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Liposome electrokinetic chromatography based *in vitro* model for early screening of the drug-induced phospholipidosis risk

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ABSTRACT

Drug-induced phospholipidosis (PLD) is a storage disorder of lysosomes characterized by the excessive accumulation of phospholipids as a result of improper medical treatments. Although few evidences have supported that PLD can induce significant pathological consequences, this potential toxicity indeed can put off the drug discovery process. In this research, a high-throughput liposome electrokinetic chromatography (LEKC) method was validated to evaluate the PLD risk of drug candidates by screening drug-phospholipid interaction, which correlates to the phospholipidosis inducing risk. A statistical analysis based on the Spearman's correlation test showed that the retention factors (log k) of the tested drugs in the LEKC system and the literature reported *in vivo* and *in vitro* PLD data were highly correlated. In order to investigate the predictability of LEKC, the effect of liposome composition such as the molar ratio of phospholipids and the addition of cholesterol were also discussed in this study. The results indicated that the LEKC method could offer a fast, reliable and cost-effective screening tool for early prediction of the PLD inducing potential of drug candidates.

1. Introduction

Drug-induced phospholipidosis (PLD) is generally considered as a storage disorder of lysosomes characterized by the excessive accumulation of phospholipids as a result of either intrinsic errors or improper medical treatments since it came to the sight of public in 1948 for the first time [1]. Under the electron microscopy, the excessive phospholipid bilayer within lysosomes mostly appears to have a multilamellar vesical structure [2]. Although few evidences have supported that PLD can induce significant pathological consequences, the situation that PLD has potential morbidic risk cannot be ruled out. PLD could cause changes in cell morphology and even alterations of functions. And in certain cases, the abnormal accumulation of drugs and/or metabolites which is caused by PLD will lead to cell injury or even disease. In the FDA database, 385 drugs were found to be PLD positive, many of which are commonly used in daily life. Seeing the potential detriment, researches toward the mechanism and screening methods of PLD have aroused substantial interest over the past decades, and a high throughput screening method for numerous new drug candidates in the early stage of drug discovery is in urgent need.

The screening methods for PLD potential have been developing rapidly. Transmission electron microscopy (TEM) diagnosis was widely accepted as the most reliable standard for identifying PLD but it suffers the disadvantages of high cost, low throughput and subjectivity [3]. Many other methods have also appeared to fulfill the needs at different stages in the drug discovery and development process. Kasahara et al. established a cell-based approach in which they measured the content of intracellular phospholipids of treated U-937 cells (a human monocyte-derived cell line) using the fluorescent probe Nile red [4]. Although this approach gives a good correlation with the PLD potential evaluation, it still cannot get rid of the common limitations of cell culture such as being time-consuming, low-throughput, high-cost and resource-demanding. Vitovic et al. reported a high-throughput langmuir-balanced approach to predict the risk of PLD, which is based on the measurement of drug-phospholipid complex

Abbreviations: CHI $IAM_{7.4}$, chromatographic hydrophobicity index values referring to immobilized artificial membrane; CMC, critical micelle concentration; IAM, immobilized artificial membrane; LEKC, liposome electrokinetic chromatography; PLD, phopholipidosis; TEM, transmission electron microscopy.

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formation by their effect on the critical micelle concentration (CMC) of a short chain acid phospholipid [5]. It is of better quality compared to computational models and more convenient than cell-based methods. However a special eight channel surface-tensiometer, which is not currently available in many labs, is required.

Chromatography, which is one of the most popular techniques applied in pharmaceutical analysis laboratories, has shown promising foreground in screening the physicochemical properties of drug candidates or their related ADMET behaviors in the early stage of drug discovery [6,7]. For example, immobilized artificial membrane (IAM) columns, which were prepared by covalently binding membrane-forming lipids to the surface of silica particles at high molecular surface densities, have been used successfully in the prediction of drug transport across cell membranes [8,9]. Similarly, liposome electrokinetic chromatography (LEKC), where phospholipid-based liposomes are added to the running buffer, has also exhibited great potential in estimating membrane affinity related properties of drug candidates, such as intestinal absorption, volume of distribution and blood–brain barrier permeability [10]. These *in vitro* chromatographic models were developed based on mimicking *in vivo* interactions between drug candidates and phospholipid molecules. Although the mechanism of drug-induced PLD is still not truly understood, the current view in favor is that the drug may inhibit phospholipases activity due to binding to the phospholipid substrate, then rendering the cell unable to break down phospholipids. This was supported by the recent demonstration of a very good correlation between phospholipidosis and the strength of drug–phospholipid interaction [10]. Consequently, the predictability of IAM for PLD risk of drug candidates was evaluated and a statistical correlation between the retention (CHI IAM7.4) of drug candidates and their PLD inducing risk was observed [7]. Another electrokinetic chromatographic approach using docusate sodium salt (AOT) vesicle as pseudostationary phase was also developed for predicting the potential of compounds to induce PLD. The results indicated that a statistical correlation does exist between the retention of the tested drugs and their PLD risk.

The LEKC technique has been widely used in different biological systems because the charged vesicle structure of liposomes can provide both hydrophilicity/hydrophobicity balance and electrostatic force, which mimics the drug–membrane interaction well. By changing the molar ratio of phospholipids or even by addition of cholesterol, the simulation of various membrane environments can be better accomplished.

In order to investigate the predictability of LEKC for the PLD inducing risk of drug candidates, a liposome system containing 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) was employed as pseudostationary phase in this research. The molar ratio of the two phospholipids in the liposome was systematically studied in order to better mimic electrostatic interactions. 77 commercial drugs assayed with various PLD risk assessment approaches providing quantitative or qualitative literature PLD data were selected as test compounds. A statistical Spearman's correlation between measured retention data on the LEKC system and literature data on various PLD risk assessment approaches are discussed in detail.

2. Materials and methods

2.1. Instruments

All CE experiments were carried out on a HP^{3D} CE system (Agilent Technologies, Waldbromm, Germany) equipped with a diode-array detector (214, 230 and 254 nm) and a temperature control system (25 °C for all experiments). An Agilent Chemstation (Hewlett–Packard, Palo Alto, CA, USA) was used for instrument control and data analysis. The fused-silica capillaries with dimensions of 50 μm I.D. (375 μm O.D.) and 43.5 cm total length (35 cm effective length) were purchased from Yongnian Optic Fiber Plant (Hebei, China). All HPLC experiments were performed on an Agilent 1050 HPLC system (Agilent Technologies, Waldbromm, Germany) equipped with a diode-array detector. A Regis IAM PC DD2 column (100 mm × 4.6 mm I.D., 10 μm particle size) was purchased from Regis Technologies Inc. (Morton Grove, Illinos, USA). The extrusion of liposomes was performed through polycarbonate membranes using a Northern Lipids Lipex extruder (Vancouver, BC, Canada).

2.2. Materials

Ammonium acetate, ammonium formate, sodium hydroxide solutions (1 M and 0.1 M), HCl solution (1 M), dimethyl sulfoxide (DMSO) and 4-(2-hydroxyethyl)- 1-piperazineethanesulfonic acid (HEPES) were purchased from Aladdin Chemicals (Shanghai, China). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) were obtained from NOF Corporation (Tokyo, Japan). All *n*-alkylphenones were purchased from Alfa Aesar (Tianjin, China). HPLC-grade methanol and acetonitrile were obtained from Merck (Shanghai, China). The distilled water was filtered using 0.22 μm membrane before use.

2.3. Methods

2.3.1. Preparation of solutions

A 25 mM HEPES (pH 7.4) buffer was used as background electrolyte in CE, while 50 mM ammonium acetate (pH 7.4) and ACN were used as mobile phase components A and B, respectively, in HPLC experiments. The pH value of both solutions was adjusted to 7.4 with 1 M NaOH or 1 M HCl. All sample solutions were prepared by dissolving the desired amount of compound in methanol to provide a final concentration around 1 mg/mL. 5–25% (v/v) of DMSO was added into the sample solution in order to aid dissolution if the compound was not readily soluble. All solutions were filtered through 0.22 μm membrane prior to the analysis. The 4 mM liposome solutions were made according to previously reported methods [6]. In brief, the appropriate amounts of POPC and POPS were dissolved in a mixture of chloroform and methanol (9:1, v/v). The resulting mixture was stirred at 40 °C for 120 min, evaporated to dryness under reduced pressure using a rotary evaporator at 60 °C and then evacuated overnight. The lipid residue was then hydrated with the desired amount of 25 mM pH 7.4 HEPES buffer, and vortexed. The hydrated liposomes were subjected five times to a freeze–thaw procedure, which consisted of freezing liposomes in dry ice containing acetone and thawing them in a 60 °C water bath. In the last step, the liposomes were extruded through 400, 200 and 100 nm polycarbonate membranes (five times each) and through 50 nm polycarbonate membranes 20 times, using the extruder which was connected to a circulating water bath maintained at 65 °C.

2.3.2. CE and HPLC conditions

New capillaries were first pretreated by flushing with 1 M NaOH for 1 h and then with acetonitrile, water, and running buffer for 30 min, respectively. Between runs, the capillaries were flushed with 0.1 M NaOH, water, ACN and the liposome running buffer at 50 psi for 2 min each. The samples were introduced by pressure (0.2 psi for 3 s) and then the separation was accomplished at 25 kV. The temperature of the capillary was maintained at 25 °C. The analytes were detected at 254 nm, 230 nm or 214 nm according to their UV absorbance spectra. The electroosmotic flow and the