

## Review

# Drug-induced Phospholipidosis —Pathological Aspects and Its Prediction

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**Abstract:** Drug-induced phospholipidosis (PLDsis) is an excessive accumulation of polar phospholipids in cells or tissues/organs caused by xenobiotics. Numerous drugs and chemicals are capable of inducing the storage disorder in animals and humans; however, despite their diverse pharmacological activities, each of these drugs shares common physicochemical properties: a hydrophobic aromatic ring structure on the molecule and a hydrophilic side chain with a charged cationic amine group and therefore are in the group of cationic amphiphilic drugs. In affected cells the appearance of membrane-bound inclusions, primarily lysosomal in origin, with a lamellar structure (lamellar bodies) is a definitive morphologic hallmark. Massive accumulations can occur in animal tissues such as the lung with little effect on organ function. The inducing drug also accumulates in association with the excess phospholipid. Although these alterations are generally reversible after cessation of drug treatment, PLDsis is of regulatory concern and an issue for drug safety for pharmaceutical companies. Thus, the assessment of potential target organ dysfunction and the identification of clinical biomarkers are important objectives for new drug development. Recent advances in biotechnology such as metabolomics and toxicogenomics have been providing novel tools to elucidate the mechanisms of PLDsis and to establish biomarkers for screening tests in the preclinical stages and for monitoring in the clinical phases in addition to conventional approaches such as morphology (lamellar bodies) and biochemical methods including assays of specific metabolites and phospholipase inhibition. (J Toxicol Pathol 2008; 21: 9–24)

**Key words:** phospholipidosis, cationic amphiphilic drug, biomarker, lamella body, metabolomics, toxicogenomics

## Introduction

Phospholipidosis (PLDsis), a phospholipid storage disorder, is defined as an excessive accumulation of intracellular phospholipids in the tissues. Phospholipids are essential and dynamic components of the plasma and intracellular membranes in normal cells<sup>1</sup>. Tiny portions of the membrane are continually sequestered by autophagosomes that fuse with lysosomes to degrade the membrane fragments and are recycled. Specific enzymes catalyze the breakdown of the membrane components such as phospholipids, glycolipids, and glycoproteins for reuse. Phospholipid fragments accumulate in the lysosomes when this membrane repair process is driven to excess or when the enzymes involved are missing or damaged. Such cell membranes contain a greater proportion of polar groups and are therefore partly soluble in non-polar solvents. The bilayer of polar lipids has been regarded as a basic structure

in biologic membranes. As mentioned above, their synthesis and metabolism are regulated by individual cells and tissues, and metabolic dysfunction induced by genetic disorders may produce lysosomal storage of phospholipids, such as Niemann-Pick and Tay-Sachs diseases<sup>2</sup>. In addition, xenobiotic drugs and chemicals, as well as hormones, cofactors, and other agents, may alter the metabolism of the cell and result in PLDsis<sup>3</sup> and, in the case of drugs, give rise to the term drug-induced PLDsis.

Historically, Nelson and Fitzhugh<sup>4</sup> firstly reported the induction of foamy macrophages in rats by long-term treatment with chloroquine in 1948. At the time of the publication, however, it was not recognized that the response was a PLDsis because this phenomenon was unknown. Subsequent research has confirmed that the foamy macrophages develop as a result of the accumulation of phospholipids within the cell and are a common response to so-called cationic amphiphilic drugs (CADs)<sup>5,6</sup>. Since that time, over 50 marketed and experimental CADs have been reported to induce PLDsis following *in vitro* and *in vivo* administration and/or under *ex vivo* conditions. The development of PLDsis during preclinical testing in animals has become a problem for the pharmaceutical industry and can result in delay or discontinuation of new drug

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development.

The phospholipids are divided into various classes. The fatty acid composition of each class of phospholipids differs widely from one tissue to another within a given species. In contrast, the corresponding fatty acid composition in a given organ or tissue in one species is remarkably similar to that of the same tissue in a different species. This fact is important to keep in mind when evaluating results from experimental animal models. Usually, there are increased levels of several classes of phospholipids, although there may be a predominant accumulation of a single phospholipid.

In PLDsis the affected cells are enlarged with a pale foamy appearance. The cytoplasm is filled with myeloid bodies or membranous lattices on electron microscopic examination. Abnormalities in any kind of phospholipid metabolism are likely to lead to myelin body accumulation and the whorled membranes represent foci of damaged or aberrant membranes of the endoplasmic reticulum. These distinctive structures that appear in cells from subjects with PLDsis have been referred to various terms such as (lysosomal) lamellar bodies, lamellated cytoplasmic inclusions, multilamellar inclusion bodies, myelin like bodies and concentric lamellar inclusion bodies, but to avoid confusion, lamellar bodies will be used throughout this review. The mechanism responsible may be either stimulated uptake of phospholipids, which is rare, or inhibition of phospholipid degradation. The latter occurs by direct enzymatic inactivation (e.g., with gentamicin) or by the binding of drugs to lysosomal membrane phospholipids, rendering them resistant to degradation (e.g., chloroquine). The morphology and mechanism of PLDsis will be described in detail in the following sections.

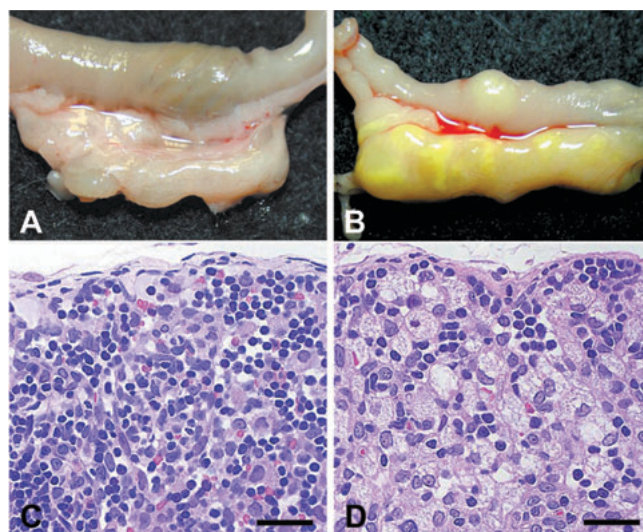
Thus, PLDsis is an old issue, and there have been numerous review articles that have described the biological features of the phenomena in detail<sup>3,7-18</sup>. However, PLDsis is still not well understood, particularly as to its toxicological significance nor are there biomarkers for the disorder. In 2006, the USA FDA outlined the form and function of the “FDA phospholipidosis Working Group” at a meeting of the Predictive Toxicology Discussion Group. The ultimate objective of the Working Group is development of a “Guidance on PLDsis”, and the FDA’s interests in this subject are in determining the incidence/prevalence of PLDsis (clinical and preclinical), understanding the toxicity implications of the phenomenon and identifying biomarker strategies<sup>19</sup>.

This review focuses on the recent advances in drug (CAD)-induced PLDsis, in particular, on possible biomarkers for PLDsis and methods of its prediction in drug development, including results obtained in our laboratories.

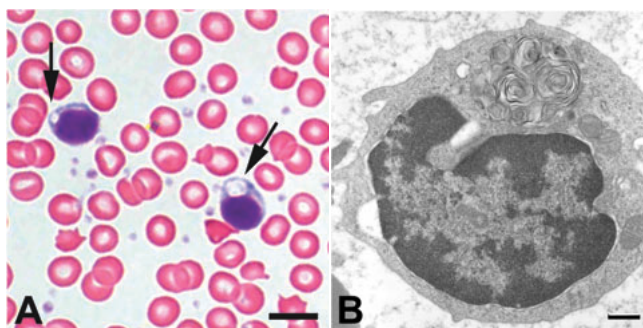
## Toxicological and Pathological Significance

PLDsis is induced by short-term or chronic administration of CADs. The induction time may be a few days to several months, depending on the affinity of CADs for susceptible cells. In cell cultures, phospholipids can

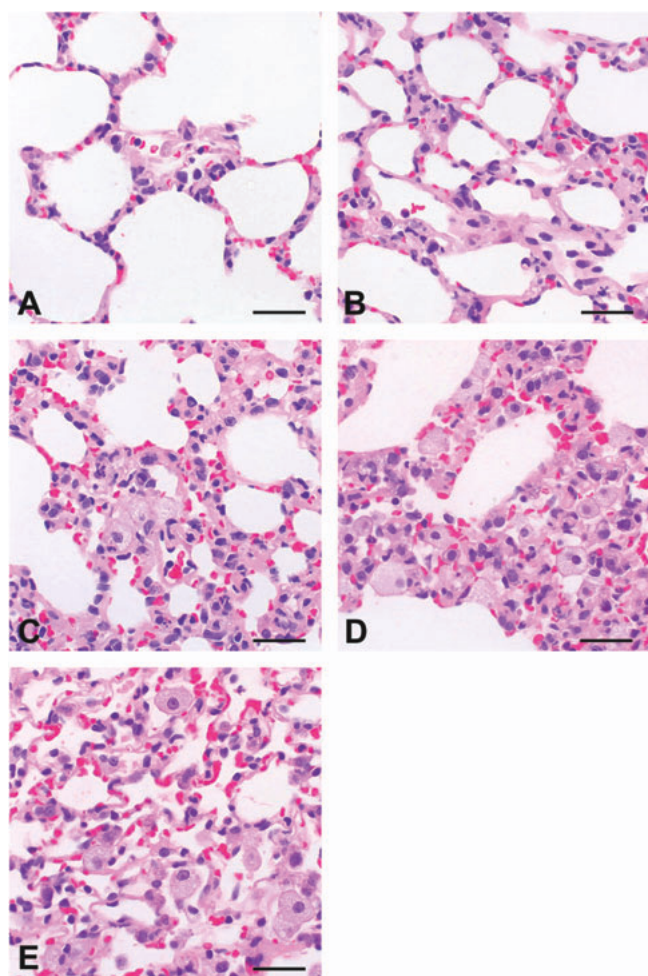
accumulate intracellularly and induce lysosomal lamellar body formation within only a few hours of exposure. In *in vivo* studies, the severity and type of drug-induced PLDsis depends on the characteristics of the causal drugs, the dosage levels, the exposure duration and the animal species used, etc. The morphological changes appear in almost all organs and tissues. In cases where a normally colored drug induces PLDsis, the organs and tissues involved in this phenomena also show the same color (Fig. 1) such that the distribution of the lesions in the whole body can be seen on gross examination. In addition, observations of peripheral blood smears from affected animals can often reveal cytoplasmic vacuolation of the lymphocytes (Fig. 2).



**Fig. 1.** Macroscopic appearance and histology of the mesenteric lymph node from rats treated with vehicle (A, C) and a GPCR antagonist for 2 weeks (B, D). In the treated animal, the mesenteric lymph nodes grossly show a yellowish color in the surface, which is the normal color of the chemical (B). In the cortical area of photo D, many macrophages and/or lymphocytes are enlarged and show a foamy appearance (D). H & E stain, Bar=30  $\mu$ m.



**Fig. 2.** Lymphocytes with PLDsis in a peripheral blood smear (A) of a rat treated with a GPCR antagonist for 2 weeks and an electron photomicrograph (B) of an affected lymphocyte. Arrows in photo A indicate vacuoles in the cytoplasm (May-Giemsa stain, Bar=10  $\mu$ m). Multi-centric lamella bodies are found in a lymphocyte in photo B (Bar = 500 nm).



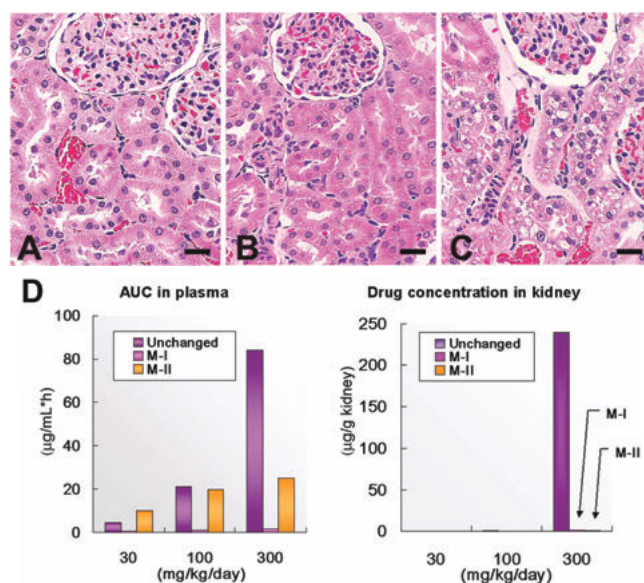
**Fig. 3.** Chronological changes of the lung from rats treated with a single dose of amiodarone (1000 mg/kg) after 6 hours (A), 1-day (B), 3-day (C), 7-day (D) and 14-day (E). Foam cell infiltration in the alveolar space or septum is evident after day 3 (C) and persists until day 14 (E). Tissue amiodarone concentration correlated with the extent and time-course of the histological changes (data not shown). H & E stain, Bar=30  $\mu$ m.

### Morphology

The morphology of PLDsis is characterized by increase and/or accumulation of cells with numerous small to large vacuoles or a foamy appearance of the cytoplasm in H.E. sections. The macrophages in the tissues are frequently affected in a generalized fashion and various parenchymal cells may be affected, too. The following are three typical patterns of lesion-distribution of PLDsis that we have encountered mainly in preclinical toxicity studies in rats:

#### (1) Macrophage-dominant PLDsis

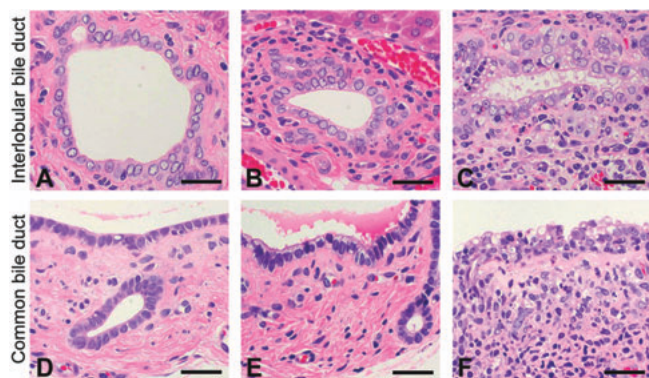
Changes in this pattern of PLDsis are initially manifested as infiltration/accumulation of foamy macrophages in the lung and lymph nodes (Figs. 1 and 3). Foamy alveolar macrophages lining beneath the pleural alveolar spaces are a good indication of treatment-related change while spontaneous alveolar macrophage accumulation tends to be focal and often occurs in angular



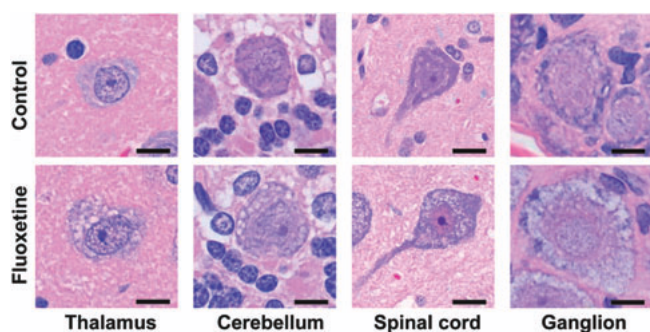
**Fig. 4.** Histology of the kidney from rats treated with vehicle (A), 100 and 300 mg/kg/day of an anti-rheumatism agent for 4 weeks (B, C). Vacuolation of the epithelial cells in the proximal tubules is evident at 300 mg/kg (C). H & E stain, Bar=30  $\mu$ m (D): AUC (left graph) of the unchanged agent and its metabolites, M-I and M-II, in plasma increased dose-dependently. The drug concentration (right) of the unchanged agent in the kidney was significantly elevated at 300 mg/kg, suggesting accumulation of the drug and/or phospholipids together in the kidney tissue.

parts of the pulmonary lobes. In advanced cases the foamy macrophages diffusely infiltrate and occupy the alveolar spaces and eosinophilic flocculent materials are seen in the air space in H.E. sections. Multi-focal accumulation may also be present, for example in the lymph nodes the increased numbers of foamy macrophages in the lymphatic sinuses correlate with the accumulation in the lung and the numbers of these cells filling the lymphatic sinuses correlate with the increase in dosage. The mesenteric lymph nodes are more frequently subject to phospholipidotic changes than the other lymphatic tissues, suggesting that some CADs which induce this pattern of PLDsis might first pass through the mesenteric lymph nodes. Tissue macrophages in the liver (i.e., the Kupffer cells), spleen, thymus and bone marrow are also affected. In almost all cases, as in the lung and lymph nodes, the macrophages in the affected tissues usually show a foamy appearance in the cytoplasm and tend to be enlarged and, *per se*, increased in number. In addition to the typical phospholipidotic foamy cells, some foamy cells which look like tangible-body macrophages and contain bluish granules may be seen. In amiodarone-induced PLDsis, large vacuoles are formed in foamy macrophages in the mesenteric lymph node and ultrastructurally, these vacuoles are unassociated with lysosomes and contain neutral fat suggesting mixed-lipid storage disorder. In advanced cases, foamy macrophages increase in the lamina propria in the gastrointestinal tract.





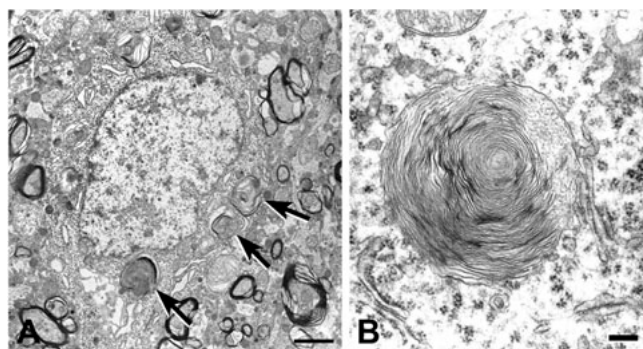
**Fig. 5.** Cytotoxic changes in the bile duct cells affected PLDsis. The interlobular and the common bile ducts from rats treated with vehicle (A, D), 30 and 100 mg/kg/day (B, E and C, F, respectively) of an anti-rheumatism agent for 2 weeks. Fine vacuoles are seen in the apical portion of the bile duct epithelium at 30 mg/kg (B, E). Vacuoles become larger at 100 mg/kg (C, F), and necrosis of the bile duct epithelium and inflammatory cell infiltration suggesting cytotoxic changes are seen. H & E stain, Bar=30  $\mu$ m.



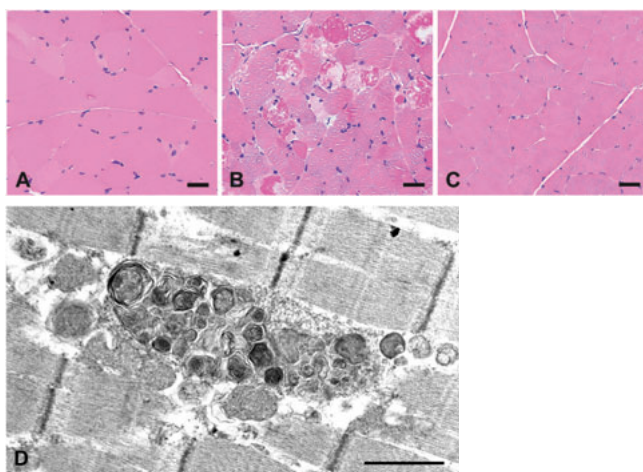
**Fig. 6.** Alterations related to PLDsis in the nervous system of rats treated with 50 mg/kg of fluoxetine for 2 weeks. The upper set of photos shows normal appearances in the controls and the lower set shows intra-cytoplasmic vacuoles suggesting PLDsis in the nerve cells at various sites. H & E stain, Bar=30  $\mu$ m.

## (2) Parenchymal cell-dominant PLDsis (generalized)

Parenchymal cells including hepatocytes, renal tubular epithelial cells (Fig. 4), bile ductal cells (Fig. 5), endocrine cells, striated and smooth muscle cells, vascular endothelial cells, nerve cells in the central and peripheral nervous systems (Fig. 6), epithelial cells in various organs may be affected. Macrophages are also affected to various degrees. The cellular distribution of phospholipid accumulation is usually associated with physiological lysosomal distribution. For example, fine to small vacuoles are prominent around the bile canaliculi between the hepatocytes and in the basal area of the renal tubular epithelial cells and the apical area in the bile ductal cells. In terms of tissue distribution of PLDsis, taking the liver as an instance, both centrilobular and periportal patterns can be found depending on the causal chemical. Some compounds may induce PLDsis in the



**Fig. 7.** Electron photomicrographs of the nerve cell from rats treated with fluoxetine. Photo A shows multiple lamellar bodies in a nerve cell in the thalamus (Bar=2  $\mu$ m). Photo B is a high power view of a lamellar body in the Purkinje cell (Bar=200 nm).



**Fig. 8.** Myopathy related to PLDsis and its reversibility. Photo A is femoral skeletal muscle from a control rat. Photo B is the same skeletal muscle from a rat treated with 300 mg/kg of an immuno-modulator for 3 weeks that shows focal degeneration/necrosis of the muscle fibers. Photo C is the same muscle tissue from a rat at 4-week recovery period after treatment. The muscle fibers show almost normal appearance (H & E stain, Bar=30  $\mu$ m). Photo D is an ultrastructural picture from the same animal as in photo B. Numerous lamella bodies are found in or between the degenerated muscle fibers. Bar=1  $\mu$ m.

convoluted part of the proximal renal tubules but others may induce it in the straight part. It is obvious that the consequences of parenchymal cells-dominant PLDsis are very important in safety evaluation, especially in correlation with the morphological changes, functional alterations and toxicity.

## (3) Localized PLDsis

This pattern of PLDsis is not major and considered to be a special type of parenchymal cell-dominant PLDsis. Bile ductal cells are one of the affected cell types in this pattern, although these cells are also potential targets in generalized PLDsis. Certain compounds can induce PLDsis in only the

bile ductal cells: numerous vacuoles are formed on the apical portion of cytoplasm in the bile ductal cells not only in those that are intra-hepatic but also those that are extra-hepatic, i.e., in the common bile duct. This predisposition to occur in the bile ductal cells may be explained by the exposure of the bile ductal cells to higher concentrations of test compounds than are other tissues when the chemicals are excreted via the bile. Renal PLDsis may also be classified into a type of localized PLDsis. Recently, we have encountered an interesting localized PLDsis showing vacuolization specifically in the nuclei of the nerve cells in the brain, though the precise mechanism is uncertain.

If the accumulation of phospholipids is extensive, it can be characterized biochemically<sup>15</sup>; however, morphological and ultrastructural changes are still the hallmark feature of the disorder<sup>20</sup>. When examined light microscopically, the cells may appear vacuolated as mentioned earlier, although this is not always evident. Ultrastructural examination is the most reliable tool for PLDsis diagnosis. The characteristic feature is the appearance of uni-centric or multi-centric lamellar bodies within the cells (Fig. 7). These lamella bodies can be solitary or exist as accumulated bodies encapsulated by the limiting membrane. These structures can also be observed as more electron-dense and non-lamellar condensed bodies or as autophagic vacuoles including cell organelles such as rough endoplasmic reticulum, distended smooth endoplasmic reticulum and glycogen granules. In addition, crystalline-like or reticular inclusion forms have also been reported<sup>15</sup>. Further, as extracellular lamella bodies, tubular myelin figures and/or membranous materials are sometimes found to be increased in the alveolar spaces or the lymphatic sinuses.

Thus, PLDsis can be diagnosed histologically by the above morphological characteristics, but cytoplasmic vacuoles induced by CAD are not always due to accumulation of phospholipids. To establish a definitive diagnosis, a specific staining method for phospholipids such as the Nile blue stain or the detection of inclusions in osmium-fixed and resin-embedded one-micron preparations is needed.

### Functional consequences

The biological consequences of phospholipid accumulation in several organs have not always been evaluated to the point where lysosomal phospholipid storage and/or consecutive lysosomal dysfunction could clearly be linked to cellular toxicity. However, *in vivo* and *in vitro* investigations have given insights into the mechanisms by which PLDsis could affect cellular functions, particularly in the lung. Lysosomal protein degradation, fusion abilities and pino- and endo-cytosis have been impaired after drug administration<sup>15</sup>. Alveolar macrophages have the ability to scavenge lamellar bodies which, when completely packed, create foamy macrophages during drug-induced PLDsis. In this process concentrations of the drugs can reach higher levels in the lamellar bodies. An enhanced phagocytic

activity in the pulmonary macrophages together with an increased production of bacterial oxygen radicals might imply a shift in the immune response towards an enhanced unspecific cellular response<sup>21,22</sup>. Indeed, the humoral immune response, in particular the delayed hypersensitivity response, the ability to activate antibody-secreting cells and the blastogenic response were significantly depressed after chlorophenthermine administration in animal models<sup>23</sup>. Based on this, the USA FDA has recommended macrophage function assays to assess the effects on the immune system when drugs produce PLDsis.

On the other hand, there is a prevailing theory that PLDsis is primarily an adaptive response to drug exposure rather than a toxic response. In other words, drug-induced PLDsis might be a part of a novel detoxification mechanism to protect the cell from xenobiotics. The central role of the lysosomes could lie in the activation of a cell survival strategy based on autophagy<sup>24</sup>. As long as the drugs are confined to the lysosomes, cells may be protected from toxicity. If so, inhibition of lysosomal enzyme activities could be a secondary effect. Thus, PLDsis could be part of a defense mechanism in which phospholipid recycle stacks accumulate with trapped toxic xenobiotics<sup>25</sup>. By releasing the lamellar bodies via exocytosis, the cell could avoid excessive oxidation and the production of reactive oxygen species, thereby preventing excessive stress. Cells of the macrophage system, such as pulmonary macrophages and hepatic Kupffer cells, scavenge lamellar bodies and facilitate their removal for further clearance of the lamellar body/xenobiotic complex. Enhanced lysosomal enzyme release and a dose-dependent clearance of drugs into the bile may be indicative of enhanced exocytotic activity<sup>26</sup>.

The secondary effects of increased cellular phospholipid, e.g., inhibition of protein synthesis, inhibition of lysosomal phospholipase (PL) C as well as impairment of protein kinase C, have been discussed elsewhere<sup>15</sup>. In fact neither PL inhibition nor phospholipid accumulation alone resulted in cell toxicity. As an example polyaspartic acid is transported to the lysosomes via endocytosis and builds complexes with the aminoglycosides gentamicin or amikacin. In such complexes aminoglycosides are stored in a non-toxic form and are prevented from interaction with phospholipids. Aminoglycosides display a broad range in their potency to induce apoptosis and interference with mitochondrial electron transport<sup>27</sup> as discussed in the next section.

### Cytotoxicity

Since elucidating the toxicological implication of PLDsis is important in order to conduct clinical trials of pharmaceutical candidates, careful histopathological attention should be paid to the cellular injuries associated with PLDsis. The role of PLDsis in cytotoxicity, however, remains speculative<sup>28</sup>. As of today, only a few cases of toxicity induced by CADs and directly linked to PLDsis have been reported: a  $\beta$ -blocker, propranolol, and a calcium-antagonist, verapamil, have been shown to decrease the

mitochondrial volume density associated with a significant dose-dependent lactate dehydrogenase (LDH) -release in hepatocytes at subtoxic concentrations<sup>29</sup>. Myopathy related to administration of CADs is another example and this has also been reported for several CADs including chloroquine, perhexiline, chlorphentermine, amiodarone, macrolide antibiotics, spinosad and dopamine D3 receptor antagonists<sup>30</sup>. Vacuoles of varying sizes were formed in myocytes from rats treated with an immuno-modulator for 3 weeks, as well as in other tissues affected by PLDis, and degeneration and necrosis developed (Fig. 8) and fragility of the lysosomes which could result in leakage of proteolytic enzymes into the cytoplasm and subsequent breakdown of the myofibrils has been considered as a cause. Once a drug exceeds certain concentrations such leakage of lysosomal enzymes into the cytosolic compartment is a likely event and might induce apoptosis<sup>31</sup>. As another instance, apoptosis, mitochondrial swelling and LDH-release during PLDis have been reported in the proximal renal tubular epithelium with treatment with aminoglycosides<sup>27</sup>. Aminoglycosides cause acute renal toxicity due to tubular necrosis. Several factors have been suggested to be responsible for this, such as mitochondrial enzyme inhibition and peroxidation of phospholipids<sup>27</sup>. Using U-937 cells, a human monocytic cell line, inhibition of cell proliferation and/or induction of apoptosis correlating with phospholipid accumulation were shown after 24 or 48 hours exposure to amiodarone and imipramine<sup>32</sup>. Although disruption of the lysosomes after phospholipid accumulation might induce the activation of caspases 2 and 3 and apoptosis, precise mechanisms of the induction of apoptosis and the *in vivo* relevance of the changes needs to be further investigated.

### Carcinogenicity

Inflammatory and degenerative changes as consequences of sustained PLDis have been often seen in chronic and long-term animal studies, but no PLDis related carcinogenicity has been known, although few reports on carcinogenicity of PLDis-inducing chemicals are available. An antidepressant, fluoxetine, showed no evidence of carcinogenicity in F344 rat and B6C3F1 mouse in 2-year carcinogenicity studies<sup>33</sup>. In addition, F344 rats given spinosad, an insecticide, at the MTD in the diet for 24 months showed PLDis and necrosis in the thyroids and inflammation in the lung and thyroids, but there were no neoplasms that were interpreted to be treatment related<sup>34</sup>. Amiodarone, an iodine-rich drug used for treatment of cardiac tachyarrhythmias, could cause overt thyroid dysfunction and may have a non-genotoxic carcinogenic potential for the thyroid; however, no carcinogenicity studies have been conducted yet. We have experience of an anti-androgenic compound which causes degenerative changes other than PLDis in endocrine systems in repeated toxicity studies in rats, but there has been no induction in any tumor in conventional rodent 2-year carcinogenicity studies.

### Reversibility

It is important that upon termination of drug administration or exposure, PLDis is generally reversible with the drug effluxing from the cell, the phospholipid levels returning to normal and morphological changes diminishing or disappearing<sup>35–40</sup>. The time course of the reversal is dependent on the dissociation rate of a CAD from the phospholipid and on the elimination rate of the CAD from the tissue, through the accumulation and efflux of active metabolites must also be considered<sup>38,41</sup>. In animal studies, reversal occurs within weeks to a few months depending on these factors. In our studies on an immuno-modulator, myopathy found in rat skeletal muscles after 4 weeks repeated dosing disappeared after a 4-week recovery period (Fig. 8). Although in general, reversibility can take several months or more in humans<sup>42–46</sup>, it is not possible to generalize the timeframe for reversibility of PLDis in humans because of the limited information available<sup>16</sup>.

### Possible Mechanisms

The accumulation of phospholipids may occur by several mechanisms depending on each CAD. There is consistent evidence that drug accumulation occurs within the lamellar bodies, i.e., the lysosomes, and that such drugs inhibit lysosomal PL activities<sup>21,47–49</sup>. Basically two hypotheses may be considered to explain the underlying mechanisms of drug-induced PLDis. The first hypothesis assumes that CADs bind directly to phospholipids to result in indigestible drug-lipid complexes, which accumulate and are stored in the form of lysosomal lamellar bodies<sup>13</sup>. The second hypothesis is based on the observation that production of lamellar bodies has been associated with the inhibition of PL activities; either due to direct inhibition, or the interaction of CADs, at the phospholipid bilayer of the lysosome<sup>15,42</sup>.

Lysosomal PLs play a critical role in degradation of the cellular membranes. Chlorpromazine, amiodarone and chloroquine inhibited PL A1, calcium-dependent and independent PL A2, and calcium-dependent PL C activities in a dose-dependent fashion<sup>1</sup>. Furthermore, chlorpromazine, propranolol and chloroquine were also found to inhibit PL C activity by direct binding to the enzyme. The affinity for PL A2 and its competitive inhibition have been variable among different amphiphilic substances<sup>3</sup>. These amphiphilic drugs have modulated the activities of other lysosomal enzymes including cathepsin B protease<sup>50</sup>. Enhanced activities of the lysosomal hexosaminidase, acid sphingomyelinase and sulfatase B in cells of the kidney cortex are considered to be due to a compensatory increase in degrading enzymes due to PL inhibition<sup>51</sup>. Interestingly, decreased sphingomyelinase activity was reported for cells of the renal cortex after gentamicin application<sup>52</sup>. Furthermore, chlorphentermine and gentamicin were shown to inhibit PL C. While gentamicin inhibited the phosphatidylcholine- and phosphatidylserin- specific variants of PL C, chlorphentermine inhibited the phosphatidylinositol specific

PL C<sup>53</sup>.

Another possible mechanism for the interference of CADs with PL activity has been proposed by Mingeot-Leclercq and co-workers, who have raised the negative charge hypothesis, by which PL A1 inhibition results from charge neutralization of the lysosomal phospholipid bilayer<sup>53</sup>. This assumption was supported by studies with an erythromycin A derivative, azithromycin and gentamicin<sup>49</sup>. Recently, Piret *et al.*<sup>55</sup> reported that the activity of PL A1 is, in fact, influenced by the charged contents of the phospholipid bilayer. Increasing concentrations of negatively-charged lipids markedly enhanced the activity of PL A1. These findings are in support of the neutral charge hypothesis. Furthermore, secondary inhibition of PL A1 could result from the accumulation of lysosomal phospholipids that influence the charge on the lysosomal bilayer in favor of neutral lipids.

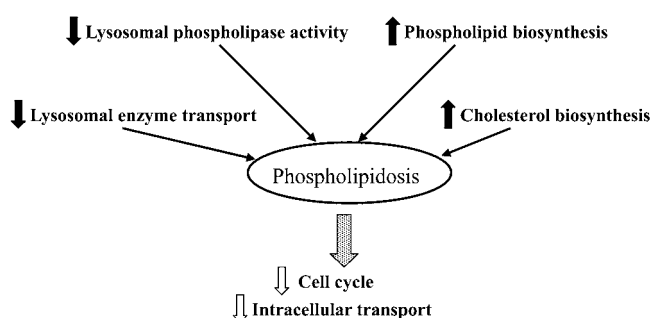
In addition, Sawada *et al.* have reported a hypothetical mechanism for drug-induced PLDsis based on their toxicogenomic approach, in which they showed up- and/or down-regulation of genes related to phospholipid and cholesterol biosynthesis, lysosomal PL activity and enzyme transport<sup>56</sup> (see the later *Toxicogenomics* section). Their hypothetical mechanism is shown in Fig. 9.

### Prediction of PLDsis in Drug Development

In new drug development, prediction of potential toxicity induced by a new drug candidate is generally first conducted using *in silico* methods, e.g., a structure-activity-relationship (SAR) analysis and a bioinformatic method such reverse pharmac/toxicogenomics. At the same time or after that, *in vitro*, *ex vivo* and/or *in vivo* assays will be carried out to detect any toxicity including the PLDsis induction potential of the compound, and then a “Go or No Go” decision for the candidate will be made based on these results, in addition to pharmacological potential, ADME data, chemical characteristics, and so on.

#### Chemical structures

Numerous drugs and chemicals are capable of inducing PLDsis in cells under conditions of *in vitro* and *in vivo* administration or *ex vivo* conditions. It is noteworthy that they do share several common physicochemical similarities despite the diverse pharmacological activities, therapeutic indications, diversity of tissue selectivity, and distinct manifestations of PLDsis that each of those compounds can induce in different species of animals. The physicochemical properties most commonly shared by phospholipidogenic compounds are a hydrophobic domain consisting of an aromatic ring structure, which may be substituted with halogen moieties, and a hydrophilic side chain consisting of aliphatic secondary or tertiary amino groups positively charged at physiological pH. These two structural properties provide the amphiphilicity that is common to these drugs and chemicals and therefore they are identified as cationic amphiphilic amines (i.e., CADs)<sup>3,57</sup>. Thus, the



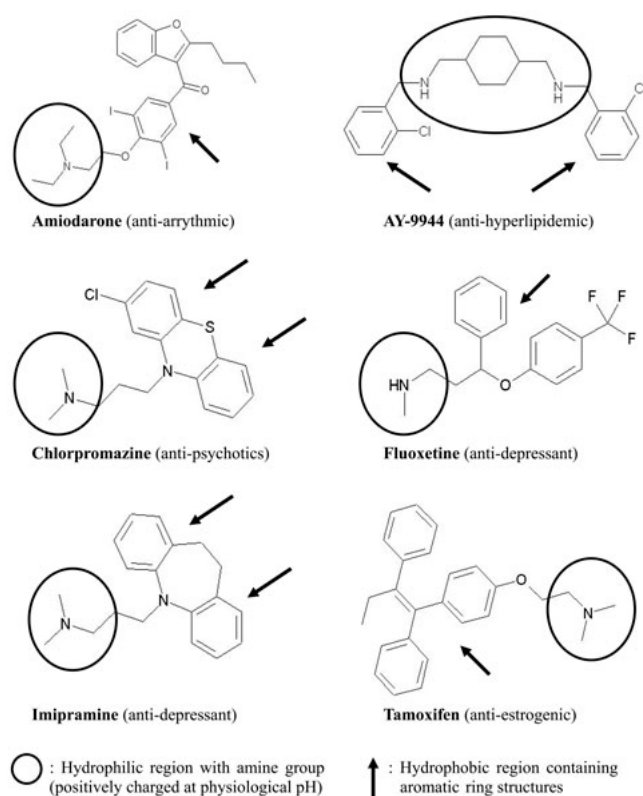
**Fig. 9.** Hypothetical mechanism of drug-induced PLDsis proposed by Sawada *et al.*<sup>56</sup> They suggest that the inhibition of lysosomal phospholipase activity and enzyme transport, and promotion of phospholipid and cholesterol synthesis (solid thick arrows) are involved in the induction of PLDsis based on data from HepG2 *in vitro* studies (see Tables 2 and 3). In addition, they speculate that cell proliferation and intracellular transport (open thick arrows) may be secondarily inhibited in this condition by increased intracellular phospholipids predicated on data from rat 3-day and 7-day studies.

phospholipidogenic potential of a compound is dependent, in part, on its physicochemical characteristics.

On the other hand, CADs appear to make “drug-like drugs” because their amphiphilic characteristics allow them to cross biological membranes easily, in particular the blood-brain barrier. The hydrophobic structure enhances the molecular ability to pass through or to penetrate plasma membranes or when CADs are not ionized<sup>58,59</sup>. The ionized form of the molecule tends to remain with the membranes and contributes to membranous changes<sup>3,57,60</sup>. Moreover, the hydrophobicity of these molecules favors their interaction with membrane receptors. The positive nature of CADs can also influence the movement of sodium or calcium ions across cell membranes, thus affecting receptor-mediated reactions. For all these reasons a number of CADs are marketed as drugs. Examples include antimalarials, antibacterials, antidepressants, antipsychotics, antiarrhythmics, antianginals and cholesterol-lowering agents<sup>61</sup>. Apparent exceptions are the aminoglycoside, aminocyclitol and macrolide antibiotics, which are cationic, but which lack the typical hydrophobic moiety. The structures of a typical CAD, amiodarone, and other representative CADs are shown in Fig. 10.

#### Calculations of hydrophobicity/hydrophilicity of compounds

Physicochemical calculations determine the hydrophobicity/hydrophilicity of compounds based on octanol/water partitioning (logP/logD), and the ionization constant of the protonatable amines (pKa) as a measure of basicity<sup>62,63</sup>. Therefore, to avoid PLDsis compounds would need decreased lipophilicity and basicity (i.e., logP and pKa) in their medicinal chemistry. In fact, Peters *et al.* could overcome PLDsis using such predictive physicochemical calculations<sup>62</sup>. Most recently, Pelletier *et al.* have improved



**Fig. 10.** Chemical structures of representative cationic amphiphilic drugs. Circles indicate the hydrophilic region with amine group, and arrows indicate the hydrophobic region containing aromatic ring structures (positively charged at physiological pH) of each compound.

the predictive ability of the published model rules and constructed a novel Bayesian model<sup>64</sup>. On the other hand, to improve the pharmacological activity of drug candidates increased lipophilicity and, frequently, an amine (aliphatic) moiety would be required as mentioned above. Such calculations provide yet some problems in actual practice. For example, the calculations might yield misleading information, particularly in the case of more complex molecules, and the actual measurement for these properties can be more problematic. The calculated properties will also miss the contribution of metabolites and do not consider the biological property of drug accumulation.

Whether CADs or not, there have probably been many more chemicals that have caused PLDis in the preclinical or developmental stages, but which have not been reported publicly. As mentioned earlier, one of the goals of the USA FDA Task Force is to create a compound database, to predict the possibility of PLDis *in silico* using the database and to reduce attrition in development programs due to PLDis-related toxicity.

## Detection of, and Biomarkers for, PLDis in Drug Development

Due to the uncertainty of whether or not PLDis can be

linked to a toxic response, there is a need to monitor for the presence of PLDis in animals and clinical trial subjects during the preclinical and clinical phases of drug development. Therefore, there has been considerable effort devoted to identifying biomarkers which could be diagnostic for the presence of this condition. In addition, although there is no clear link to toxicity, a novel screening method for detecting the phospholipidogenic potential of candidate compounds prior to candidate selection would provide an advantage during drug development programs. If valid and reliable biomarkers for PLDis were found, they could also provide useful parameters to monitor the induction of PLDis both in the preclinical and the clinical phases. An ideal and suitable biomarker for detecting not only PLDis, but also any related alterations, would need: 1) to be present in an easily accessible compartment such as blood or urine (to access it invasively) 2) to change consistently and quantitatively in correlation with the extent of the condition regardless of the target tissue 3) to have an adequate half-life, so that it could serve as an index of any effects over an extended prior to time 4) to utilize reliable and convenient analytical methods for measurement 5) to be specific for the exposure and 6) give an accurate indication of the health risks of exposure. Due to the variation in tissue and species responses to CADs, efforts have not, so far, identified such a valid and ideal biomarker. However, most recently, potential biomarkers for the detection of, and to monitor for, PLDis have been reported, especially the measurement of an unusual lysosomal metabolite, lyso-bis-phosphatidic acid (LBPA), and methods using metabolomic and toxicogenomic technology as described later<sup>52,56,65</sup>.

## Traditional and conventional approaches to early detection of PLDis

There are several methods that have been used to detect PLDis in toxicology, e.g., 1) conventional histopathology and ultrastructural examinations in toxicity studies (single-dose and repeated-dose) and in blood smear observations, 2) alveolar lavage fluid examinations (macrophage observations and measurements of phospholipids in the lavage), 3) *in vitro* screening using human liver beads, HepG2 cells, lymphocytes, fibroblasts, and so on. One to 3 day-preliminary toxicity studies can also be used as practical screening tests in the early stage of new drug development. In addition, flow cytometry and morphological assays using fluorescent probes and biochemical approaches have been also applied such as measurement of phospholipids in blood and tissues/organs and inhibition assays of lysosomal PL activities *in vitro*. Most recently, by using PL A2-deficient mice, which were derived by the systemic deletion of exon 5 of the *Lpla2* gene, it has been demonstrated that PL A2 is one of the key enzyme contributing to PLDis induction, at least in the lung<sup>66</sup>. We measured the inhibitory potential of 33 chemicals including CADs on the activity of rat liver lysosomal PL A1, A2 and C (IC<sub>50</sub>) *in vitro* and examined whether the intensity of inhibition of PL activities was correlated with the occurrence of pulmonary and/or hepatic



**Table 1.** Summary of Correlations between Phospholipase A1 Inhibition (IC50) and Phospholipidosis Induction in Various Compounds

PLDsis*	Phospholipase A1 Inhibition (IC50, $\mu\text{mol/L}$ )			(Subtotal)
	<30	30–300	>300	
Occurred	18 <sup>1)</sup>	3 <sup>2)</sup>	1 <sup>3)</sup>	(22)
Not occurred	1 <sup>4)</sup>	4	6 <sup>5)</sup>	(11)
Total No. of Compounds				33

\*: PLDsis in the liver and lung in rats.

1) Amiodarone, clomipramine, fluoxetine, tamoxifene and perhexiline are included in this category.

2) Quinacrine and imipramine are assigned in this category.

3) Amantadine is the only exception that induces PLDsis among chemicals having a higher IC50 value (more than 300  $\mu\text{mol/L}$ ).

4) Chlorpromazine is a false positive compound in this study, although this has been reported that can induce PLDsis.

5) Although chloroquine has been known to induce ocular PLDsis, the compound is assigned in this category in this study.

PLDsis detectable on histology. To summarize, there seemed to be a relatively good correlation between them in PL A1 (Table 1), however, due to the large amount of the chemicals needed for the assays and the higher cost, the measurement of the inhibition rates of these enzyme activities was not considered to be practical as a first-stage screening method. However, the measurement of the inhibition of PL activities will provide valuable information in our consideration of mechanisms of PLDsis.

Several studies have focused on the development of fluorescence screening methods to detect and stratify the PLDsis potential for drug candidates. A cell culture-based fluorescence assay using 1-acyl-2-(12[(7-nitro-2-1-,3-benzoxadiazol-4-yl)amino]dodecanoyl (NBD)-labeled phospholipids was developed by Ulrich *et al.*<sup>67</sup> in 1991 and subsequently used to study the effects of benzamides<sup>68</sup>, amiodarone<sup>69</sup> and matrix metalloproteinase inhibitors<sup>40</sup> on cultured hepatocytes. This cellular NBD-phospholipid assay has been adapted into a high-throughput format<sup>70</sup> and the fluorescent probe, Nile red, has been utilized to assess PLDsis<sup>71,72</sup>. Nile red fluorescence is directly dependent on the hydrophobicity of drugs and distributes into the intracellular phospholipids induced by CADs. Halstead *et al.* used flow cytometry to assess the uptake of Nile red by peripheral leukocytes from rats treated with maprotiline, imipramine, tilorone and amikacin. The accumulation of Nile red correlated well with the ultrastructural lamellar bodies in the cells and the phospholipid levels in the liver for maprotiline and tilorone.

Among these traditional and conventional approaches electron microscopic examinations to detect lamellar bodies has been thought the most reliable and definitive method for identifying phospholipidotic cell damages, but its use is impractical as a rapid screening tool as mentioned in the next section in details.

### Lamellar bodies

Currently, the most definitive biomarker is the presence of the characteristic lamellar bodies in cells as this is one of the hallmark features of the condition. Light microscopic examination of cells and tissues can identify abnormal

accumulations of lipids, but is not sensitive enough to detect the ultrastructural features. Hence examination by transmission electron microscopy (TEM) is required. Peripheral blood cells have been suggested as a biomarker for the development of PLDsis in animals and humans<sup>7,72,73</sup>. These cells are easily removed by lysis allowing examination of lymphocytes, monocytes and granulocytes. For example, Lüllmann<sup>7</sup>, Fedorko<sup>74</sup> and Dake *et al.*<sup>75</sup> reported the presence of lamellar bodies in lymphocytes from humans and animals. Such examination of the peripheral blood cells is technically feasible because of the relative ease of obtaining samples. Examination of other tissues is problematic because of the need for more invasive procedures. Electron microscopic evaluation is time consuming, expensive and non-quantitative and such an approach is not feasible for large numbers of samples in clinical studies. In addition, it is possible that PLDsis will not be present in peripheral blood cells when it is present elsewhere in the body; thus the potential for false negatives will be of concern. Further, there is one study that showed that lamellar bodies in lymphocytes can be observed in 10% of control subjects and the authors pointed out the need to have accurate histories from the subjects enrolled in such a study<sup>76</sup>. Nevertheless, even with all of the problems described, it appears that for fuller evaluation of the peripheral blood cells as a potential biomarker more CADs need to be tested in the manner reported and evaluated in humans when possible by Halstead *et al.*<sup>72</sup>.

### Specific metabolites

Some investigations have focused on unusual metabolites of biological molecules as biomarkers for drug-induced PLDsis. One unusual metabolite, a lysosomal phospholipid, lyso-*bis*-phosphatidic acid (LBPA), also known as *bis* (monoglycerol) phosphate, has been noted in studies on humans and animals to increase markedly in tissues with PLDsis. From the 1970s to early in the 1980s, the appearance of this unusual metabolite in the tissues of rats or humans treated with 4,4'-diethylaminoethoxyhexestrol or chloroquine was reported by several researchers by Halstead *et al.*<sup>77–80</sup>. A liquid

chromatography-coupled electrospray ionization-mass spectrometry/mass spectrometry method was employed for quantification of LBPA and it was suggested that this was a high-throughput method that could easily be transferred to the clinic for the analysis of samples from subjects. These authors postulated that LBPA might increase because of increased delivery of phospholipids to the lysosomes during the development of PLDsis coupled with decreased catabolism of phospholipids. The high levels of phosphatidylinositol that occur in the lysosomes during this condition may be responsible, in part, for the increased concentrations of LBPA because it has been identified as an acyl donor in the synthesis of LBPA<sup>80</sup>. Studies on trospectomycin also revealed hepatic accumulation of LBPA along with another unusual lysosomal phospholipid, acylphosphatidylglycerol, and increases in phosphatidylcholine and phosphatidylinositol<sup>81</sup>. LBPA was shown to increase not only in the tissues but also in the sera from rats treated with amiodarone<sup>61</sup>. This means that measurements of serum LBPA could become a useful tool for monitoring PLDsis in humans. Taken together, further evaluation of LBPA should be continued to get evidence of its value as a potential biomarker for drug-induced PLDsis. Thus, although the use of metabonomics (or metabolomics)<sup>82</sup> in the investigation of biomarkers of drug-induced PLDsis is still in its infancy, this approach has the potential to identify useful biomarkers for PLDsis (see the following *Metabonomics*).

### Metabonomics

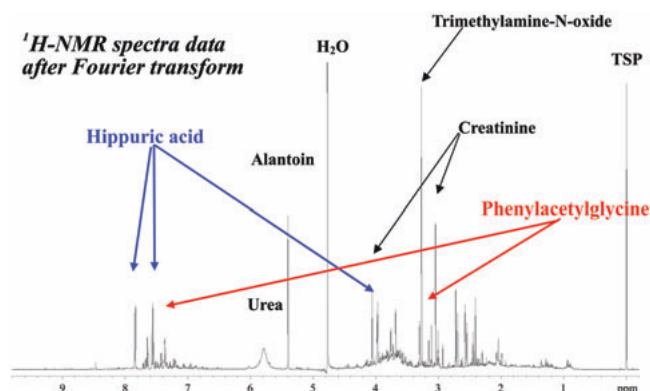
Metabonomics is an emerging technology that enables rapid *in vivo* screening for toxicity, disease state, or drug efficacy. The technology combines the power of high-resolution nuclear magnetic resonance (NMR) techniques with statistical data analysis methods to rapidly evaluate the metabolic status of an animal. Using NMR-based metabonomics techniques, urinary and plasma phenylacetylglutamine (PAG) has been evaluated to identify biomarkers for PLDsis<sup>83–86</sup>. Although all the results indicated a perturbation in PAG levels with the induction of PLDsis, the results have been equivocal even using PAG:creatinine ratios. In fact, levels of PAG can change solely as a result of altered food consumption. Furthermore, the mechanistic link between the induction of PLDsis and changes in PAG is unclear. If properly controlled, the application of PAG as a biomarker is limited to animals because this metabolite is not produced in humans.

More recently, Horinouchi *et al.*<sup>65</sup> have carried out an investigation to identify potential surrogate markers for drug-induced PLDsis in urinary samples of CAD-treated rats using NMR techniques, like those conducted by Nicholls *et al.*, described above. The authors, however, paid attention to not only PAG itself, but also the ratio of PAG and other phenylalanine-related metabolites. In their experiments, 6 typical CADs known to induce PLDsis were administered orally to rats at 3 dosage levels for 3 consecutive days. After the final dosing, urine samples were collected in cooled

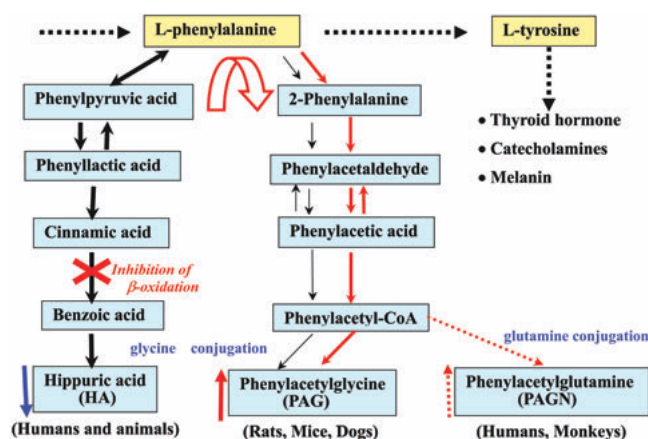
plastic bottles from the treated rats and age-matched controls for 6 hours and metabolites analysis was carried out by NMR techniques (Fig. 11-1). Induction of PLDsis was confirmed by measurement of the vacuolated lymphocyte ratio in blood smears, and by histopathological examination of the following organs: liver, lung, mesenteric lymph nodes, spleen, kidney and brain. Vacuolated lymphocyte ratios increased with increasing dose levels in all CAD-treated rats. Histological changes related to PLDsis were noted in one or more organs from all the CAD-treated rats. In the NMR analysis, increased PAG levels and decreased hippuric acid (HA) levels were observed with dose-dependency among all the CAD-treated rats. An increase in the ratio of PAG/HA, which is a measure of two metabolites derived from the essential amino acid, phenylalanine, may be a good index for PLDsis. Further, all the changes in the urine were also observed in the plasma from rats treated with amiodarone with a good correlation ( $R^2=0.8999$ ). The possible mechanism for the increased ratio is shown in Figure 11-2. A metabolic shift in L-phenylalanine from HA dominant to PAG dominant, probably due to inhibition of beta-oxidation, most likely contributed to this change. Thus, an increased ratio of PAG/HA, which reflects an altered phenylalanine metabolism, may be a useful surrogate marker for PLDsis at least in animal studies.

### Toxicogenomics

A toxicogenomic approach to detect the PLDsis-inducing potential of new drug candidates has been reported by Sawada *et al.*<sup>56</sup>, recently. They performed a large-scale gene expression analysis using DNA microarrays on human hepatoma HepG2 cells after treatment with 12 compounds known to induce PLDsis. The authors tried to 1) understand the molecular mechanisms of CAD-induced PLDsis and 2) obtain specific gene markers for *in vitro* screening tests or the prediction of the PLDsis-inducing potential of drug candidates. Ultrastructural and flow cytometric analyses revealed the formation of lamellar myelin-like bodies in the lysosomes and increased levels of phospholipids, respectively, in the HepG2 cells after treatment with each of the 12 compounds for 72 hours. In the DNA microarray analysis at 6 and 24 hours in the studies, alterations of gene expression reflecting the inhibition of lysosomal PL activity and lysosomal enzyme transport, and the induction of phospholipid and cholesterol biosynthesis were observed (Tables 2 and 3). In addition, 17 genes that showed similar expression profiles following treatment were selected as candidate markers for PLDsis detection. Furthermore, real-time PCR and pathological analysis confirmed that 12 gene markers showed significant concordance with the lamellar body formation observed by transmission electron microscopy. The degree of the average change in the values of these markers correlated well with the magnitude of the morphological changes. Functional annotation and categorization of these genes that were up- or down-regulated by at least 6 compounds could lead to them consider mechanisms for CAD-induced PLDsis and it is



**Fig. 11-1.** Metabonomics analysis on normal rat urine. Urinary samples were collected in cooled plastic bottles from normal rats that treated with physiological saline for 3 consecutive days. Metabonomics analyses of urine samples were carried out by NMR techniques. Original NMR data obtained are transformed by the Fourier transform method to spectra data (overview). These NMR spectra data show quantitatively main components in rat urine samples including hippuric acid (blue arrows) and phenylacetyl-glycine (red arrows), a main metabolite of L-phenylalanine and a minor one, respectively.



**Fig. 11-2.** Possible shift of surplus L-phenylalanine metabolism in phospholipidotic conditions. L-phenylalanine is one of amino acids utilized in protein synthesis and also metabolized by hydroxylation to L-tyrosine that is the mother source of thyroid hormone, catecholamines and melanin. Under physiological conditions surplus L-phenylalanine is metabolized through both the left major (thick black arrows) and right minor (thin black arrows) pathways showed in the figure. However, when the major pathway is disrupted for some reason in rodents, for example inhibition of  $\beta$ -oxidation (red cross mark), a shift in the metabolism of L-phenylalanine will occur (open red arrow), that is, the usual minor pathways will become the major pathway (red arrows). As a result, the terminal metabolite phenylacetyl-glycine (PAG) is increased and hippuric acid (HA) is decreased. Thus, the PAG/HA ratio in urine will increase in phospholipidotic conditions in rodents. In monkeys, and probably in humans, phenylacetylglutamine will increase instead of PAG in phospholipidosis (unpublished data).

hypothesized that four processes are involved in the induction of PLDsis under these conditions: 1) inhibition of lysosomal phospholipase activity, 2) inhibition of lysosomal enzyme transport, 3) enhanced phospholipid biosynthesis, and 4) enhanced cholesterol synthesis (Fig. 9). Using this platform, the authors claim this approach could be used as a rapid and sensitive screening test for drug-induced PLDsis.

Another toxicogenomic study on gentamicin conducted by Amin *et al.*<sup>87</sup> also examined gene expression changes associated with gentamicin treatment, but focused on the identification of toxicity markers and not biomarkers for PLDsis. Several studies have been reported using toxicogenomic techniques to examine tissue responses to toxicants<sup>88-90</sup>. Thus, now this technology is readily available it will be used more for this field of research in the near future.

## Risk Assessment and Conclusion

There are many marketed drugs that are categorized as CADs and associated with inducing PLDsis in experimental animals and, in some instances, in humans. The ability to induce PLDsis is principally dependent on the cationic, amphiphilic structure of the molecule or metabolites and not on their pharmacological activity. The interaction with membrane function and the inhibition of lysosomal phospholipase activities such as PL A1, PL A2 and PL C, play important roles in PLDsis development, and the characteristics of a drug's metabolism, its binding affinity to phospholipids and its lysosomotropic properties may also be determinants in the induction of PLDsis. These alterations may be also observed during new drug development and could raise concerns regarding drug safety for the regulatory authorities and pharmaceutical companies. Therefore, attention should first be paid to the chemical structures of drugs, as this can be important in predicting their potential to induce PLDsis.

Despite the impressive morphological features associated with PLDsis (i.e., the lamellar bodies), the toxicological significance of and mechanisms for PLDsis are not well understood. PLDsis in some cases could be simply a physiological response to intracellular accumulation of drugs with no associated adverse effects. In other cases, however, induction of the lamellar bodies could precede a decrease in cell function and result in cell death. Essentially, a depot effect could result in the slow release of drugs and the alteration could either enhance or reduce the toxicity of the trapped drugs. Further, PLDsis is usually reversible after drug administration or exposure is terminated, and the drug effluxes from the cell. The phospholipid levels may return to normal and the ultrastructural changes will diminish or disappear.

Although a number of studies have been published in this field, there is no conclusive evidence to directly link the presence of drug-induced PLDsis to impaired cell or tissue function. Efforts have been directed at evaluating functional consequences of drug-induced PLDsis. Tissue accumulation

**Table 2.** Category/Function of Up-Regulated PLDis-Related Representative Genes\*

Category/Function	Gene Title	Number of compounds that up-regulated	
		6 hours	24 hours
Lipid metabolism/phospholipid degeneration	N-acylsphingosine aminohydrolase (acid ceramidase) 1	0	7
	Sphingomyelin phosphodiesterase, acid-like 3A	3	6
	Hypothetical protein MGC4171	0	7
Lipid metabolism/cholesterol biosynthesis	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble)	10	4
	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase	11	5
	Squalene epoxidase	7	3
	Lanosterol synthase	4	6
	7-Dehydrocholesterol reductase	4	6
Lipid metabolism/regulation of cholesterol metabolism	Nuclear receptor subfamily 0, group B, member 2	0	8
Lipid metabolism/cholesterol metabolism	Lipase A, lysosomalacid, cholesterol esterase	0	6
Lipid metabolism/fatty acid biosynthesis	ELOVL family member 6, elongation of long chain fatty acids Stearoyl-CoA desaturase	5	6
Lipid metabolism/fatty acid alpha-oxidation	Phytanoyl-CoA desaturase	8	7
Lipid metabolism/fatty acid transport	Fatty acid binding protein 1, liver	0	9
Lipid metabolism/lysosome enzyme	Ceroid-lipofuscinosis, neuronal 2, late infantile	0	6
Cell cycle, proliferation, death	Prostate differentiation factor	2	9
	Activin beta A	5	8
	p8 protein (candidate of metastasis 1)	7	7
	Chromosome 20 open reading frame 97	6	6
	connective tissue growth factor	0	6
	Granulin	0	6
	Insulin-like growth factorbinding protein 1	3	6
	Optineurin	0	6
	B-cell translocation gene 1, anti-proliferative	9	3
	Cyclin G2	8	3
Transport	Solute carrier family 7, (cationic amino acid transporter, y+ system) number 11	8	6
	Solute carrier organic anion transporter family, number 4C1	4	6
	Syntaxin 3A	1	7
	Transient receptor potential cation channel, subfamily V, number 2	1	6
	Transthyretin, (prealbumin, amyloidosis type I)	0	8
Proteolysis and peptidolysis	Calpain 3, (p94)	0	6
	Coagulation factor VII (serum prothrombin conversion accelerator)	0	7
	Hepsin (transmembrane protease, serine 1)	0	10
	Protease, serin, 8 (prostasin)	1	7
Endopeptidase inhibition	Pre-alpha (globulin) inhibitor, H3 polypeptide	0	7
	Serine (or cysteine) protease inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), number 3	0	8
	Serine protease inhibitor, Kazal type 1	0	7
Miscellaneous	Asparagine synthetase	0	8
	Argininosuccinate synthetase	0	7
	Yippee protein	2	7
	Deiodinase, iodothyronine, type I	4	6
	Interleukin 6 receptor	2	6
	J domain containing protein 1	3	6
	Lipin 1	8	4
	S100 calcium binding protein P	0	6
	Spondin 2, extracellular matrix protein	0	6
	Sulphotranspharase family, cytosolic, 2A, dehydroepiandrosterone (DHEA) – preferring, number 1	0	7
	Transmembrane 7 superfamily member 2	0	6



**Table 2.** Continued

Category/Function	Gene Title	Number of compounds that up-regulated	
		6 hours	24 hours
Unknown/EST	Chromosome 10 open reading frame 10	4	9
	Chromosome 22 open reading frame 20	6	5
	Delta-like 1 homolog (Drosophila)	0	6
	Hypothetical protein FLJ10055	3	7
	Likely ortholog of mouse fibronectin type III repeat containing protein 1	2	9
	KIAA1199	0	6
	Hepatocellular carcinoma-associated gene TD26	6	6
	Hypothetical protein MGC13024	2	7
	Hypothetical protein MGC2650	0	7
	HIF-1 responsive RTP801	7	4

(Modified with permission from Ref. 58, Table 4; Sawada *et al.* 2005)

\* Category/function and gene title are grouped by each probe set obtained from the NetAffx and HumanPSD databases. The two columns on the far right indicate the number of compounds out of 12 that induced PLDsis and up-regulated genes in HepG2 cells after 6 or 24 hours of treatment.

**Table 3.** Category/Function of Down-Regulated PLDsis-Related Representative Genes\*

Category/Function	Gene Title	Number of compounds that down-regulated	
		6 hours	24 hours
Transport	Adaptor-related protein complex 1, sigma 1 subunit	0	7
	Solute carrier family 2 8(facilitated glucose transporter) member 3	6	12
	Solute carrier family 2 8(facilitated glucose transporter) member 14	8	11
Cell cycle, proliferation, death	Immediate early response 3	6	3
	Dickkopf homolog 1 ( <i>Xenopus laevis</i> )	2	6
Miscellaneous	Transgelin	2	7
	Metallothionein IX	0	6
	Endothelin 1	6	0
Unknown/EST	KIAA0779 protein	0	8

(Modified with permission from Ref. 58, Table 5; Sawada *et al.* 2005)

\* Category/function and gene title are grouped by each probe set obtained from the NetAffx and HumanPSD databases. The two columns on the far right indicate the number of compounds out of 12 that induced PLDsis and down-regulated genes in HepG2 cells after 6 or 24 hours of treatment.

of drugs and/or their metabolites, which is coincident with PLDsis, is another great concern to which toxicologists should pay attention. Probably most problematic is the lack of a mechanistic understanding of the toxicity thought to be associated with PLDsis. A better understanding of the functional consequences of PLDsis will facilitate regulatory considerations.

Thus, it is clear that PLDsis, *per se*, is not a sufficient cause for discontinuing the development of a drug candidate and any toxicity data should be used in the risk assessment or for “Go or No Go” decisions for the candidate. In this regard, the relationship of the dose level that induces PLDsis and that inducing systemic organ toxicity should first be considered then, if there is no difference between those two levels, the usual risk assessment of the candidate drug should be applied in the toxicological evaluation. Hence, the risk and benefit of the candidate, the therapeutic indication, intended dosage, exposure duration, toxicity profiles such as target organs, reversibility and non-observed-adverse-effect-

levels (NOAEL) of the candidate should be considered. If there is no or little margin between the pharmacologically effective dose and the NOAEL, (on comparison of the two blood relevant concentrations), then termination of the development of the drug candidate should be considered. However, if there is an “appropriate” difference between the dose levels at which PLDsis appears and the therapeutic level, PLDsis should be assessed based on the affected cell or tissue function and its mechanisms. The target organs affected by PLDsis should be also considered. For example, renal or hepatic PLDsis may be more manageable than CNS or pulmonary PLDsis, because there are reliable biomarkers for detecting of early renal or hepatic injury. Therefore, there would be great value in having valid and ubiquitous biomarkers to monitor the presence of this condition. Ideally, multiple (not single) biomarkers would be desirable. Unfortunately there are, at present, no valid markers that can be used in animal and human studies. However, recent advances in biotechnology such as metabonomics and

toxicogenomics could provide us with novel tools to elucidate the mechanisms of PLDis and to establish biomarkers for screening tests in the preclinical stages and for monitoring in the clinical phases, in addition to conventional approaches such as morphology (lamellar bodies) and biochemical methods including assays of specific metabolites and PL inhibition as described above.

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