been reported.[34,35] Since atropine, lidocaine, mianserin, propranolol, and sertraline are subjected to extensive metabolism in humans and animals in vivo, the differences in their clearance can be responsible for the different behavior observed between in vivo and in vitro data. Greater attention should be devoted to the description of the in vitro and in vivo procedures to increase the knowledge in this field. The major known metabolites for compounds 20−24 are reported in Table 3.

Indeed, the effects of drug metabolism on PLD induction have rarely been investigated.[41,42] Quaglino et al.[41] compared the effects of amiodarone, and two of its main metabolites MDEA (mono-N-desethylamiodarone) and DDEA (di-N-desethylamiodarone), on the formation of vacuoles in macrophages and found significant differences in potency among the

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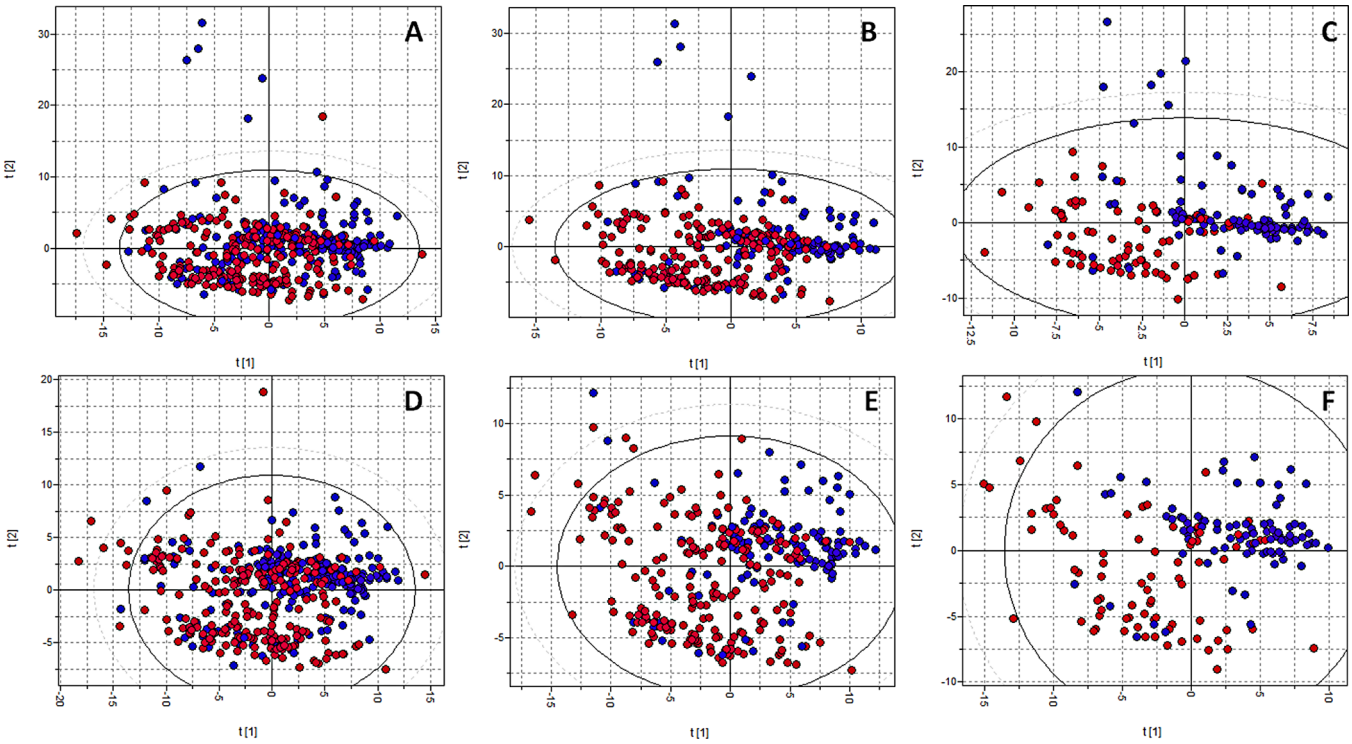

Figure 1. Discriminant PLS t1-t2 score plot for the databases from O2012 and L2010 using VolSurf+ descriptors. In parts A and D O2012 data including *high-* and *medium-confidence* compounds were modeled, in the presence (A) or in the absence (D) of the aminoglycosides, macrolide, and aminocyclitol antibiotics. Similarly parts B and E show the models generated using the "modified O2012 database", in the presence (B) or in the absence (E) of aminoglycosides, macrolide, and aminocyclitol antibiotics. Finally, parts C and F show the model for the L2010 database with or without the antibiotics subset, respectively.

three compounds. In fact, the ability to induce the formation of vacuoles in macrophages appears to rank in the following order: amiodarone > MDEA ≫ DDEA, even though MDEA has higher lipid binding potential compared to its parent compound.[41] Thus, both the N-dealkylation of the amino nitrogen in CAD-like compounds and the rate of decomposition of extensively metabolized drugs need to be further assessed for their effects on the development of PLD. In the present study entries 20−24 were considerd as PLD+ due to the lack of information about the in vivo data; the rate of the N-dealkylation reactions between different species could make the difference, according to Quaglino et al.[41]

In summary, compounds 1−4, 10−13, 15, 16, and 18 in Table 1 were removed from our PLD database. They were removed due to lack of evidence of their abilities to induce PLD, or because of major discrepancies uncovered between the various data sets that were analyzed. Clearly, more evidence is required, in order to identify these compounds as PLD+ or PLD−. Thus, this study revealed that, although published databases appear to be strictly linked, a careful comparison is required to accurately assess the information and remove possible error sources prior to modeling efforts.

Data Selection: Finding a Compromise between Quantity and Quality. A critical issue in the construction of a database for modeling purposes is whether it is better to focus on using the largest number of available compounds or on selecting the most reliable data. This point is particularly important when, as in the case of PLD induction, reliable data are not easy to obtain because 1) the most accepted method for detecting PLD is electron microscopy, which is a low-throughput technique; 2) tests are performed in a variety of species; 3) there is a lack evidence for PLD− compounds, because it is common to test only those that are likely to be PLD inducers. In order to minimize these problems, we focused our attention on two of the selected databases: O2012 and L2010, because they are the two largest databases available and they were built following different strategies.

The aim of Orogo and co-workers (O2012) was the construction of an enhanced version of the U.S. Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER) Phospholipidosis Working Group (PLWG) database to be used for (quantitative) structure−activity relationship [(Q)SAR] modeling.[20] PLD data were donated by pharmaceutical companies, which were then combined with existing data from FDA/CDER archives and the published literature. A significant effort was made to find a balance between PLD+ and PLD− compounds, which is important in QSAR modeling. The final database, including proprietary data, contains 743 compounds.

The most useful information in the O2012 database is the classification into *high-* and *medium-confidence* categories, based upon the types of keywords found and the source of the data. Keywords that relate to electron microscopy confirmation of PLD were considered *high-confidence*, whereas those relating to only the presence of foamy macrophages, cytoplasmic vacuolations, cytoplasmic granules, lipidosis, dyslipidosis, and histiocytosis were considered *medium-confidence*. The absence of PLD keywords in the New Drug Applications (NDA) documents was considered *high-confidence* for negative compounds, while *medium-confidence* was addressed based solely on a search of Investigational New Drug (IND). Therefore, for example, the mention of "*high*" and "*medium*"

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Table 4. Statistical Parameters for the Models in Figure 1

	accuracy	sensitivity	specificity	precision	Matthew's CC	hit rate @5%
A	0.70	0.69	0.70	0.68	0.58	86
B	0.77	0.67	0.83	0.71	0.62	100
C	0.83	0.87	0.78	0.83	0.71	100
D	0.71	0.69	0.72	0.68	0.58	85
E	0.77	0.64	0.84	0.70	0.61	93
F	0.84	0.90	0.77	0.81	0.73	100

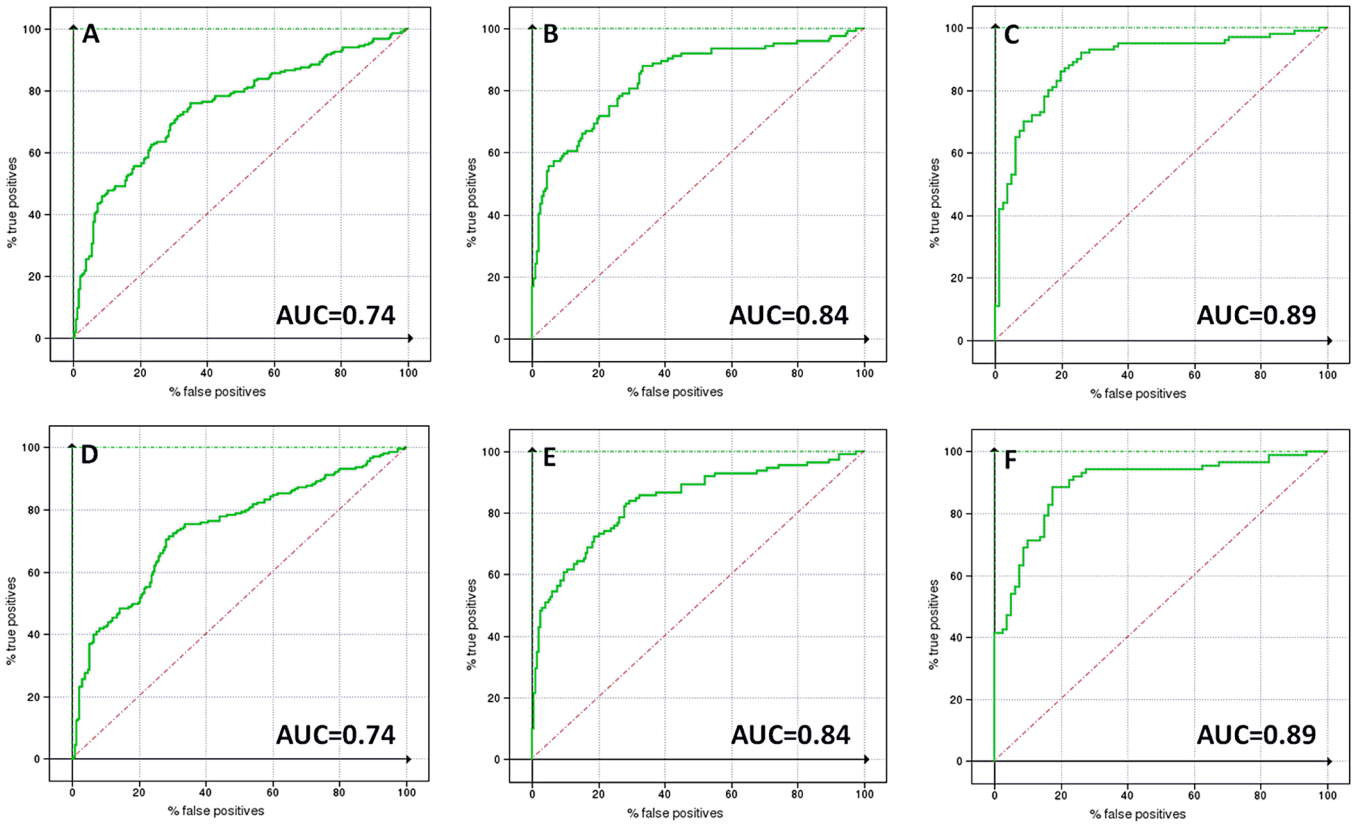

Figure 2. ROC curves for the PLS-DA models whose the t1-t2 plots are reported in Figure 1. The curves and the AUCs are calculated at the second latent variable. Letters A–F correspond to databases as reported in Figure 1.

in Table 2 are not related to the potency of the PLD induction but to the reliability of the data. Among the 743 compounds collected in O2012, 424 were labeled as "*high*", meaning *high-confidence* data, and 319 as "*medium*".[20]

The database constructed by Lowe and co-workers[17] contained total of 185 compounds, of which 102 were positive for phospholipidosis (PLD+) and 83 were negative (PLD−). In this case the data were considered reliable, because 68 out of 102 PLD+ compounds were confirmed by electron microscopy, while 34 were reported to be PLD+ due to the presence of foamy macrophages or vacuolations. Most importantly, all of the 83 PLD− compounds were reported negative by electron microscopy.

A preliminary modeling of the two databases was performed. Partial Least Squares Discriminant (PLS-DA) approach and VolSurf+ descriptors were applied to the two databases of compounds labeled with a categorical score. Details on the chemical nature of the VolSurf+ descriptors are reported in the Methods section. Two significant latent variables emerged from the two cross-validated PLS models. The PLS t1-t2 score plot of the resulting O2012 model and L2010 model are reported in Figure 1. Compounds are color-coded by their activity values, using a scale from red (PLD−, inactives) to blue (PLD+, actives). Two models were generated for O2012. In the first model the entire database (*high- and medium-confidence data*) was used (Figure 1A). In the second model, with the aim to find a compromise between the quantity and the quality of the data, we extracted from the O2012 database all the "*high-confidence data*" together with those "*medium-confidence*" data for which the literature source was reported. We decided not to include any *medium-confidence* data included in the O2012 database which are from undisclosed sources. The PLS t1-t2 score plot for this "modified O2012 database" is reported in Figure 1B. Finally, the model for L2010 is reported in Figure 1C.

The aminocyclitols and aminoglycosides formed a cluster in the three models (Figure 1 A-C), and macrolide antibiotics are located between the PLD+ CADs and that cluster. As previously discussed, this is not surprising, because these PLD+ inducers do not belong to CADs family. Thus, the aminocyclitols, aminoglycosides, and macrolide antibiotics should be considered separately. Although they are all PLD+

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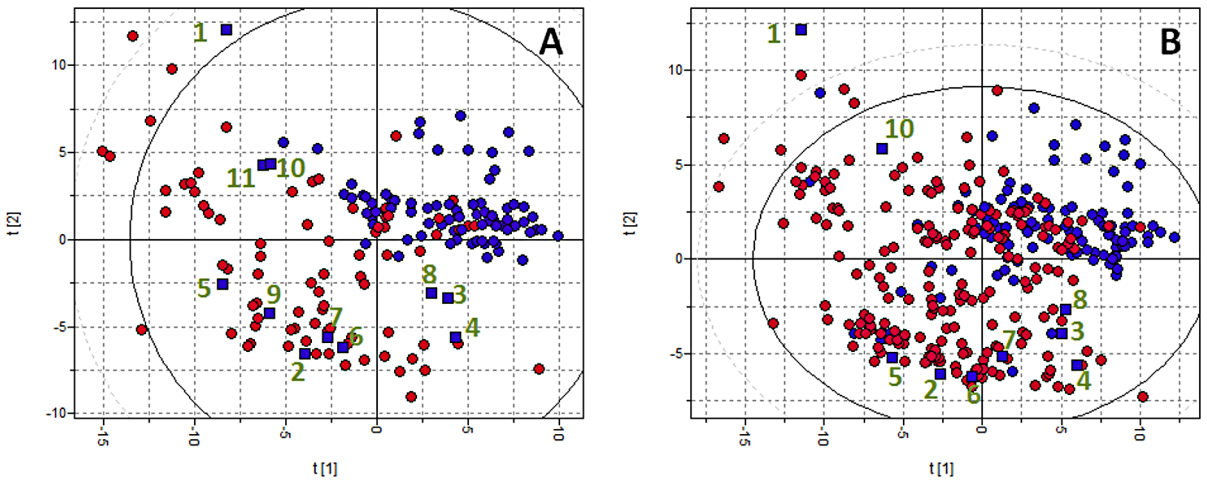
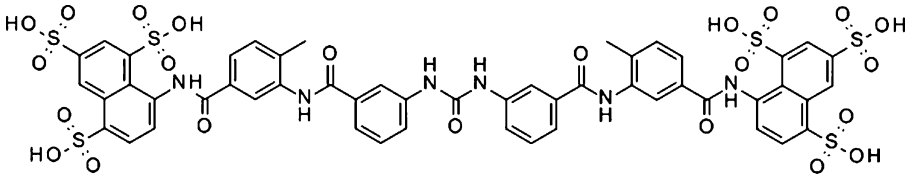


Figure 3. Main false negatives obtained by modeling the L2010 database (A) and their position in the model based on the "modified" O2012 database (B). Suramin (1), benzamide (2), ketoconazole (3), loratadine (4), allopurinol (5), phenacetin (6), ethyl loflazepate (7), lysergic acid diethylamide (8), chloramphenicol (9), 5-hydroxydopamine (10), 6-hydroxydopamine (11). Chloramphenicol and 6-hydroxydopamine are not included in the original O2012 database.

Chart 1. Structure of Suramin



inducers, their use in the model does not provide any important information to discriminate between PLD+ and PLD− compounds. Figure 1 D-F represents the new recalculated PLS models, following removal of the aminoglycosides, macrolide, and aminocyclitol compounds. It appears that the removal of this subclass of PLD+ compounds allows a clearer view of the plot, although the statistical parameters reported in Table 4 are almost insensitive to the outliers.

The comparison of Figures 1A-1B and 1D-1E and the correspondent statistical parameters in Table 4 also indicates that the quality of the PLS model seems to improve when the "modified O2012 database" is used rather than the whole O2012 data set, with or without the aminoglycosides, macrolide, and aminocyclitol compounds. Except for sensitivity, statistical parameters in Table 4 are greater for the "modified O2012 database", and this behavior is confirmed by a comparison of the ROC curves (Figure 2).

A different set of van der Waals Surface Area (VSA) descriptors (PEOE_VSA, SMR_VSA, and SlogP_VSA MOE-type descriptors from the RDKit package)[43] was also used to generate the six PLS-DA models. VSA descriptors have been introduced by Labute,[44] and, despite their reduced number, they are considered high-quality and weakly correlated descriptors, successfully used also in challenging studies.[45,46] However, in our study, the majority of the statistical parameters obtained using the VolSurf+ or the VSA descriptors were similar, but VolSurf+ descriptors performed better in terms of ROC curves and hit-rate. All data for the models generated using the VSA descriptors are reported in the Supporting Information.

The aim of this paper was not to test the predictivity of a new in silico model but to illustrate how the construction of a database can be critical to evaluate the quality of a model. The PLS model built using the L2010 database better discriminates between PLD+ and PLD− compounds, but, of course, the number of objects used is much lower. Regardless, the "modified O2012 data set" may provide a good compromise between quantity and quality. However, in the next paragraph we use the model built from the L2010 database to illustrate how models can be useful for a further refining process.

Model Refining. Although the model based on the Lowe's database showed the best discrimination between PLD+ and PLD− compounds, we focused on some false negative points, shown in Figure 3A. The same compounds are also false negatives in the database generated using the "modified O2012 database" (Figure 3B).

Among the PLD+ compounds, suramin (1) was most distant from the other phospholipidosis inducers. Suramin is a polyanionic drug, unlike the common PLD inducers (see Chart 1).

Pelletier et al. (P2007) first reported suramin as a phospholipidosis inducer.[13] Afterward, more recent databases, that are known to include P2007 data (L2010, O2012), also report suramin to be PLD+, although the O2012 database labels it as a *medium-confidence* PLD inducer, citing P2007 as reference. The suramin-induced formation of myelin-like figures in the lysosomes of albino rat testes[47] provided the only experimental finding leading to that classification. In addition, suramin is a strong inducer of another lysosomal storage disorder, mucopolysaccharidosis.[48] Thus, the PLD+ classification for this compound may be unreliable. For this reason suramin was removed from the database.

Benzamide (2) is reported to be PLD+ in L2010 and O2012, and it presents an interesting issue. The source reported in O2012 indicates that this compound is an antiarrhythmic agent.[2] Searching the literature using "benzamide", "antiar-

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rhythmic", and "phospholipidosis" as key words, led to a study by Cramer et al.[49] referring to "benzamide antiarrhythmic agents". However, Cramer and co-workers did not study the benzamide "as is" but rather a "CAD-like" derivative of the benzamide, which possesses a protonable amino group (benzamide,N-[2-aminocyclohexyl]-4-bromo-,*trans*-,monomethanesulfonate).[49] Figure 4 clearly shows that this structure fits

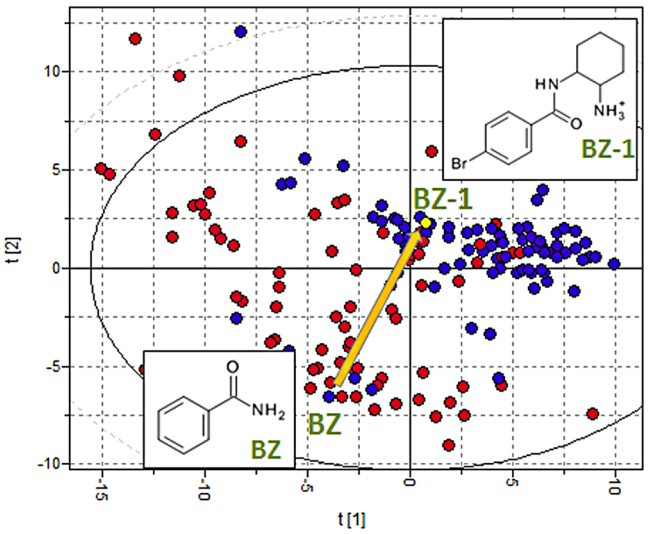
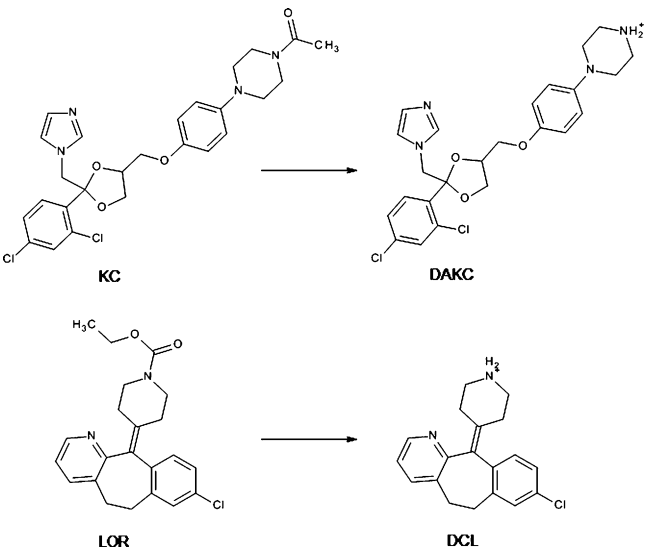


Figure 4. Effect of the correction of the structure for the "benzamide" antiarrhythmic agent. BZ-1 is the structure of the compound originally studied by Cramer et al.,[49] while BZ is the structure associated with the PLD effect in O2012 and L2010.

the model. An analogue effect was observed using the VSA descriptors to generate the models (see the Supporting Information). So, in the following database it has been used to replace benzamide.

A third case regards ketoconazole (3). It is known that ketoconazole itself does not induce PLD, and the induction of this disease in vivo is due to the de-N-acetyl ketoconazole (DAKC) metabolite (see Chart 2).[50] Models published so far

Chart 2. Major Metabolites for Ketoconazole (KC) and Loratadine (LOR)



have not taken this biotransformation into account, which is why most in silico methods fail to predict the PLD induction by ketoconazole.[51] It is clear that the structure of the metabolite should be used in cases where it is the metabolite, rather than the parent compound, which is responsible for inducing PLD. Figure 5 shows the projection of DAKC, the metabolite, into

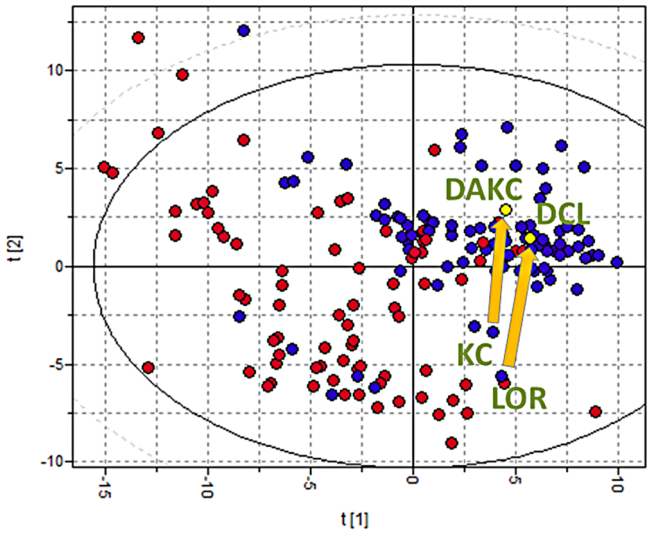


Figure 5. Effect of metabolism on the prediction of PLD. Ketoconazole (KC) is not an inducer but is reported PLD+ (blue circle) because its main metabolite is an inducer of phospholipidosis. The model correctly locates KC in the red point region (PLD− region). When the de-N-acetyl-ketoconazole (DAKC) is projected into the model, it is correctly located in the inducer region (blue region). The same is true for loratadine (LOR) and descarboethoxyloratadine (DCL).

the PLS model. In agreement with the experimental evidence, DAKC locates in the PLD+ region, along with structurally related PLD inducers. Again, a similar assignation for DAKC was observed using the VSA descriptors, although models based on VolSurf+ descriptors resulted to be more sensitive to the metabolic effect reported (see the Supporting Information).

The role of loratadine (4) in PLD induction may be analogous to that of ketoconazole. Although the effect of its metabolism has not been reported, it experiences first-pass hepatic biotransformation, and its product, descarboethoxyloratadine (DCL), is an active metabolite.[52] DCL is also named desloratadine and is marketed as Clarinex (see Chart 2).

Desloratadine is reported to be a PLD+ compound in the O2012 database, and, interestingly, one of the references cited by Hanumegowda et al.[14] for loratadine-induced PLD is actually the Clarinex (desloratadine) Drug Approval Package. Thus, desloratadine was projected into the PLS model (Figure 5), and the loratadine/desloratadine shift is similar to the one obtained for ketoconazole and its metabolite.

In our database the KC structure was substituted with the DAKC structure, while DCL was left in the database and LOR was removed, because of the above findings. Currently, we are performing metabolic studies to determine if LOR should be included in the database.

Among the six false negative compounds highlighted in Figure 3, three compounds (allopurinol, lysergic acid diethylamide, and chloramphenicol) have been reported to be *medium-confidence* data according to the labeling of Orogo and co-workers,[20] while three compounds (ethyl loflazepate (or

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ethyl flucozepate), phenacetin, and 5-hydroxydopamine) have been reported to be *high-confidence* data. All these compounds were included in our database, with the exception of chloramphenicol, which was among the "uncertain compounds" reported in Table 2. Still, wrong predictions for these compounds may also be related to the potency of the PLD induction. For example, ethyl loflazepate is reported to induce pulmonary PLD in rats but only after a long period of treatment (6 months) and at high doses.[53] Finally, considerations must be reported for 5-hydroxydopamine and 6-hydroxydopamine in the L2010 database. They have been reported to induce phospholipidosis.[13,16,17] However, the cited references[54,55] only refer to 6-hydroxydopamine, which was the only one tested. Thus, the collected database includes only the 6-hydroxydopamine.

An analogue approach should be used to study the false positive compounds, thus PLD− compounds located in the PLD+ region in our models. As previously mentioned, the PLD− assignation is generally not due to experimental evidence but rather the lack of PLD+ data. Until now, only Lowe and co-workers[17] reported a database (L2010) where compounds were confirmed to be noninducers by electron-microscopy. Unfortunately the references for these data are not available, making accurate assessments difficult.

CONCLUSIONS

In the recent years, a large effort has been devoted to the creation of predictive PLD models. In fact, at least eight different PLD models have been published in the past two years. Currently, the tendency is to move toward more complex in silico approaches. This requires increasing the number of descriptors, or the mathematical model complexity, with an increased risk of overfitting the experimental data. Moreover, less care has been applied to assessing data quality and including physiological models. The latter is important, because modeling the independent steps and mechanism of drug metabolism play an important role in understanding the property under consideration. In contrast, the databases currently used for modeling purposes are usually collections of previous literature sources and proprietary compounds. Rarely new PLD tested compounds were added to these sets.

In this paper, we focused our attention on publicly available compounds and reported an overview on the difficulties and errors encountered when comparing seven recently published databases. As a result of this study, a new database of 466 compounds was constructed. Here, 19 compounds reported in other databases were added, with respect to the largest database published by Orogo et al.[20] Although there is a strong correlation on PLD effects among all the published databases, 24 compounds were labeled differently in the literature sources. These compounds were critically analyzed, and some were referred to as "doubtful" for correct classification, at least based on literature sources. A further data analyses were performed using the VolSurf+ software to generate PLS models. The focus was not on the discriminant capability of the model but on the study of the false negative compounds, since VolSurf+ is capable of generating dynamic cheminformatic models that are easy to interpret. This approach defined some inconsistencies and identified a number of doubtful PLD+ and PLD− assignations. In addition, it became clear that metabolism must be taken into account, in order to generate useful methods for predicting the potential for compounds to induce PLD. Similar results were obtained using VSA descriptors, supporting our conclusions. On the basis of these findings, we constructed a second database of 331 compounds, with all identified inconsistencies corrected and in which *medium-confidence* data with undisclosed sources from the Orogo et al.[20] database were removed. Both the data set collections are publicly available in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Data set collections discussed in this study and PLS-DA models generated using VSA descriptors. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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