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# Screening for phospholipidosis induced by central nervous drugs: Comparing the predictivity of an *in vitro* assay to high throughput *in silico* assays

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ABSTRACT

Drug-induced phospholipidosis is a side effect for which drug candidates can be screened in the drug discovery phase. The numerous *in silico* models that have been developed as a first line of screening are based on the characteristic physicochemical properties of phospholipidosis-inducing drugs, e.g. high log *P* and p$K_b$ values. However, applying these models on a predominantly high lipophilic, basic CNS chemistry results in a high false positive rate and consequently in a wrong classification of a large number of valuable drug candidates. Here, we tested 33 CNS-compounds (24 *in vivo* negative and 9 *in vivo* positive phospholipidosis-inducers) in our in house developed *in vitro* phospholipidosis screening assay (Mesens et al., 2009) and compared its predictivity with the outcome of three different, well established *in silico* prediction models. Our *in vitro* assay demonstrates an increased specificity of 79% over the *in silico* models (29%). Moreover, by considering the proposed plasma concentration at the efficacious dose we can show a clear correlation between the *in vitro* and *in vivo* occurrence of phospholipidosis, improving the specificity of prediction to 96%. Through its high predictive value, the *in vitro* low throughput assay is thus preferred above high throughput *in silico* assays, characterized by a high false positive rate.



## 1. Introduction

Phospholipidosis is a drug-induced phospholipid storage disorder, resulting in a massive accumulation of both phospholipids and the inducing drug into the typical drug-phospholipid complexes in affected tissues (Hein et al., 1990). Whether the occurrence of phospholipidosis is an adaptive mechanism of the tissues or a toxicological phenomenon is debatable (Lüllmann-Rauch, 1979; Reasor et al., 2006). Since few organ-specific safety biomarkers exist to track potential toxicological consequences, phospholipidosis occurring in test animals can restrain risk assessment and complicate drug development (Monteith et al., 2006). Many pharmaceutical companies apply phospholipidosis screening assays on their drug candidates to tackle the phospholipidosis-issue earlier in the development process (Fujimura et al., 2007; Kasahara et al., 2006). Because of the nature of drug discovery today, characterized by automated HTS (high throughput screening) in which thousands of compounds can be assayed in a single day; *in silico* assays are favoured above the more time consuming *in vitro* assays (Ling, 2008; Kenny et al., 1998). Numerous robust, easily handling *in silico* assays are developed that predict the occurrence of drug-induced phospholipidosis by evaluating mainly two physicochemical properties of the drug: the log *P* (lipophilicity) and p$K_b$ (dissociation constant of the base) (Ploemen et al., 2004; Pelletier et al., 2007; Tomizawa et al., 2006).

Potential phospholipidosis-inducing drugs are characterized by a high log *P* and a high p$K_b$ as they render the drug lysosomotrophic (De Duve, 1968) and will concentrate the drug in the lysosomes by pH-partitioning (Fig. 1). The highly lipophilic drug easily penetrates the membranes of the lysosomes, wherein the basic nitrogen group is protonated due to the acidic environment. Protonation impedes membrane diffusion and consequently traps the drug in the lysosomes (Kaufmann and Krise, 2007). By inhibiting phospholipases or binding to phospholipids, phospholipid degradation is inhibited directly or indirectly and phospholipidosis occurs (Hein et al., 1990).

Apart from the last component in the mechanism of phospholipidosis-induction, many CNS drugs undergo the same fate. The majority of all drugs contain at least one basic nitrogen (Kaufmann and Krise, 2007), and to reach therapeutic concentrations in the brain most CNS drugs are highly lipophilic to permeate the blood–brain barrier. Lysosomal sequestering of CNS drugs is a well-known phenomenon (Daniel et al., 1999; MacIntyre and Cutler, 1988), but this is not always accompanied by phospholipidosis-induction. Screening CNS drugs for phospholipidosis is, however,

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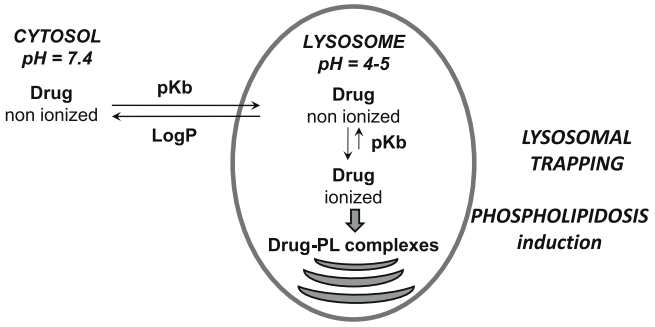


**Fig. 1.** Mechanism of phospholipidosis-induction and lysosomal trapping of drugs. Log *P*: partition coefficient. p$K_b$: diffusion constant of the base. Adapted from Hein et al. (1990). For explanation see Section 1.

advantageous since the CNS lacks safety markers to follow potential phospholipidosis-related toxicological consequences (Monteith et al., 2006) and since phospholipidosis occurring in the nerves resembles the pathology of the genetic lipid storage disorders Niemann-Pick, Gaucher disease and Tay-Sachs disease (Guggenbuhl et al., 2008; Risch, 2001; Thomas, 1988; Vance, 2006).

An *in silico* assay for phospholipidosis based mainly on the p$K_b$ and the log *P* can be expected to have a high false positive rate when applied on a predominantly basic, lipophilic chemistry. To explore this, we composed a set of nine in house drugs that are known to cause phospholipidosis in test animals and can be decoded for publication. In addition, we randomly chose 24 negative in house drugs, mainly CNS drugs, which have reached the clinic and for which phospholipidosis has so far not been observed to occur preclinical or clinical. Based on the physicochemical properties, the positive and negative compounds were randomly distributed (Fig. 2). Both sets were tested in various *in silico* screening assays for phospholipidosis and in our in house developed *in vitro* phospholipidosis screening assay (Mesens et al., 2009). The results show that physicochemical properties alone are indeed insufficient to distinguish between lysosomal trapping and phospholipidosis-induction. In line with these findings we propose a risk assessment approach in which the outcome of the *in vitro* assay is extrapolated to the plasma concentration of the efficacious dose of the drug.

## 2. Materials and methods

### 2.1. Materials

Amiodarone was purchased from Sigma–Aldrich (Bornem, Belgium). The other drugs used were obtained from the internal J&J library and are identified by their generic name, IUPAC name (when available) or chemical name. In addition, the CAS-number and pharmacological action are provided (Table 1). All other materials were purchased from the same suppliers as describes previously (Mesens et al., 2009).

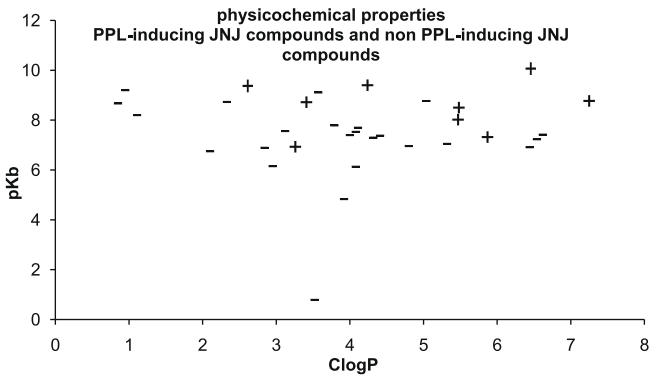


**Fig. 2.** Physicochemical properties of the 39 JNJ compounds. '+' *In vivo* positive phospholipidosis-inducers. '–' *In vivo* negative phospholipidosis-inducers. *Clog P*: calculated partition coefficient. p$K_b$: diffusion constant of the base.

### 2.2. Cell culture and drug treatment

THP-1 cells, human monocytes, were cultured as described previously (Mesens et al., 2009). Cells were incubated in a 96-well plate in triplicate with a serial dilution of each drug ranging from 100 to 0.22 μM (nine concentrations, dilution factor $f = 2.15$). In addition two negative controls: the unstained control cells (not incubated with the fluorescent phospholipids) and the stained controls cells (incubated with the fluorescent phospholipids) and one positive control (amiodarone 12.5 μM) were added to the plate.

### 2.3. Ninety six-well phospholipidosis assay and data analysis

The *in vitro* phospholipidosis assay was performed as described previously (Mesens et al., 2009). In short, cells were incubated for 24 h with the test compound and the fluorescent phospholipids, which were then removed from the medium during a washing step. Cells were then re-incubated with the test compound in the same dilution series for another 24 h and phospholipids' fluorescence was analysed on the Guava easycyte™ system.

In addition, cells were incubated with the same dilutions of the test compound in parallel for 48 h to perform a cytotoxicity test on the Guava easycyte™ system.

Data were analysed as previously described by Mesens et al. (2009). In short, results of fluorescence and cytotoxicity of the treated cells were expressed as the percentage of the vehicle control (DMSO 0.25%). Dose curves of phospholipidosis-induction and cytotoxicity were generated with the curve fitting EXCEL program XL-fit™, version 2.4. The cytotoxicity dose response curve was fitted using the dose response model no. 205, $y = A + [(B - A)/(1 + ((C/x)\text{^}D))]$.

From this curve the $IC_{50}$- and $IC_{80}$-values were calculated. Fitting fluorescence increase was then performed according to the exponential model no. 500, $y = A * \exp(B * x)$. The highest concentration used for fitting fluorescence increase was the concentration that caused a reduction in the viability of 20% ($IC_{80}$-value). From this curve the 2-fold increase value (the concentration at which the drug induces the first relevant fluorescent phospholipid accumulation compared to the control cells or an increase in the fluorescence of 200% compared to the control cells) was calculated.

### 2.4. In vitro prediction of phospholipidosis

The *in vitro* prediction of phospholipidosis was based on the classification method established in Mesens et al. (2009). All compounds were classified into three different categories based on the 2-fold increase value. The compound is categorized according to the following scheme:

2-Fold increase value $\leqslant$ 10 μM → STRONG.
10 μM < 2-fold increase value of $\leqslant$ 50 μM → WEAK.
50 μM < 2-fold increase value → NON.

For the ease of calculating both the sensitivity and specificity, strong compounds are judged as positive and compounds in the weak-class and non-class are judged as negative phospholipidosis-inducers.