CHAPTER 25

Drug-Induced Phospholipidosis

Peter R. Bernstein*, **Paul Ciaccio****** and **James Morelli**†**

Contents

1. INTRODUCTION

1.1. What is drug-induced phospholipidosis?

Drug-induced phospholipidosis (PLD) is a condition in which drugs cause the excessive accumulation of phospholipids in cells [1]. It is characterized by the accumulation of phospholipid–drug complexes as intracellular concentric lamellar bodies that are visible within lysosomes by electron microscopy [2]. In most instances, the drugs that induce this

* PhaRmaB LLC, 14 Forest View Road, Rose Valley, PA 19086, USA
** AstraZeneca Pharmaceuticals, 35 Gatehouse Drive, B2.85, Waltham, MA 02451, USA
† AstraZeneca Pharmaceuticals, 1800 Concord Pike, Wilmington, DE 19850-5437, USA

Annual Reports in Medicinal Chemistry, Volume 46
ISSN: 0065-7743, DOI: 10.1016/B978-0-12-386009-5.00001-1

condition are those that have both a hydrophilic domain, with one or more positively charged nitrogen groups, and a hydrophobic domain that frequently contains an aryl group. Generally such compounds are called cationic amphiphilic drugs (CADs).

1.2. What is the mechanism of PLD?

There is no single causative mechanism for induction of PLD, as diverse drugs seem to induce it in different ways [3] and others may involve multiple causative mechanisms [4]. Some CADs are attracted to negatively charged phospholipids and accumulate in membranes, changing the susceptibility of the membranes to breakdown. Others cause phospholipid buildup by directly inhibiting the action of phospholipases, and some appear to increase phospholipid synthesis [5].

There are numerous PLD-responding cell types and tissues. Cells of the macrophage system (*e.g.*, bronchiolar macrophages and Kupffer cells) are prominent responders that act to scavenge lamellar bodies and facilitate the lamellar body/drug complex removal. Such cells are particularly lysosome rich, and the CADs ionize and partition into the more acidic environment of the lysosome which serves to protect the cell from cytotoxicity. PLD induction seems to be a dose-dependent process with manifestation of PLD being related to accumulation of the CADs.

1.3. What are the toxicological implications?

Numerous (>50) marketed drugs are known to induce PLD in preclinical species at doses that are significantly higher (>10-fold) than are routinely prescribed in the clinic [6]. PLD is generally considered to be reversible, but the rate of reversibility varies widely within drugs, tissues, and species. It appears more commonly preclinically than clinically, and in one important study, CNS PLD did not yield electrophysiological functional deficits [7]. Viewed this way, PLD would not be considered adverse or high risk for drug development.

However, PLD has been circumstantially associated with toxicities *in vivo* both preclinically and clinically [8]. Although there are no comprehensive published reviews of pharmaceutical industry data to sort these risks, various published reports include: PLD observed *sometimes* concurrently with cataracts and corneal opacities, pneumonitis, myodegeneration, neurodegeneration and neuropathies, and liver toxicity [8]. Additionally, the physicochemical properties of phospholipogenics *may* predispose them to additional toxicities apart from direct PLD, such as mitochondrial toxicity and increased risk of QT prolongation.

2. EVOLVING REGULATORY AND INDUSTRY VIEWS

Since the linkage between toxicological responses and PLD remains unclear, there are currently no regulatory guidelines. In an effort to address this issue, the Food and Drug Administration (FDA) announced that guidance on PLD was being developed and established a ''FDA Phospholipidosis Working Group'' [9]. Although a formal report has not been issued, the challenges are documented in presentations given at a recent FDA advisory committee meeting [10]. These include the questions: Why is it that only a small number of compounds that show PLD also show toxicity and why is it that PLD seen in animals is not predictive of clinical results?

Multiple reports reveal that PLD-inducing compounds have either a higher incidence of histological findings in toxicity studies than PLD negative (non-phospholipogenics) compounds [11] or a strong correlative relationship between PLD and preclinical toxicities (toxicity lowest effect level [LOEL] *vs.* PLD LOEL) [12]. A high proportion of drugs known to cause PLD in preclinical animal models are known to cause QT prolongation clinically [13,14]. The mechanistic basis for this increased QT risk is unproven since (a) the intrinsic PLD potencies and hERG blockade potencies of agents that induce Torsades des Pointes [Torsadogens] do not appear to correlate [11] and (b) the risk is not directly correlated with hERG potency as QT effects are found with PLD positive agents at much higher hERG IC_{50} values than those found for PLD negative ones [15]. Specifically, induction of Torsades des Pointes is usually associated with potent hERG blockers; however, if the Torsadogen is also a known PLD inducer, then the cardiac effects manifest themselves even if the direct effect at hERG is very weak [12]. While it is not known why this occurs and although only a small number of phospholipogenics also cause cardiac PLD, a potential hypothesis for the increased risk of QT prolongration is pharmacokinetic, that is, amphiphilic drugs may partition into cardiac tissue, reaching concentrations that exceed plasma levels and hERG IC_{50} values.

Given these considerations, discovery of PLD during preclinical *in vivo* toxicology has the potential to cause significant delays and increased expenditures in the drug development process. On many occasions, drug development of potentially beneficial compounds showing PLD has been terminated [16]. These decisions are made not because of a proven toxicological problem but because of the uncertainty a PLD finding introduces and the increased hurdle/cost of developing such molecules [17]. The possibility of discovering PLD late in the drug discovery process has caused significant effort to be spent on strategies to minimize the potential for compounds to induce PLD as evidenced by numerous recent publications [6,18].

It is incumbent upon drug makers to challenge views that assume a low risk and adaptive nature for PLD, or assume a low PLD risk for compounds that are CADs. They must be prepared to support risk assessments after obtaining experimental evidence of PLD-inducing potential prior to pivotal toxicology study support [19]. Some companies may profile phospholipogenic compound safety risks on a case-by-case basis. Others are developing more sophisticated risk avoidance and management strategies that include: (a) *in silico* and *in vitro* screening of compounds in discovery; (b) identification of both *in vitro* and *in vivo* intrinsic PLD potency and associated tissue degeneration *in vivo* before pivotal studies are conducted; (c) confirmatory *in vivo* tools (electron microscopy, measurement of phospholipid content, Lamp2 [lysosome-associated membrane protein 2] immunohistochemistry, and gene expression profiling), as well as mechanistic investigations by which PLD affects cell function (*e.g.*, inhibition of autophagy and impact on lysosomal protein degradation and release, fusion abilities and endocytosis; apoptosis; lysosomal *vs*. mitochondrial role in cytotoxicity); (d) drug accumulation and PK association with responses over time; and (e) functional response evaluations (*e.g.*, QT, supporting clinical need for thorough evaluations, and neurologic effects).

In their comprehensive review of an internal Pfizer risk assessment strategy, Chatman *et al*. [20] emphasized the importance of demonstrating reversibility in pivotal studies and used a tiered testing approach, starting in discovery with *in silico* and *in vitro* tools, followed by thorough characterization of responses *in vivo*. Pharmaceutical sponsors might also consider additional points, including (1) understanding the correlation of response severity to intrinsic phospholipogenic potency or tissue levels, (2) examining the presence of supraproportionate tissue levels relative to plasma exposure, and (3) investigating the time dependency of proposed biomarkers to discriminate toxic effects from PLD-only responses. A particularly promising noninvasive PLD biomarker is bis(monacylglycerol)phosphate (BMP), a lysosomal membrane phospholipid that marks membranes for degradation, a feature that distinguishes it mechanistically from perimeter membrane phospholipids [8].

3. SCREENING METHODS

3.1. Introduction

In an effort to reduce the risk of drug attrition due to toxicity associated with physicochemical parameters, the drug industry has been introducing numerous methods that will allow companies to select compounds that are less likely to fail for reasons other than lack of efficacy [21].

To reduce the risk of failure *in vivo* due to PLD, the methodology has included *in silico* models and *in vitro* assays.

3.2. *In silico* techniques

Although many computational models have been disclosed, a common shortcoming is that many models are proprietary and built with limited datasets [22,23]. An openly accessible, effective *in silico*, model that yielded rapid and accurate predictions of PLD would be of great value to the drug discovery industry. A recent review provides a detailed description of most *in silico* approaches [18]. Amphiphilicity of drug molecules is linked to their ability to induce PLD. This is because binding with both the polar phosphate region and the lipophilic core of phospholipid membranes requires specific spatial distribution of the molecule's lipophilic and charged groups. Recognizing this fact, a group at Roche developed an *in silico* program (CAFCA) that calculated the free energy of amphiphilicity for molecules [24]. Picking the correct conformations for these calculations was important for this approach to successfully provide good agreement with experimentally determined values. Each conformation produces a different value and although the reason was not clear, ''in all cases the lowest calculated free energies of amphiphilicity came closest to the measured values.'' In an early attempt to produce an *in silico* model, a group at Organon published a method for predicting PLD-inducing potential that was based on ClogP and calculated pK_a [25]. The result was a prediction that cationic amphiphilic compounds would be phospholipogenic provided that p$K_a > 8$ and ClogP > 1 and also that Eq. (1) is satisfied.

$$(\text{ClogP})^2 + (\text{calculated pK}_a)^2 > \quad 90 \tag{1}$$

The limitations of this approach were discussed in a published analysis by a Pfizer group that also described modifications to the Organon model that significantly improved the percentage of compounds that were correctly diagnosed as not inducing PLD [26]. QSAR models based on two commercially available software programs MC4PC-QSAR and MDL-QSAR have been described by an FDA-based team [27]. Although the statistics for the single MC4PC-QSAR model were modest, addition of the second model and seeking consensus prediction by the two models significantly increased the accuracy of the tool. Another approach to increasing the predictability of *in silico* models based on ClogP and pK_a was illustrated by a Bristol-Myers Squibb team, that showed the addition of volume of distribution (V_d) resulted in improved concordance with PLD [28]. Most recently, a team at the University of St. Andrew's reviewed the status of predicting PLD using machine learning [23].

The Pfizer, FDA, and St. Andrew's team all cited improved accuracy as the size of the datasets they used to build and validate their models were increased.

3.3. *In vitro* techniques

An early *in vitro* screen for PLD was described in 1991 using human foreskin fibroblasts treated with a fluorescently labeled surrogate phospholipid, NBD-PC [29]. NBD-PC consists of phosphotidylcholine (PC) linked to the fluorescent moiety, nitrobenzoxadiazole (NBD). Comparison of control to treated samples using a fluorescent spectrophotometer following cell disruption yielded an insufficient signal-to-noise ratio for screening purposes. Despite this limitation, the methodology laid the ground work for later assay development by several groups using other methods, including high content biology, which was not available at the time.

In 2001, a team at Abbott described an assay, using rat and human hepatocytes and phosphoethanolamine (NBD-PE), combined with laser scanning confocal microscopy, as part of an effort to find alternatives for matrix metalloproteinase inhibitors that caused PLD [30]. The authors underscored the importance of assaying metabolites of the parent drug, as initial attempts to detect PLD using hepatocytes with the parent drug alone yielded negative results, but use of the amine metabolite resulted in strong detection of NBD-PE accumulation.

Kasahara *et al*. found that treatment of the CHO-K1 cell line with NBD-PC and drug combined with fluorescent spectrophotometer readings gave good results; it correctly identified a large set of known positive and negative phospholipogenic compounds with high specificity and sensitivity [31].

In 2006, a team at AstraZeneca described the use of a mouse splenic macrophage cell line, NBD-PE, and the Cellomics Arrayscan High Content Screening (HCS) instrument to develop an assay with high dynamic range, sensitivity, and specificity [32]. Elimination of individual dead cells from quantitation using Sytox Orange Dye allowed for treatment of cells through a wide range of drug concentrations (typically up to 300 μM), while measuring for effects only on live cells.

In 2009, a team at JNJ developed a flow cytometric-based method for detecting PLD in a high-throughput 96-well format [33]. This method uses the human monocyte cell line THP-1, Invitrogen's LipidTox reagent for phospholipid staining, and a live-dead cell stain combination for assessment of cell toxicity. The group claims that one significant advantage of the method is its sensitivity, exemplified by its ability to identify compounds which cause only a twofold increase in LipidTox retention over controls.

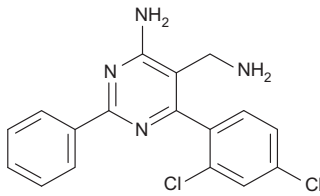
PLD assays based on toxicogenomics have also been described. In 2005, Sawada analyzed RNA from treated HepG2 cells using Affymetrix microarray technology [4]. Analysis of the microarray studies yielded 12 marker genes which could be used as signatures to predict the potential for PLD. Shortly after another team also published a gene expression-based assay [34]. While these assays provide important contributions to understanding the mechanisms occurring during PLD, their broader use is restricted due to high cost and low throughput.

In conclusion, many methods for assaying compounds for the potential to cause PLD exist. Each has advantages and disadvantages in terms of cost, required instrumentation, and desired throughput. Despite these diverse options, *in vitro* methods have many limitations. *In vitro* assays cannot predict multiorgan PLD, which tissues will be affected, which species will be affected, or even if there will be a human response to the drug. For this, additional knowledge gathering is needed.

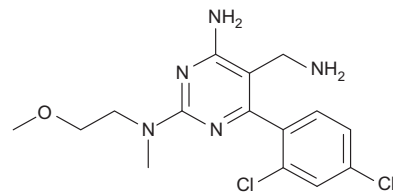
4. EXAMPLES OF PROJECT RESPONSES TO FINDING PLD

The impact on drug development of finding drug-induced PLD during toxicological studies has been documented in many disease areas and projects. In most cases, the induction of PLD has been traced to a direct effect of the parent molecule. Following the discovery of PLD, teams have used a variety of approaches to develop new compounds with different physicochemical properties that retain potency at the pharmacologic target but which have reduced probability of inducing PLD. Several examples follow and additional description and examples may be found in a recent review [18].

At Roche, a team working to develop DPP-IV inhibitors found that compound **1** had many favorable properties but induced PLD in a concentration-dependent manner in cultured fibroblasts (at 2.5–20 μM) [35]. Knowing that the ''CAFCA'' program [25] shows reduced potential for inducing PLD for compounds with a calculated free energy of amphiphilicity ($\Delta\Delta G_{AM}$) greater than -6 kJ/mol and that **1** had $\Delta\Delta G_{AM} = -6.6$ they prepared a set of compounds that were predicted to be less amphiphilic. This effort led to compound **2**, which matched the DPP-IV potency of **1** but which had $\Delta\Delta G_{AM} = -5.6$ and which did not induce PLD in the fibroblast assay at its highest tested concentration [20 μM].



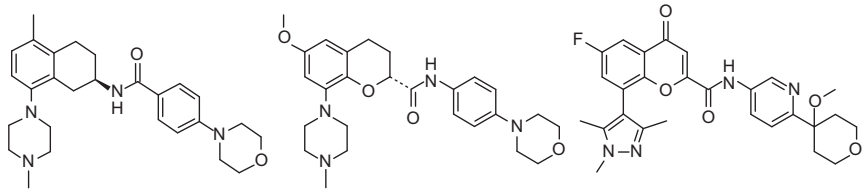
1



2

A team at AstraZeneca disclosed that a series of 5-HT_{1B} antagonists, exemplified by AZD8129 (**3**, Table 1), had exhibited PLD in preclinical rat and dog toxicology studies [38]. They used computational modeling and an *in vitro* assay [32] to develop a follow-up compound (AZD3783, **4**) that had improved 5-HT_{1B} potency to PLD ratios, both *in vitro* and *in vivo*, compared to **3** (as well as many reference drugs, including amiodarone and fluoxetine). A comparison of the *in vivo* dorsal root ganglia and sciatic nerve responses to these two candidate drugs is shown in Table 1. The apparent improvement of **4** over **3** illustrates early success in this program [36]. Dorsal root ganglia [DRG] are a structural unit that is vulnerable to toxicants, mainly due to fenestrated endothelium and discontinuous capillary basement membranes that enable higher exposures than surrounding tissues. DRG are linked functionally to the sciatic nerve. At lower exposures in a 1-month repeat dose rat study, **3** administration yielded greater dorsal root ganglia PLD, sciatic neurodegeneration and intrinsic PLD potency than **4** even as animals were exposed to higher amounts of **4** than **3**.

Unfortunately, AZD3783 was also withdrawn from development due to other toxicities potentially linked to buildup of compound and PLD (data not shown). So, the team designed **5**, a picomolar 5-HT_{1B} antagonist [37]. Since **5** no longer contained the basic piperazine found in **4**, it was no longer a CAD and was predicted to not be phospholipidogenic. This was confirmed by its inactivity at 300 μM in the *in vitro* PLD assay at which concentration **4** showed 81% inhibition.



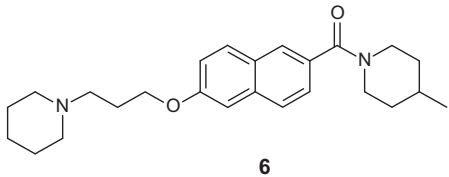
3 **4** **5**

Table 1 AUC levels and *in vitro/in vivo* PLD potency of 5-HT_{1B} antagonists [36,37]

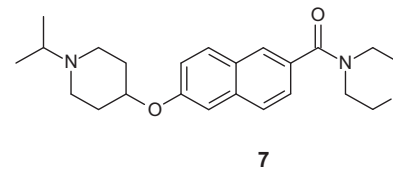
Compound	Total AUC exposures μmol*h	*In vitro* PLD potency (EC_{50}) μM	Rat dorsal root ganglia PLD response severity(Incidence)[a]	Rat sciatic nerve degeneration severity (Incidence)
3	145	10	+++ (5/6)	+ (2/7)
4	764	164	++ (5/8)	− (0/8)

[a] Severity (−, no incidence: +, slight; ++ minimal; +++, moderate; ++++, severe)/incidence (# rats responding/# rats tested).

Teams at several companies developing H3 antagonists have described needing to overcome PLD. A group at Roche discovered a naphthalene based series exemplified by **6** [39]. While this compound had many positive attributes, it was strongly positive in an *in vitro* PLD assay at 5 μM and above. Analysis *via* CAFCA showed that this compound was highly amphiphilic and had $\Delta\Delta G_{AM} = -10.4$. They explored replacements for all three regions of the molecule: piperidinyl propyl ether, amide, and naphthyl core. The new compounds were designed to decrease amphiphilicity but would still fit an H3-pharmacophore model. This led to the discovery of **7**, a compound which retained most of the H3 potency of **6**, and its other positive features but which had $\Delta\Delta G_{AM} = -4.27$ and did not trigger a PLD flag. Efforts to further reduce $\Delta\Delta G_{AM}$ by replacing the naphthalene core with quinolone led to unacceptable loses in H3 potency.

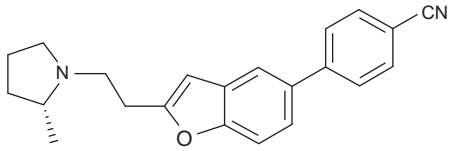


6

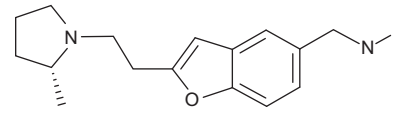


7

At Abbott a team working on backups to the H3-antagonist ABT-239 (**8**) disclosed **9**, an analog with a basic side chain that was more potent than amiodarone in a PLD assay in cultured rat hepatocytes [40]. This was shown to be due to the inhibition of phospholipases upon the binding of dibasic amines with membranes [41]. By either reducing the basicity of the second amine by arylating it (*e.g.*, **10**) or removing the amine side chain, they were able to prepare potent H3 antagonists with reduced PLD-inducing potential.



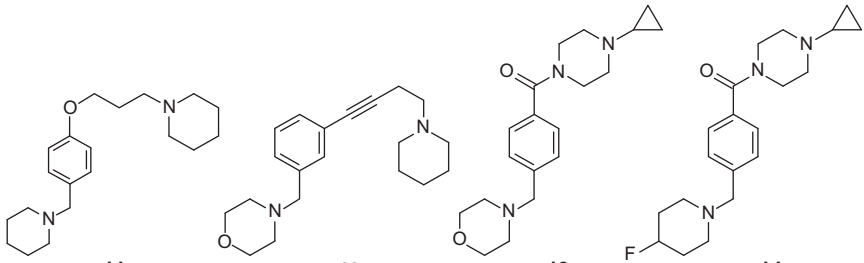
8



9 R = H
10 R = pyrimidin-2-yl

Similarly, JNJ researchers disclosed that JNJ-5207852 (**11**), a potent selective H3 antagonist, produced PLD following prolonged high exposure in rodents [42]. Since **11** was a dibasic CAD with a very high volume of distribution at steady state (Vdss) and a long $t_{1/2}$ (multiple days in brain), the approach used to produce compounds with an improved safety profile included reducing both the ClogP and the pK_a of the two amines [43]. This initially led to JNJ-10181457 (**12**), a compound in which one of the two piperidine groups was replaced with a morpholine. It had a

much shorter $t_{1/2}$, with no detectable brain levels remaining after 24 h. Replacement of the second piperidine and increasing polarity, *via* incorporating an amide linker, led to compounds (**13**) and (**14**). These were still active *in vivo* and had reduced Vdss, improved clearance (in rats), and did not exhibit PLD [44].



An additional complication that teams face is that in rare cases PLD has been found during *in vivo* studies even though the compound had not been predicted to show PLD by earlier assays [19]. In several of these cases, it has been possible to show that circulating metabolites do profile *in vitro* (or *in silico*) as probable inducers of PLD, thereby providing a link.

5. CONCLUSION

The original view that PLD is just an adaptive/reversible response has been replaced by a cautionary one that considers it a sign of a potential toxicological response. Unfortunately, there is no clear understanding why some PLD responses, and CAD chemistries, are linked with concurrent toxicities and others are not. Until clarification of this occurs, it is incumbent upon the drug discovery and development community to minimize the potential for PLD to manifest.

This presents a problem that could potentially be solved by avoiding all CADs. However, given the number of marketed CADs that is safe and effective, this option could hinder access to many potentially valuable treatments. One approach that is illustrated in this review is to use *in silico* models and *in vitro* assays to maximize the target potency/PLD induction ratio, then confirm with *in vivo* studies that PLD has been minimized or eliminated, and that more importantly, related concurrent toxicities are not occurring. An alternative approach, which targets the same receptors/disease but avoids CADs and is the subject of much current research in the field of 7TM-GPCR receptors, is to develop allosteric modulators [45].

ACKNOWLEDGMENTS

The authors gratefully acknowledge Anna-Lena Berg (AstraZeneca Pharmaceuticals) for her seminal pathology contributions to the AstraZeneca 5-HT_{1B} project and for directly influencing our general understanding of the phospholipidotic response.

REFERENCES

[1] W. H. Halliwell, *Toxicol. Pathol*, 1997, **25**, 53.
[2] F. A. de la Iglesia, G. Feuer, E. J. McGuire and A. Takada, *Toxicol. Appl. Pharmacol.*, 1977, **34**, 28.
[3] U. P. Kodavanti and H. M. Mehendale, *Pharmacol. Rev.*, 1990, **42**, 327.
[4] H. Sawada, K. Takami and S. Asahi, *Toxicol. Sci.*, 2005, **83**, 282.
[5] B. D. Wilson, C. E. Clarkson and M. L. Lippman, *Am. Rev. Resp. Dis.*, 1991, **143**, 1110.
[6] M. J. Reasor, K. L. Hastings and R. G. Ulrich, *Expert Opin. Drug Saf.*, 2006, **5**, 567.
[7] M. E. Cartwright, J. Petruska, J. Arezzo, D. Frank, M. Litwak, R. E. Morrissey, J. MacDonald and T. E. Davis, *Toxicol. Pathol.*, 2009, **37**, 902.
[8] E. A. Tengstrand, G. T. Miwa and F. Y. Hsieh, *Expert Opin. Drug Metab. Toxicol.*, 2010, **6**, 555.
[9] Pink Sheet: August 16, 2004 | Volume 66| Number 033 |p. 24: FDA Phospholipidosis Guidance Being Developed.
[10] For slides presented at the FDA PS&CP Advisory Committee meeting on April 14th 2010, see: http://www.fda.gov/downloads/AdvisoryCommittees/CommitteesMeetingMaterials/Drugs/AdvisoryCommitteeforPharmaceuticalScienceandClinicalPharmacology/UCM210798.pdf.
[11] L. Barone, S. Boyer, J. Damewood, J. Fikes, S. Matis, G. Gipson and P. Ciaccio, Are Phospholipogenic Compounds Associated with More Toxicities than Non-Phospholipogenic Compounds?, Society of Toxicology Meeting, Baltimore, MD, March, 2009.
[12] J. M. Willard, FDA PLWG Activities on Phospholipidosis: Data Mining, Modeling and Laboratory Research, *Phospholipidosis Working Group and Division of Cardiovascular and Renal Products—CDER. FDA ADVISORY MEETING*, April 14, 2010. see Ref.[10] for slide set.
[13] B. G. Small, L. R. Barone, J. P. Valentine, C. Pollard and P. J. Ciaccio, *J. Pharmacol. Toxicol. Methods*, 2007, **56**(2), e70.
[14] L. R. Barone, P. J. Ciaccio, B. G. Small, J.-P. Valentin and C. E. Pollard, Society of Toxicology Meeting, Charlotte, NC, March, 2007.
[15] See Slides 77 and 78 in: J. M. Willard, Ref.[12], FDA ACPS meeting, April 14, 2010.
[16] M. J. Reasor, Overview of Drug Induced Phospholipidosis, *Phospholipidosis Working Group and Division of Cardiovascular and Renal Products—CDER. FDA Advisory Meeting*, April 14, 2010, see Ref. [10] for slide set.
[17] B. R. Berridge, L. A. Chatman, M. Odin, A. E. Schultze, P. E. Losco, J. T. Meehan, T. Peters and S. L. Vonderfecht, *Toxicol. Pathol.*, 2007, **35**, 325.
[18] A. J. Ratcliffe, *Curr. Med. Chem.*, 2009, **16**, 2816.
[19] K. L. Hastings, Phospholipidosis Industry Perspective, *Phospholipidosis Working Group and Division of Cardiovascular and Renal Products—CDER. FDA Advisory Meeting*, April 14, 2010. see Ref. [10] for slide set.
[20] L. A. Chatman, D. Morton, T. O. Johnson and S. D. Anway, *Toxicol. Pathol.*, 2009, **37**, 997.
[21] P. D. Leeson and J. R. Empfield, *Ann. Rep. Med. Chem.*, 2010, **45**, 393.
[22] D. K. Monteith, R. E. Morgan and B. Halstead, *Expert Opin. Drug. Metab. Toxicol.*, 2006, **2**, 687.
[23] R. Lowe, R. C. Glen and J. B. O. Mitchell, *Mol. Pharm.*, 2010, **7**, 1708.

[24] H. Fisher, M. Kansy and D. Bur, *Chimia*, 2000, **54**, 640.
[25] J.-P. H. T. M. Ploemen, J. Kelder, T. Hafmans, H. van de Sandt, J. A. van Burgsteden, P. J. M. Salemink and E. van Esch, *Exp. Toxicol. Pathol.*, 2004, **55**, 347.
[26] D. J. Pelletier, D. Gehlhaar, A. Tilloy-Ellul, T. O. Johnson and N. Greene, *J. Chem. Inf. Model.*, 2007, **47**, 1196.
[27] N. L. Kruhlak, S. S. Choi, J. F. Contrera, J. L. Weaver, J. M. Willard, K. L. Hastings and L. F. Sancilio, *Toxicol. Mech. Methods*, 2008, **18**, 217.
[28] U. M. Hanumegowda, R. Yordanova, G. Wenke, J. P. Corradi, A. Regueiro-Ren and S. P. Adams, *Chem. Res. Toxicol.*, 2010, **23**, 749.
[29] R. G. Ulrich, K. S. Kilgore, E. L. Sun, C. T. Cramer and L. C. Ginsberg, *Toxicol. Methods*, 1991, **1**, 89.
[30] R. J. Gum, D. Hickman, J. A. Fagerland, M. A. Heindel, G. D. Gagne, J. M. Schmidt, M. R. Michaelides, S. K. Davidsen and R. G. Ulrich, *Biochem. Pharmacol.*, 2001, **62**, 1661.
[31] T. Kasahara, K. Tomita, H. Murano, T. Harada, K. Tsubakimoto, T. Ogihara, S. Ohnishi and C. Kakinuma, *Toxicol. Sci.*, 2006, **90**, 133.
[32] J. K. Morelli, M. Buehrle, F. Pognan, L. R. Barone, W. Fieles and P. J. Ciaccio, *Cell Biol. Toxicol.*, 2006, **22**, 15.
[33] M. Natalie, S. Margino, H. Erik, P. Annelieke, V. Geert and V. Philippe, *Toxicol. In Vitro*, 2009, **23**, 217.
[34] P. Nioi, B. K. Perry, E.-J. Wang, Y.-Z. Gu and R. D. Snyder, *Toxicol. Sci.*, 2007, **99**, 162.
[35] J. U. Peters, D. Hunziker, H. Fischer, M. Kansy, S. Weber, S. Kritter, A. Muller, A. Wallier, F. Ricklin, M. Boehringer, S. M. Poli, M. Csato and B.-M. Loeffler, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 3575.
[36] A. L. Berg and P. Ciaccio, Compounds Causing Phospholipidosis, Including Effects on the Central Nervous System, Classic examples in Toxicologic Pathology XVI. Seminar for the European Society of Toxicologic Pathology and the Graduate School for Biomedical Sciences at the University of Veterinary Medicine in Hannover, February 27–28, 2009.
[37] D. A. Nugiel, J. R. Krumrine, D. Hill, J. R. Damewood, P. R. Bernstein, C. D. Sobotka-Briner, J. Liu, A. Zacco and M. E. Pierson, *J. Med. Chem.*, 1876, **2010**, 53.
[38] P. R. Bernstein, 3rd RSC/SCI Symposium on GPCRs in Medicinal Chemistry, MSD, Oss, The Netherlands, September 20–22, 2010.
[39] R. M. R. Sarmiento, M. T. Nettekoven, S. Taylor, J. M. Plancher, H. Richter and O. Roche, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 4495.
[40] M. Sun, C. Zhao, G. A. Gfesser, C. Thiffault, T. R. Miller, K. Marsh, J. Wetter, M. Curtis, R. Faghih, T. A. Esbenshade, A. A. Hancock and M. Cowart, *J. Med. Chem.*, 2005, **48**, 6482.
[41] J. P. Montenez, F. Van Bambeke, J. Piret, R. Brasseur, P. M. Tulkens and M. P. Mingeot-Leclercq, *Toxicol. Appl. Pharmacol.*, 1999, **156**, 129.
[42] P. Bonaventure, M. Letavic, C. Dugovic, S. Wilson, L. Aluisio, C. Pudiak, B. Lord, C. Mazur, F. Kamme, S. Nishino, N. Carruthers and T. Lovenberg, *Biochem. Pharmacol.*, 2007, **73**, 1084.
[43] See discussion pages 114–116: S. Celanire, F. Lebon and H. Stark, in *The Third Histamine Receptor: Selective Ligands as Potential Therapeutic Agents in CSN Disorders* (eds. D. Vohora), CRC Press, Boca Raton, 2009, pp. 103–166.
[44] N. I. Carruthers, (1-[4-(3-Piperidin-1-ylpropoxy)benzyl]piperidine): A Template for the Design of Potent and Selective Non-Imidazole Histamine H3 Receptor Antagonists, 33rd Meeting of the European Histamine Research Society, Cologne, Germany, April 28th–May 2nd, 2004.
[45] P. J. Conn, A. Christopoulos and C. W. Lindsley, *Nat. Rev. Drug Discov.*, 2009, **8**, 41.