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Biomarkers to monitor drug-induced phospholipidosis

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Abstract

Di-docosahexaenoyl (C22:6)-bis(monoacylglycerol) phosphate (BMP) was identified as a promising phospholipidosis (PL) biomarker in rats treated with either amiodarone, gentamicin, or azithromycin. Sprague–Dawley rats received either amiodarone (150 mg/kg), gentamicin (100 mg/kg) or azithromycin (30 mg/kg) once daily for ten consecutive days. Histopathological examination of tissues by transmission electron microscopy (TEM) indicated different degrees of accumulation of phospholipidosis in liver, lung, mesenteric lymph node, and kidney of drug-treated rats but not controls. Liquid chromatography coupled to mass spectrometry (LC/MS) was used to identify levels of endogenous biochemical profiles in rat urine. Urinary levels of di-docosahexaenoyl (C22:6)-bis(monoacylglycerol) phosphate (BMP) correlated with induction of phospholipidosis for amiodarone, gentamicin and azithromycin. Rats treated with gentamicin also had increased urinary levels of several phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) species.
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Keywords: Drug-induced phospholipidosis; Amiodarone; Azithromycin; Gentamicin; Mass spectrometry; Biomarker; Phospholipids; Cationic amphiphilic drugs

Introduction

Cationic amphiphilic drug-induced phospholipidosis is a phospholipid storage disorder resulting in excessive accumulation of phospholipids in tissues. More than 50 cationic amphiphilic drugs (CADs), including antidepressants, antianginal, antimalarial, and cholesterol-lowering agents, have been reported to induce phospholipidosis. Phospholipidosis has been observed as a recurrent pathological feature in toxicity studies in both animals and humans. Several cationic amphiphilic compounds, such as amiodarone (and its major metabolite desethylamiodarone), azithromycin, perhexiline, gentamicin, fluoxetine, chlorpromazine, tamoxifen, etc., are known to induce phospholipidosis. CADs may induce formation of lamellar (phospholipid bilayer) inclusions in the lens and cornea of eyes (chloroquine, amiodarone), lung (amiodarone), kidney (gentamicin), or spleen lymphocytes (chlorphentermine). However, after cessation of drug administration, the phospholipidosis is reversible with drugs effluxing from cells. The presence of phospholipidosis is currently not considered a toxicologic effect. To date, the determination of phospholipidosis relies on transmission electron microscopy (TEM) and light microscopy (LM) (Josepovitz et al., 1985; Laurent et al., 1990;Mazue et al., 1984; Montenez et al., 1999; Mortuza et al., 2003; Reasor et al., 1996; Reasor and Kacew, 2001; Van Bambeke et al., 1996).

Amiodarone is an antiarrhythmic drug. An effect of amiodarone therapy is pulmonary phospholipidosis. Amiodarone or its major metabolite (mono-*N*-desethylamiodarone) may cause the development of pulmonary phospholipidosis evidenced by the presence of alveolar foamy macrophages containing multilamellar whorls (also termed myelin bodies) (Mortuza et al., 2003; Reasor et al., 1988, 1989, 1996). Phospholipid response to amiodarone has also been characterized in lung, heart, skeletal muscle, and kidney (Rabkin, 2006). Phospholipid accumulation may provide evidence that amiodarone initiates a specific and nearly complete inhibition of phospholipid degradation by inhibiting lysosomal phospholipase A1 and A2. Some potential phospholipidosis markers from *in vivo* amiodarone-treated rat studies were identified as bis (monoglycerol)phosphate (BMP) (Mortuza et al., 2003) and phenylacetylglycine (Delaney et al., 2004). BMP is a structural

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isomer of phosphatidylglycerol (PG) and is often referred to as lyso-bis-phosphatidic acid, LBPA.

Azithromycin, derived from erythromycin, is a macrolide antibiotic for treating bacterial infections. Azithromycin deeply penetrates the lipophilic core of the phospholipid membrane and binds to phospholipids to induce phospholipidosis. Subsequently, the ultrastructural appearance of membranous lamellar inclusions is observed in the cytoplasm of enterocytes, the cytoplasm of lung alveolar macrophages, and the cytoplasm of mesenteric lymph node macrophages (Montenez et al., 1999; Van Bambeke et al., 1996, 1998; Montenez et al., 1996).

Aminoglycosides, such as gentamicin and streptomycin, are polyaminated compounds and are too hydrophilic to freely diffuse across proximal tubular cell membranes. These drugs accumulate in lysosomes, bind to negatively charged phospholipids, and then cause the inhibition of lysosomal phospholipidases. The lysosomal phospholipidosis may be associated with aminoglycoside induced necrosis of proximal tubular cells (Beauchamp et al., 1990; El Mouedden et al., 2000; Josepovitz et al., 1985, 1986; Kishore et al., 1992; Laurent et al., 1990; Toubeau et al., 1986; Zakim et al., 1988). In a four-day phospholipidosis study by Josepovitz et al. (1986), rats were dosed with the aminoglycosides neomycin, gentamicin, tobramycin, and netilmycin at a level of 100 mg/kg for 1 to 4 days. Phospholipid accumulation in the renal cortex and phospholipid excretion in the urine were observed.

The clinical pharmacokinetic profiles of amiodarone, azithromycin, and gentamicin are shown in Table 1. The plasma half-life of amiodarone is long. Amiodarone is highly plasma protein bound and excreted via the liver and gastrointestinal tract by biliary excretion. However, the volume distribution of amiodarone and azithromycin is higher than that of gentamicin (Hardman and Limbird, 2001; Latini et al., 1984; Luke and Foulds, 1997).

In addition to the *in vivo* study, levels of phospholipidosis biomarkers from *in vitro* assays may be correlated with the amount of phospholipid accumulation in human hepatocellular carcinoma (HepG2) cells. Sawada et al. (2005) identified some potential toxicogenomic phospholipidosis markers by using HepG2 cells, which were treated with cationic amphiphilic drugs.

Table 1
Clinical pharmacokinetic data of amiodarone, azithromycin, and gentamicin

	Amiodarone	Azithromycin	Gentamicin
Bioavailability (%)	46±22	37	
Urinary excretion (%)	0	12	>90
Plasma protein binding (%)	99.98±0.01	7–50	<10
Clearance (ml/min kg)	1.9±0.4	9	0.82
Volume distribution (l/kg)	66±44	31	0.31±0.01
Half-life	14–59 days	40 h	2.5 h
Major metabolite (s)	Desethylamiodarone	Descladinose and 9-*N*-desmethyl azithromycin	

The metabolic profiles of cells, tissues and biological fluids provide an integrated fingerprint of both physiological and pathophysiological processes. Comparative analysis of metabolic profiles using mass spectrometry can identify biomarkers related to the effects of drug administration, such as drug-induced phospholipidosis. Biomarkers may be used non-invasively to measure phospholipidosis after exposure to CADs. Metabolic profiles may assist in examining mechanisms of action that contribute to the development of phospholipidosis. The resulting changes in the metabolic signature may be correlated with the magnitude of pathological change due to the drugs' effects.

The focus of this study was to relate a universal biomarker to tissue phospholipidosis induced by amiodarone, azithromycin, and gentamicin from *in vivo* studies using Sprague–Dawley rats. Biomarkers in urine related to drug-induced phospholipidosis in tissues could be used for animal-to-human phospholipidosis predictions. An additional effort was to determine changes in the urinary phospholipid profile of gentamicin treated rats. Gentamicin is excreted >90% in urine and has been shown in the literature to induce changes in levels of kidney and urinary phospholipids.

Materials and methods

Test compounds. Amiodarone hydrochloride, gentamicin sulfate, and azithromycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dosing solutions were prepared fresh daily in 0.5% hydroxypropyl methylcellulose (HPMC)/0.2% Tween 80 in deionized water.

Chemicals and reagents. Chloroform and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium hydroxide (28.0–30.0%), formic acid (90%), methanol, and acetonitrile were purchased from JT Baker (Phillipsburg, NJ, USA). Hexane was purchased from EMD Chemicals (Gibbstown, NJ, USA). Deuterated phospholipid (PL) standards and PL mixtures were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Deuterated PL standards included 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine-1,1,2,2-D4, 1,2-dimpalmityl-*sn*-glycero-3-phosphocholine-1,1,2,2-D4, and 1,2-distearoyl-*sn*-glycero-3-phosphocholine-1,1,2,2-D4. PL mixtures used as reference standards included L-α-phosphatidylinositol (PI) from bovine liver, L-α-phosphatidylcholine (PC) from chicken egg, and L-α-phosphatidylethanolamine (PE) from chicken egg. Trump fixative and sodium cacodylate buffer (0.2 M, pH 7.4) were purchased from Electron Microscopy Sciences (Hatfield, PA, USA).

Animals and treatment conditions. Male Sprague–Dawley rats (8–10 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). After an acclimation period (5 days), animals were randomly assigned into control and treatment groups based on body weight. Rats from each treatment group received either amiodarone (N=15, 150 mg/kg via oral gavage), gentamicin (N=10, 100 mg/kg via subcutaneous injection), or azithromycin (N=10, 30 mg/kg via oral gavage) once daily for either 5 or 10 consecutive days (Table 2). The dose selection and route of administration were based on existing data reported in the literature for induction of tissue phospholipidosis (Delaney et al., 2004; Mortuza et al., 2003; Van Bambeke et al., 1998; Feldman et al., 1982). Control animals (N=5) were administered 0.5% HPMC/0.2% Tween 80 via oral gavage. Urine was collected via overnight cage pan collection on dry ice. Urine was collected randomly from 5 rats prior to treatment to establish baseline values. Urine was collected 24 h after final treatment (Day 6 and Day 11). Animals were humanely euthanized on Day 6 and Day 11 by exsanguination following induction of general anesthesia via an IM injection (Ketamine HCL: 75 mg/kg, Xylazine: 2.5 mg/kg, Acepromazine: 2.5 mg/kg).

Table 2
In vivo study design

Group number	Number of animals		Test article	Dosage level (mg/kg)	Dosage (ml/kg)	Dose concentration (mg/ml)	Route, dosing regimen	Necropsy
	Males	Females						
1	10	0	0.5% HPMC/0.2% Tween 80	0	5	0	PO, once daily for 10 Days	5 rats on Day 6 and 5 rats on Day 11
2	15	0	Amiodarone	150	5	30	PO, once daily for 10 Days	5 rats on Day 6 and 5 rats on Day 11
3	10	0	Azithromycin	30	5	6	PO, once daily for 10 Days	5 rats on Day 6 and 5 rats on Day 11
4	10	0	Gentamicin	100	1	100	SC, once daily for 10 Days	5 rats on Day 6 and 5 rats on Day 11

Electron microscopic examination of tissues. Kidney, liver, lung, and mesenteric lymph node tissues were collected from one rat per group at necropsy on Day 11. Tissue sections (3–4 mm cube) were refrigerated (4 °C) in Trump fixative for 24 h. After removing the fixative, samples were submerged in 1:1 cacodylate buffer:water and delivered to New England Medical Center, Boston MA, USA for examination by electron microscopy.

Magnetic resonance imaging. As an *ex vivo* proof-of-concept study for identifying phospholipid accumulation by magnetic resonance imaging (MRI), liver, lung, and kidney tissues were extracted on Day 11 from 5 rats treated with amiodarone and 2 control rats for MRI analysis.

Biomarker discovery. Urine samples were prepared for analysis using a standard phospholipid extraction method described by Mortuza et al. (2003). An internal standard mixture (12.5 µl) containing 25 µM of each deuterated PL was added to each urine sample (250 µl). Cold 3:1 chloroform/methanol (v/v) containing an antioxidant BHT (50 µg/ml) was added (500 µl). The mixture was vortexed before adding 100 µl 0.1% formic acid (aq.). The sample was vortexed again, allowed to sit for 5 min, and centrifuged for 15 min. The bottom (organic) layer was carefully removed and concentrated to approximately 25 µl. The samples were reconstituted to 125 µl with 0.05% formic acid in 2:1 acetonitrile/methanol (v/v).

The molecular profile of each urine sample was acquired using liquid chromatography coupled to mass spectrometry (LC/MS). Injections were made onto a 150 × 2.00 mm Synergi Hydro-RP column (Phenomenex, Torrance, CA, USA) using an a Gilson 235 auto-injector (Gilson, Inc., Middletown, WI, USA) and an Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA, USA). Mobile phase A was 0.25% ammonium hydroxide, 0.05% formic acid in 88:12 methanol/water (v/v). Mobile phase B was 0.25% ammonium hydroxide, 0.05% formic acid in 80:20 methanol/hexane (v/v). The flow rate was 0.25 ml/min. Initial conditions were 85% A and 15% B. The % B was increased to 90% over a 45 min period.

An MDS Sciex API QStar Pulsar quadrupole time-of-flight (Q-TOF) mass spectrometer (Applied Biosystems, Foster City, CA) was used for detection. Data were acquired in full scan TOF MS mode (*m/z* 100 to 2000) with negative electrospray ionization (ESI). The Turbo IonSpray interface was set at 425 °C and maintained at an ionspray voltage of −4.2 kV with a declustering potential of −50 V. Ionization was assisted with a nebulizer and ionspray gas (nitrogen) set at 55 and 75 (arbitrary units) respectively.

Statistical analysis. The reproducibility of sample preparation and LC/MS system performance was evaluated prior to data analysis using three deuterated PLs as internal standards (LC/MS peak area, ±15% RSD). The parameters of *m/z* and retention time were combined to form a unique identifier for each spectral component of the sample. The SAS was used for statistical analysis and biomarker selection. Comparisons were made for (Control Day 11, $N=5$) vs. (Amiodarone Day 11, Azithromycin Day 11, Gentamicin Day 11, $N=15$) and (Control Day 11, $N=5$) vs. (Gentamicin Day 11, $N=5$) by independent-sample *t* test ($p \leq 0.05$).

Biomarker identification. Potential biomarkers were identified based on comparison to reference standards of naturally occurring PLs. Species assignment was based on exact mass assignment, isotope distribution, and MS/MS fragmentation pattern. The exact mass and isotopic peak distribution were determined in full scan TOF MS mode (*m/z* 100 to 2000) with negative electrospray ionization (ESI). The exact mass of the analyte was determined by averaging peak centroid data (25 scans each) for five injections (one rat from each group). The measured isotopic peak distribution was determined by averaging peak centroid data (25 scans) for a single injection. The theoretical isotopic peak distribution was determined using Analyst QS software isotopic distribution calculator (Applied Biosystems, Foster City, CA). On-line tandem mass spectrometry (API QStar) was used to determine molecular species of PLs. Product ion scanning of deprotonated ions $[M-H]^-$ or formate ion adducts $[M+HCO_2]^-$ in the negative mode was used. The collision energies used were −35 V, −45 V, and −55 V.

Results

Electron microscopic examination of tissues

Ultrastructural examination of rat tissues (kidney, liver, lung, and mesenteric lymph node) revealed the presence of phospholipidosis in cytoplasm of affected cells. As shown in Table 3, for the conditions of this study, the relative accumulation of lamellar inclusions in kidney, liver, lung, and/or mesenteric lymph node was determined: gentamicin > amiodarone > azithromycin.

Magnetic resonance imaging

MRI scans did not identify phospholipid accumulation in tissues from the proof-of-concept liver, lung, and kidney tissues.

Common biomarker for phospholipidosis induced by 3 different drugs

Levels of an unidentified analyte were increased in urine of amiodarone, azithromycin, and gentamicin treated rats compared of controls (Fig. 1). Based on the exact mass, the analyte

Table 3
Histological examination of rat tissues by electron microscopy (Day 11)

Tissue	Control	Amiodarone	Azithromycin	Gentamicin
Liver	−	+	−	+++
Lung	−	+++	++	++
Kidney	−	+++	++	+++
Lymph node	−	+	−	+

Relative accumulation of lamellar inclusions: − not observed, + slight (very few), ++ mild (some), +++ moderate (several).

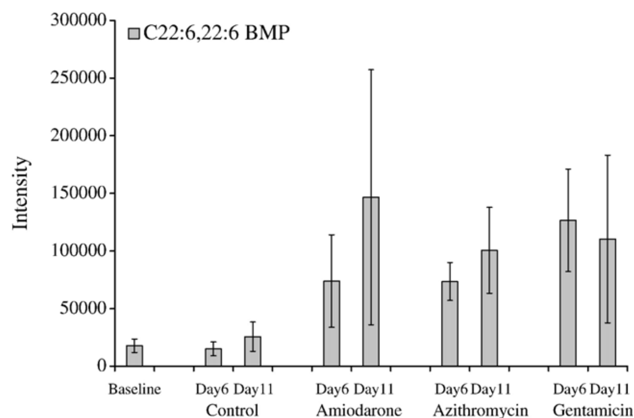

Fig. 1. Levels of di-C22:6-BMP in rat urine. Data represent mean LC/MS peak area ± 1SD, $N=5$ rats per group. Treatment groups: control, amiodarone (150 mg/kg/day), azithromycin (30 mg/kg/day), gentamicin (100 mg/kg/day). Day 6 = overnight collection after 5 consecutive daily treatments. Day 11 = overnight collection after 10 consecutive daily treatments. Compared to baseline and control levels, the levels of di-C22:6-BMP were significantly increased (p value ≤ 0.05).

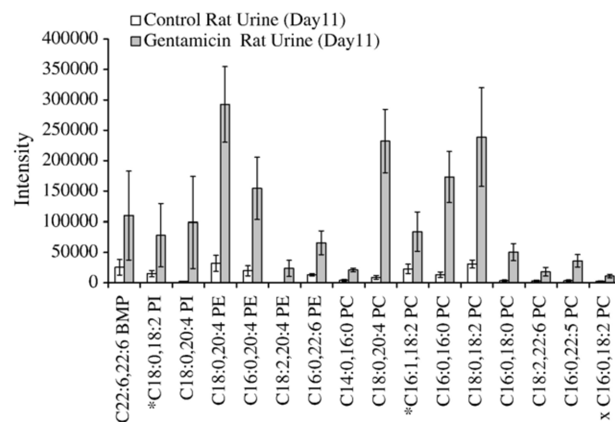

Fig. 3. The effect of gentamicin treatment (100 mg/kg/day via subcutaneous injection for 10 days) on urinary phospholipids in Sprague–Dawley rats. Data represent mean LC/MS peak area ± 1SD, $N=5$ rats per group (p value ≤ 0.05). *Intensity value ×10, X C16:0-alkyl linkage.

was expected to be di-C22:6-BMP ($C_{50}H_{75}O_{10}P$, MW 866.5098, $[M-H]^-$ 865.5025) (Fig. 2). The isotopic peak distribution for m/z 865.5 matched the theoretical isotope peak ratio for di-C22:6-BMP (data not shown). The ESI-MS/MS fragmentation pattern shows the presence of C22:6 anion (m/z 327.2), loss of $-$C22:6 + H_2O $-$ glycerol (m/z 463.2), loss of $-$C22:6 + H_2O (m/z 537.2), loss of $-$C22:6 (m/z 555.2), and other characteristic peaks of di-C22:6-BMP in agreement with observations by Ito et al. (2002).

Changes in urinary phospholipid profile of gentamicin treated rats

The levels of many PLs excreted in urine were increased significantly ($p \leq 0.05$) for gentamicin treated rats compared to controls. Fig. 3 shows the intensity response (i.e., LC/MS peak area) for select lipid species in rat urine. The PL species were identified by on-line tandem mass spectrometry (Koivusalo et al., 2001). Several PLs showed a significant increase (approximately 10-fold increase, $p \leq 0.05$) in treated compared to control rats. These included arachidonic acid (C20:4) containing PI, PE, and PC species (C18:0,20:4 PI, C18:0,20:4 PE, C18:2,20:4 PE, C18:0,20:4 PC). Several other PI, PE, and PC phospholipids were increased between 2- and 10-fold ($p \leq 0.05$). Some of these contained docosahexaenoyl acid (C22:6) (C16:0,22:6 PE and C18:2,22:6 PC), a feature similar to di-docosahexaenoyl (C22:6)-BMP.

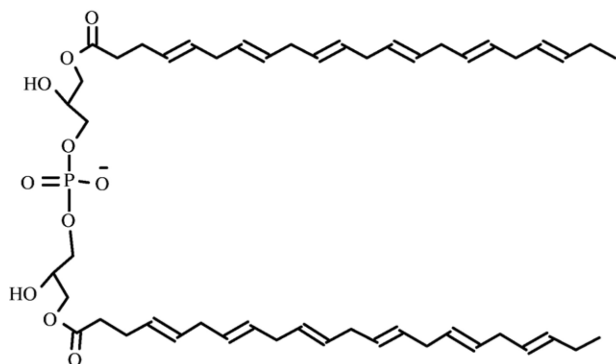

Fig. 2. Structure of di-docosahexaenoyl (C22:6)-BMP ($C_{50}H_{75}O_{10}P$, $[M-H]^-$ m/z 865.5).

Discussion

Di-docosahexaenoyl (C22:6)-bis(monoacylglycerol)phosphate (BMP) was identified in this study as a potential marker of drug-induced phospholipidosis. Di-C22:6 BMP was significantly increased in the urine of rats treated with amiodarone, azithromycin, or gentamicin. BMPs are structural isomers of phosphatidylglycerol (PG) phospholipids. BMPs are found in most cell types and tissues. They usually represent <1% of the total phospholipid mass (Luquain et al., 2001). The structure of di-C22:6-BMP is shown in Fig. 2. Levels of total BMP have previously been shown to increase in serum, lymphocytes, lung, and liver of amiodarone treated rats, administered with 150 mg/kg amiodarone daily for 7 days, compared to controls (Mortuza et al., 2003). Specifically, oleic acid (C18:0) represented more than 50% of the total acyl chains of BMP extracted from lung of rats treated with amiodarone. Polyunsaturated fatty acid (PUFA) moieties such as arachidonic (C20:4) and docosahexaenoic (C22:6) acids were found at much lower relative abundance (<0.5%) in lung. Cox et al. (1989) report BMP levels in liver account for 21% of the increase in phospholipids in a rat model of trospectomycin (an aminocyclitol antibiotic) induced phospholipidosis.

Several possibilities for the function of BMP have been suggested (Schmid and Cullis, 1998). The fatty acid composition of BMP varies with tissue and cell type from almost only oleic acid (C18:0) to almost only docosapentaenoic acid (C22:5) and C22:6 (Brotherus et al., 1974; Luquian et al., 2000; Tjong and Debuch, 1978). BMP is localized specifically in intra-vesicular vesicles of late endosomes/lysosomes where it may take part in protein and cholesterol sorting (Kobayashi et al., 1998; Simons

and Gruenberg, 2000). BMP may be a secretory phospholipase A_2 (PLA_2)-resistant phospholipid (Amidon et al., 1995; Kobayashi et al., 1998). However, a variety of PLA_2 isozymes exist. Some forms may be able to hydrolyze BMP (Ito et al., 2002).

Di-docosahexaenoyl (C22:6)-BMP may have potential as a useful indicator to assess for the presence or absence of phospholipidosis in some clinical circumstances. Phospholipidosis cannot currently be assessed non-invasively in the clinic. In some circumstances, it may be useful to assess for the occurrence of phospholipidosis because the phospholipid accumulation may be injurious. This biomarker could be especially useful for effects in tissues where no sensitive indicators of adverse effects exist.

Several mechanisms of aminoglycoside induced phospholipidosis have been proposed. These include drugs directly decreasing the rate of PL catabolism (decrease activity of phospholipase A and C) and drugs altering the physiochemical properties of PLs (binding interactions) perturbing catabolism. Gentamicin is thought to bind to PI in the proximal tubular cell membrane, accumulate within lysosomes, and inhibit the degradation of lysosomal PLs. This increases the accumulation of PI and PC in the lysosome. Accumulation of PC inhibits the activity of enzymes that hydrolyze PI to phosphoinositol. The renal cortex of Sprague–Dawley rat is estimated to contain approximately 36% PC, 32% PE, 15% SPH (sphingomyelin), 9% PS, 7% PI, and 1% PA expressed as percent tissue PL composition based on μmol/g dry weight tissue (Feldman et al., 1982). Gentamicin treatment (100 mg/kg/day for 2 days) significantly increases ($p \leq 0.05$) the levels of PC (+22%) and PI (+45%) in renal cortex. These changes are detected within 24 h of drug treatment and before signs of nephrotoxicity (elevation of blood urea nitrogen and serum creatine). The levels of PS and PE phospholipids showed approximately 10% increase, whereas levels of SPH and PA remain the same (Feldman et al., 1982).

Nonclerq et al. (1992) suggest that gentamicin induced tubular injury elicits a process of renal tissue repair that involves an increase of cell turnover in tubular epithelium. Exocytosis of lysosomal contents into the tubular lumen and sloughing of injured cells may contribute to an increase in phospholipid excretion. In a study by Josepovitz et al. (1986), Sprague–Dawley rats injected with gentamicin (100 mg/kg/day for 4 days) excreted a progressive increase of phospholipids in the urine compared to controls. The total amount of phospholipids in urine increased approximately 5-fold.

Results of this study show increases in specific species of PI, PC, and PE phospholipids in urine of gentamicin treated rats. These observations are in agreement with changes within PL profile of renal cortex (Feldman et al., 1982). However, changes in levels of PS were not observed. The levels of several phospholipid species containing arachidonic acid (C20:4) and docosahexaenoyl acid (C20:6) were increased. This possibly suggests that there is decreased sensitivity towards hydrolysis of long chain polyunsaturated fatty acids.

Treatment with amiodarone (150 mg/kg/day), azithromycin (30 mg/kg/day), and gentamicin (100 mg/kg/day) induced phospholipidosis in Sprague–Dawley rats after 10 consecutive days of treatment. When examined by electron microscopy, tissues (liver, lung, kidney, mesenteric lymph node) show abnormal accumulation of lamellated membranous inclusions (myeloid bodies). The degree of accumulation was treatment and tissue dependent. Each treatment likely has a different affinity for phospholipids and effect on lipid metabolism. Tissue differences may be due to cellular lipid profile, rate of cellular membrane restructuring, and rate of cellular turnaround (lifetime).

Histological examination of tissues from gentamicin treated rat shows a relatively moderate accumulation of lamellar inclusions in kidney and liver, a mild accumulation in lung, and a slight accumulation in lymph node. Results for kidney are similar to observations made by Beauchamp et al. (1990) showing prominent myeloid bodies in kidney proximal tubular cells of Sprague–Dawley rats treated with a single dose (100 mg/kg) gentamicin. Toubeau et al. (1986) and Kishore et al. (1992) also report the characteristic appearance of multilamellar myeloid bodies in renal cortex of Sprague–Dawley rats treated with gentamicin. Morphological alterations are accompanied by changes in total PL levels. Gentamicin treatment (100 mg/kg/day for 2 days) was reported to increase total PL levels in Sprague–Dawley rat renal cortex by about 15% (Feldman et al., 1982). Results of a study by Josepovitz et al. (1986) indicate that gentamicin treatment (100 mg/kg/day) increases total renal cortical PLs in Sprague–Dawley rats after 24 h. A plateau at approximately plus 10% was reached after three consecutive daily injections of gentamicin.

Examination of tissues from amiodarone treated rat shows a relatively moderate accumulation of lamellar inclusions in lung and kidney and slight accumulation in liver and lymph node. These results are similar to observations made by previous studies. Mortuza et al. (2003) detected lipid accumulation (foamy macrophages) in lung and liver of Sprague–Dawley rats administered amiodarone (150 mg/kg) daily for 7 consecutive days. Mazue et al. (1984) detected the accumulation of phospholipids in several tissues from Fisher and Sprague–Dawley rats receiving 150 mg/kg/day amiodarone for 2 consecutive weeks. Phospholipid accumulation appeared to start in the jejunal mucosa and Peyer's patches followed by accumulation in the mesenteric lymph nodes and then other organs, particularly lungs. Fisher rats showed increased sensitivity compared to the more moderate response of Sprague–Dawley rats. Similarly, Reasor et al. (1996) detected accumulation of lamellar inclusions in alveolar macrophages of Fisher rats (numerous inclusions) and Sprague–Dawley rats (occasional inclusions) treated with amiodarone (100 mg/kg/day, p.o.) for 7 consecutive days. Results of our study are also similar to observations made for amiodarone treated Wistar rats by Rabkin (2006). Total phospholipid content of lung, kidney, and skeletal muscle was increased after 3 consecutive weeks of amiodarone treatment (20 mg/kg/day, i.p.).

Azithromycin treated rats show a mild accumulation of lamellar inclusions in lung and kidney. Phospholipidosis was not observed in liver or lymph node of azithromycin treated rats. Previous azithromycin studies in rats show accumulation of phospholipids in some tissues (eye, liver, gall bladder, kidney,

spleen, and pancreas). The phospholipidosis effect was reversible after termination of azithromycin treatment.

In conclusion, di-docosahexaenoyl (C22:6)-BMP in urine was identified as a potential common biomarker of tissue phospholipidosis in rats induced by amiodarone, azithromycin, and gentamicin at the dosages used in this study. Compared to controls, gentamicin treated rats showed changes in levels of other urinary phospholipid species (PI, PE, PC) in addition to di-C22:6-BMP. With the development of a suitable assay, di-C22:6-BMP may have potential as an indicator of drug-induced phospholipidosis in some circumstances. However, a mechanism based relationship has yet to be determined between the accumulation of lamellar inclusions in tissue and increased levels of di-C22:6-BMP levels in urine. The relationship may be tissue and treatment dependent. Because BMP is localized specifically within lysosomes, it has a potential to play an early role in the development of lysosomal lamellar inclusions. A time course experiment would assess the value of di-C22:6-BMP as an early predictor of the accumulation of lamellar inclusions in tissues. Likewise, a recovery period experiment would determine whether the development of inclusions and change in urinary di-C22:6 BMP is reversible after cessation of drug treatment. An understanding of these relationships must be established before the full potential of di-C22:6-BMP as an indicator of tissue phospholipidosis can be realized for clinical applications.

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