CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Imaging Mass Microscopy of Kidneys from Azithromycin-Treated Rats with Phospholipidosis

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Accepted for publication
May 29, 2018.

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Drug-induced phospholipidosis is a lysosomal storage disorder characterized by the excess accumulation of tissue phospholipids. Although azithromycin can be used to induce phospholipidosis, no experimental studies evaluating the relationship between drug accumulation and phospholipid localization have been performed. In this study, azithromycin was orally administered to rats for 7 days, and the relationship between drug and phospholipid accumulation was performed using imaging mass microscopy. The administration of azithromycin induced tubular epithelial vacuolation in the inner stripe of the outer medulla of the kidney, consistent with the lamellar bodies that are typical manifestations of drug-induced phospholipidosis. Azithromycin and phospholipid tissue levels were extensively elevated in the kidneys of azithromycin-treated rats. Imaging mass microscopy revealed that both azithromycin and its metabolites were found in the kidneys of azithromycin-treated rats but not in control animals. The vacuolated areas of the kidneys were primarily found in the inner stripe of the outer medulla, consistent with the areas of high azithromycin concentration. Azithromycin was colocalized with several phospholipids-phosphatidylinositol (18:0/20:4), phosphatidylethanolamine (18:0/20:4 and 16:0/20:4), and possibly didocosahexaenoyl (C22:6)-bis(monoacylglycerol) phosphate, a putative biomarker of drug-induced phospholipidosis. In summary, we found correlations between regions of kidney damage and the accumulation of azithromycin, its metabolites, and phospholipids using imaging mass microscopy. Such analyses may help reveal the mechanism and identify putative biomarkers of drug-induced phospholipidosis. (*Am J Pathol* 2018, 188: 1993–2003; <https://doi.org/10.1016/j.ajpath.2018.05.018>)

Phospholipidosis is a lysosomal storage disorder that is characterized by the excess accumulation of phospholipids in the kidney, liver, lung, heart, and other organs in both humans and animals. The amphiphilic regions of cationic drugs enter the phospholipid bilayer of the plasma membrane and become localized to the lysosome during phagocytosis, pinocytosis, and plasma membrane recycling. Many cationic drugs cause drug-induced phospholipidosis (DIPL); it is well known that DIPL can be induced by macrolide antibiotics, such as azithromycin (AZM),¹ erythromycin,² and telithromycin.³ AZM was the first clinically developed antibiotic in the azalide subclass of macrolides; these molecules are characterized by the expansion of the 14-membered aglycone ring containing an endocytic ionizable nitrogen.⁴ Compared with erythromycin, AZM displays several interesting properties, such as

exceptionally high levels of tissue accumulation and retention.⁵ Cell fractionation studies revealed that most AZM accumulates in lysosomes,^{6,7} which can cause typical lysosomal phospholipidosis.⁸ Indeed, treatment with AZM has been shown to induce phospholipidosis in Sprague-Dawley rats after 10 consecutive days of treatment. When examined by electron microscopy, kidney sections display an abnormal accumulation of lamellated membranous inclusions,¹ which are the morphologic hallmarks of DIPL.⁹ Although these previous studies showed that treatment with AZM can induce typical DIPL in rat kidneys, it is not clear that the primary insult occurs in this tissue.^{1,8,9} DIPL involves the trapping of drugs within late endosomes/

Disclosures: All authors are employees of Taisho Pharmaceutical Co, Ltd.

lysosomes, followed by the gradual accumulation of drug-phospholipid complexes within the internal lysosomal membrane complex.¹⁰ Thus, it may be possible that AZM and drug-phospholipid complexes accumulate in kidney proximal tubular cells, which have highly developed endocytic complexes.¹¹

Although phospholipids play important roles in myriad biological processes, current phospholipid visualization methods are unable to discriminate the specific molecular lipid moieties. Recently, Tanaka et al¹² used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) imaging mass spectrometry (IMS) to detect biomolecules and drugs without significant fragmentation.¹³ To date, MALDI-TOF IMS has been successfully applied to the analysis of lipids,¹⁴ glycolipids,¹⁵ low-molecular-weight compounds,^{16,17} peptides, and proteins¹⁸ in animals. Specifically, phospholipids that are ionized as either phosphate anions or nitrogen-centered cations (and thus generate abundant positive or negative ions) during the MALDI process¹⁹ are best used as analytical targets for MALDI-TOF IMS. Moreover, the use of MALDI-TOF IMS for the label-free detection and differentiation of compounds and metabolites is a major advantage over autoradiography, which is the standard method used to investigate the spatial distribution of drug candidates.²⁰

Using MALDI-TOF IMS, it was examined whether the oral administration of AZM to rats for 7 days would result in a correlation between the morphologic damage to the kidney and the accumulation of AZM and/or its metabolites. A microscope was coupled to a high-resolution atmospheric pressure—MALDI-TOF analyzer,²¹ and the precise kidney regions in which the drug was distributed were visualized. In addition, the distribution of several specific phospholipids associated with DIPL and AZM was assessed.

Materials and Methods

Chemicals and Animals

AZM was purchased from the Tokyo Chemical Industry Co, Ltd (Tokyo, Japan). Phosphatidylinositol (PI) (18:0/20:4), phosphatidylethanolamine (PE) (16:0/20:4), and PE (18:0/20:4) standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Six-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan) and maintained under a 12-hour light/dark cycle (lights on at 7:15 AM), controlled temperature ($23^{\circ}\text{C} \pm 2^{\circ}\text{C}$), and relative humidity ($50\% \pm 20\%$); they were fed a commercial diet (MF; Oriental Yeast Co, Ltd, Tokyo, Japan) and water ad libitum. The care of rats and the present protocols complied with the General Consideration for Animal Experiments and were approved by the Ethics Committee for the Treatment of Laboratory Animals at Taisho Pharmaceutical Co, Ltd (Saitama-shi, Japan).

Sample Collection

Rats were divided into three groups of five animals each and orally administered 800 or 1600 mg/kg AZM or vehicle (0.5% methyl cellulose; MC400; Wako Pure Chemical Industries, Ltd, Osaka, Japan) for 7 days. Twenty-four hours after the final dose was administered, the rats were anesthetized using isoflurane and sacrificed by severing the abdominal aorta. Blood samples were then collected and centrifuged to obtain plasma or serum samples for blood chemistry analysis. Determination of phospholipids was performed using the clinical analyzer 7180 (Hitachi High-Technologies Corp., Tokyo, Japan).

The kidneys were dissected for histopathological, immunohistochemical, and ultrastructural study, as well as for MALDI-TOF IMS and subsequent bioanalyses. For both the histopathological and immunohistochemical studies, samples were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries Ltd) and embedded in paraffin. For the ultrastructural study, samples were fixed in 2% paraformaldehyde (Sigma-Aldrich Japan G.K., Tokyo, Japan) and 2.5% glutaraldehyde (TAAB Laboratories Equipment Ltd, Berks, UK), post-fixed in 1% osmium tetroxide (Merck Millipore, Billerica, MA) and 1.5% potassium ferrocyanide (Wako Pure Chemical Industries Ltd), stained *en bloc* in 1% uranyl acetate (Merck Millipore), and embedded in Epon 812 (TAAB Laboratories Equipment Ltd). For IMS and subsequent bioanalyses, samples were rapidly frozen in dry ice or liquid nitrogen and stored at -80°C until use.

Histopathological and Immunohistochemical Studies

Routine histology was performed using hematoxylin and eosin staining. To assess the levels of lysosomal-associated membrane protein (LAMP)-2 and aquaporin (AQP)-2, an immunohistochemical analysis of serial kidney sections was performed. For LAMP-2 antigen retrieval, dewaxed sections were boiled in 0.01 mol/L citrate buffer at pH 6.0 (00-5000; Invitrogen, Carlsbad, CA) for 40 minutes and allowed to cool to room temperature. After blocking nonspecific binding with 1% bovine serum albumin in phosphate-buffered saline, the sections were incubated with rabbit anti-LAMP-2 polyclonal antibody (51-2200; 1:200 dilution; 1.25 $\mu\text{g/mL}$; Invitrogen) or rabbit anti-AQP-2 polyclonal antibody (AQP-002; 1:8000 dilution; 0.075 $\mu\text{g/mL}$; Alomone Labs, Jerusalem, Israel) overnight at 4°C , followed by treatment with avidin-biotin peroxidase. Negative controls were incubated with normal rabbit IgG (20304; 1.25 or 0.075 $\mu\text{g/mL}$; Imgenex Corp., San Diego, CA) instead of primary antibodies.

Ultrastructural Study

Ultrathin sections from Epon 812-embedded blocks were generated using an ultramicrotome (Ultracut E;

Reichert-Jung, Vienna, Austria) and stained with uranyl acetate and Sato's lead. The specimens were then subjected to transmission electron microscopy (H-7500; Hitachi, Tokyo, Japan).

MALDI-TOF IMS Study

Frozen kidney samples from AZM- and vehicle-treated rats were sectioned (5 μ m thick) with a cryostat (CM3050; Leica Microsystems, Tokyo, Japan) and then thaw mounted onto an indium tin oxide-coated glass slide (Sigma-Aldrich Japan G.K.). Either a dihydroxybenzoic acid (DHB; 50 mg/mL in 70% methanol and 30% water plus 0.1% trifluoroacetic acid) or a 1,5-diaminonaphthalene (DAN; 10 mg/mL in 80% acetone and 20% water) matrix solution was sprayed (DHB) or sublimated (1,5-DAN) onto the kidney sections. Imaging data were acquired in the positive (DHB) or positive and/or negative (1,5-DAN) ionization mode using an iMScope atmospheric pressure-MALDI-quadrupole ion trap-TOF-MS equipped with a 355-nm Nd/YAG laser (Shimadzu Corp., Kyoto, Japan). The laser was irradiated at 100 shots/spectrum at a frequency of 1000 Hz. The IMS data were collected and analyzed using Imaging MS Solution software version 1.10 (Shimadzu Corp.).

Bioanalysis

Analysis of PI (18:0/20:4), PE (18:0/20:4), and PE (16:0/20:4)

The kidney samples obtained from AZM- and vehicle-treated rats were homogenized in four volumes of distilled water using a MultiBeads Shocker (Yasui kikai Corp., Osaka, Japan). Plasma samples were diluted twofold with distilled water, and the kidney homogenates were diluted 200-fold with distilled water. Methyl-tert-butyl ether/methanol (10:3, v/v, 400 μ L) was added to a 100-mL aliquot of the diluted sample in a polypropylene tube. The tubes were vortexed, placed on a rotary shaker, and mixed for 30 minutes at room temperature. The samples were centrifuged at $3000 \times g$ for 5 minutes at room temperature, and the organic layers were collected into a glass tube and dried under nitrogen. Extracted lipids were dissolved in 100 μ L methanol. Plasma and kidney levels of PI (18:0/20:4), PE (18:0/20:4), and PE (16:0/20:4) were analyzed by liquid chromatography-high-resolution MS (LC-HRMS) on an LTQ Orbitrap XL Hybrid Ion Trap-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The LC system that was used in this study was an Agilent 1260 series high-performance LC (Agilent Technologies, Santa Clara, CA) equipped with an L-column2 ODS (2.0-mm i.d. $\times$ 150 mm, 3.0- μ m particle size, metal free; CERI, Tokyo, Japan). The gradient consisted of 10 mmol/L ammonium formate/formic acid (5000:1, v/v) as solvent A and acetonitrile/2-propanol (1:9, v/v) as solvent B, starting at 50% B, holding for 2 minutes, ramping

to 98% B over 38 minutes, holding for 10 minutes, decreasing to 50% B in 0.01 minutes, and holding for 10 minutes. The flow rate was 0.2 mL/minute, and the column was maintained at 40°C. The samples were subjected to lipid analysis in negative ion-heated electrospray ionization mode. The MS conditions were set as follows: spray voltage, 2500 V; scanning range, m/z 100 to 1000; resolving power, 60,000 for a full scan and 15,000 for a product ion scan; and normalized collision energy for a product ion scan, 35%. The MS data were acquired and analyzed using Xcalibur software version 3.0 (Thermo Fisher Scientific).

Quantitative Analysis of Plasma and Kidney Levels of AZM
Plasma and kidney tissue concentrations of AZM were determined using an LC-tandem MS (MS/MS) system, which consisted of an LC-20AD liquid chromatograph (Shimadzu Corp.) and an API4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA). AZM and an internal standard (erythromycin) were extracted from the samples by protein precipitation with acetonitrile/methanol (9:1, v/v) containing the internal standard, and the mixture was centrifuged at $3639 \times g$ at 4°C for 10 minutes. The resulting supernatant was injected into the LC-MS/MS system. Chromatographic separation was performed on a Shim-pack XR-ODS analytical column (3.0-mm i.d. $\times$ 30 mm, 2.2- μ m particle size; Shimadzu Corp.). The gradient consisted of water/formic acid (1000:1, v/v) as solvent A and acetonitrile as solvent B, starting at 2% B, ramping to 80% B over 1.2 minutes, stepping up to 98% in 0.01 minutes, holding for 0.3 minutes, decreasing to 2% B in 0.01 minutes, and then holding for 0.5 minutes. The flow rate was 1.3 mL/minute, and the column was maintained at 50°C. Mass spectral detection was performed in positive turbo-ion spray ionization mode using multiple reaction monitoring transitions of m/z 749.5 to m/z 591.5 and m/z 734.5 to m/z 158.0 for AZM and the internal standard, respectively. The peak area ratios of the analyte to the internal standard were plotted as a function of the nominal concentrations of the analyte. Quantitation was performed by linear regression with $1/x^2$ weighting. The calibration range was 10 to 10,000 ng/mL in plasma and 50 to 50,000 ng/g in kidneys. The MS/MS data were acquired and analyzed using Analyst software version 1.5.1 (AB Sciex).

Statistical Analysis

All statistical analyses were performed using SAS software version 9.2 (SAS Institute Inc., Cary, NC). All values are reported as means $\pm$ SD. The LC/MS peak areas of plasma and kidney phospholipids were analyzed using the F-test, followed by the *t*-test. Values represent the results of the *t*-test; $P < 0.05$ was considered statistically significant.

Figure 1 Morphologic analysis of azithromycin (AZM)- or vehicle-treated rat kidneys. **A** and **B**: Hematoxylin and eosin staining of kidneys in rats treated with either 800 mg/kg AZM (**A**) or vehicle (**B**). **A**: Mild tubular epithelial vacuolations within the inner stripe of the outer medulla in AZM-treated rats (asterisk). **C**: Electron microscopy of collecting duct epithelial cells in 800 mg/kg AZM-treated rats. Arrows indicate multilamellar bodies in collecting duct epithelial cells. Scale bars: 200 μ m (**A** and **B**); 1.4 μ m (**C**). N, nucleus.

Results

Histopathological Study of Kidney Sections

It was determined whether AZM induces phospholipidosis in rat kidneys by histopathological and ultrastructural analyses. The histopathological study showed that mild vacuolations were observed in the renal tubules of the inner stripe of the outer medulla (Figure 1A) as well as in the renal tubules of cortex, glomerulus, and transitional epithelium of the kidney, muscular layer of the pelvis, and small arteries in AZM-treated rats. However, there were no obvious vacuolations of similar regions in vehicle-treated rats (Figure 1B). Moreover, the ultrastructural study using transmission electron microscopy confirmed that these vacuolations represent dense lamellar bodies, which are the morphologic hallmarks of DIPL (Figure 1C). Thus, AZM induced typical phospholipidosis in rat kidneys.

Determination of Plasma and Renal AZM and Serum Phospholipid Levels

Twenty-four hours after the last dose on day 7, plasma and renal AZM and total serum phospholipid levels were determined (Table 1). In AZM-treated rat groups, plasma and kidney AZM levels were elevated in a dose-dependent manner, with concentrations in the kidney approximately 200-fold higher than those in plasma. Serum total phospholipids were also increased in a dose-dependent manner in AZM-treated rats compared with vehicle-treated rat.

MALDI-TOF IMS Study

An HRMS spectrum of the AZM standard shows only its protonated molecular ($[M+H]^+$) ion at m/z 749.499 in positive-ion mode with DHB precoated targets (Supplemental Figure S1A). In kidney sections obtained from AZM-treated rats (800 and 1600 mg/kg), two major ions were observed at m/z 749.51 and m/z 591.42, corresponding to the $[M+H]^+$ ions of AZM and the possible descladinose form of AZM, respectively (Supplemental Figure S1, B and C). In addition, an ion at m/z 573.411, likely an $[M+H]^+$ ion of the descladinose dehydroxy-2-ene form of AZM,²² was detected in 1600 mg/kg AZM-treated rat (Supplemental Figure S1C). In the kidney sections of vehicle-treated rat, no drug-related ions were observed (Supplemental Figure S1D).

To evaluate the spatial distribution of AZM and its metabolites in the kidney, low-magnification MALDI-TOF IMS analysis was conducted in positive-ion mode with a DHB precoated matrix. In AZM-treated rats, the $[M+H]^+$ ions of AZM and its two metabolites were observed in the renal cortex and outer medulla, irrespective of the AZM dose used; particularly strong signals from these ions were

Table 1 Plasma and Renal Exposures of Azithromycin and Serum Phospholipid Levels at 24 Hours after 7-Day Repeated Oral Administration of Azithromycin in Rat (800 or 1600 mg/kg per Day) on Present IMS Study

Dose, mg/kg per day*	Azithromycin [†]		Total phospholipids [‡]
	Plasma, ng/mL	Kidney, ng/g	Serum, mg/dL
0 [§]	NC	1160 ± 1320 [¶]	72.4 ± 6.0
800 [§]	7370 ± 5140	2,810,000 ± 870,000	186.0 ± 27.8
1600 [§]	18,500 ± 8720	4,230,000 ± 2,480,000	194.0 ± 49.4

Data are expressed as means ± SD. Lower limit of quantification: 10 ng/mL (plasma) and 50 ng/g (kidney).

*Azithromycin was administered once a day for 7 days.

[†]Concentrations of azithromycin were determined using liquid chromatography–tandem mass spectrometry.

[‡]Serum phospholipids were measured enzymatically on a Hitachi 7180 clinical analyzer.

[§]For 0 and 800 mg/kg per day, *n* = 5; for 1600 mg/kg per day, *n* = 3 because of animal deaths.

[¶]Contamination occurring at necropsy from experimental instruments (eg, scissors and knives).

IMS, imaging mass spectrometry; NC, not calculated (all five samples were below the lower limit of quantification).

detected at 1600 mg/kg AZM (Figure 2B) compared with 800 mg/kg AZM (Figure 2A). No drug-related ions were detected in vehicle-treated rat (Figure 2C).

High-magnification MALDI-TOF IMS analysis of the cortex-medulla boundary zone was conducted in positive-

ion mode with a 1,5-DAN precoated matrix. AZM and its metabolites were detected, with strong signals at *m/z* 749.512 (AZM) and *m/z* 591.420 (descladinose AZM) and weak signals at *m/z* 573.413 (descladinose dehydroxy-2-ene AZM) and *m/z* 735.4986 obtained from 800 mg/kg AZM-

Figure 2 Optical and low-magnification imaging mass spectrometry images of azithromycin (AZM) and its metabolites in renal sections obtained from AZM-treated rats [800 mg/kg (A) or 1600 mg/kg (B)] and vehicle-treated rat (C). Scale bars = 1000 μ m (A–C).

Figure 3 Optical and high-magnification imaging mass spectrometry images of azithromycin (AZM) and its metabolites in renal sections obtained from 800 mg/kg AZM-treated rat (**A**) and vehicle-treated rat (**B**). The asterisks indicate the outer medulla. Scale bars = 200 μ m (**A** and **B**).

treated rat (Figure 3A). The ion detected at *m/z* 735.4986 was not observed in the DHB matrix and is probably the desmethyl form of AZM. No drug-related ions were detected in vehicle-treated rat (Figure 3B). All signals from AZM and its metabolites were extensively distributed in the tubules of the inner stripe of the outer medulla and were generally consistent with the regions in which mild vacuolations were observed (Figure 1A). Furthermore, AZM was either linearly or radially distributed in the renal cortex (Figure 3A).

Morphologic Properties of the Renal Cortex in Which AZM Was Localized

To evaluate the morphologic properties of the region in which AZM was distributed in the renal cortex, serial tissue sections were obtained and analyzed. An investigation of hematoxylin and eosin–stained sections showed evidence of linear vacuolation of tubules within the renal cortex in 800 mg/kg AZM-treated rats, consistent with the histopathological finding of DIPL (Figure 4A); analogous changes were not observed in vehicle-treated rats (Figure 4B). Subsequently, LAMP-2 immunohistochemical analysis showed that linearly vacuolated tubules in 800 mg/kg AZM-treated rats were strongly positive for LAMP-2 (Figure 4C). However, nonvacuolated tubules in vehicle-treated rats were either faintly positive or negative for this protein (Figure 4D). In addition, an immunohistochemical analysis of AQP-2, a marker of the renal collecting duct, revealed that linearly vacuolated tubules in 800 mg/kg AZM-treated rat (Figure 4E) and nonvacuolated tubules in vehicle-treated rat (Figure 4F) were positive for AQP-2, confirming

the location of the collecting duct. From these results, the region of linear or radial distribution of AZM in the renal cortex was consistent with the vacuolated collecting duct.

Figure 4 Serial kidney sections from azithromycin (AZM)-treated rats (**A**, **C**, and **E**) and vehicle-treated rats (**B**, **D**, and **F**). **A**: Vacuolations in renal tubules in AZM-treated rats (arrows). **C** and **D**: Lysosomal-associated membrane protein-2 immunohistochemical analysis in AZM-treated rats (arrows; **C**) and vehicle-treated rat (arrows; **D**). **E** and **F**: Aquaporin-2 immunohistochemical analysis in AZM-treated rat (arrows; **E**) and vehicle-treated rat (arrows; **F**). Scale bars = 50 μ m (**A–F**).

Figure 5 Optical and imaging mass spectrometry images of specific phospholipids at the cortex-medulla boundary zone of kidney sections obtained from 800 mg/kg azithromycin (AZM)-treated rat and vehicle-treated rat. Colocalization of some phospholipids with AZM (m/z 749.512) in 800 mg/kg AZM-treated rat. The **asterisks** indicate the outer medulla. Scale bars = 200 μ m. 22:6 BMP, di-docosa-hexaenoyl (C22:6)-bis(monoacylglycerol) phosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

Relationship between the Spatial Distribution of AZM and Specific Phospholipids

A MALDI-TOF IMS analysis of kidney sections in positive- or negative-ion mode with a 1,5-DAN precoated matrix showed the presence of a strong AZM signal in the inner stripe of the outer medulla (Figure 5). Furthermore, hierarchical cluster analysis using Imaging MS Solution software revealed that several deprotonated molecular ($[M-H]^-$) ions of possible phospholipids colocalized with AZM, including m/z 885.545, m/z 766.526, m/z 738.493, and m/z 865.480, which correspond to PI (18:0/20:4), PE (18:0/20:4), PE (16:0/20:4), and di-docosa-hexaenoyl

(C22:6)-bis(monoacylglycerol) phosphate (BMP),¹ respectively (Figure 5). These ions were not observed or evenly distributed within the inner stripe of the outer medulla of vehicle-treated rat under similar conditions (Figure 5). In addition, some unidentified substances, potentially lipids, also colocalized with AZM within the outer medulla (Supplemental Figure S2). Furthermore, ion was detected: at m/z 760.580 in the tubules of the inner stripe of the outer medulla in both groups. On the basis of their theoretical masses and spatial distribution patterns, it is conceivable that they were the $[M+H]^+$ ion for phosphatidylcholine (34:1).²³ Many unidentified substances, potentially lipids, noncolocalized with AZM within the outer medulla (Supplemental Figure S3).

LC-HRMS Analysis of PI (18:0/20:4), PE (18:0/20:4), and PE (16:0/20:4)

To confirm the presence of PI (18:0/20:4), PE (18:0/20:4), and PE (16:0/20:4), which are known to increase the rate of urinary excretion in rats with DIPL,¹ renal homogenates were subjected to LC-HRMS analysis. As a result, the lipids with peak retention times of 33.2, 35.4, and 32.9 minutes were identical to those for PI (18:0/20:4), PE (18:0/20:4), and PE (16:0/20:4), respectively (Figure 6, A and B), after comparing both HRMS and MS² spectra with those of authentic standards, along with their chromatographic behaviors (Figure 6, C and D). Among these phospholipids, kidney PI (18:0/20:4) and plasma PE (16:0/20:4) were increased significantly (1.44- and 1.65-fold increases, $P < 0.001$ and $P < 0.05$, respectively) after treatment with AZM (Figure 6, E and F).

Discussion

The present study showed that the spatial distribution of AZM and its metabolites is highly correlated with the damaged regions of the rat kidney. Although there are IMS drug delivery studies in which anticancer drugs were reportedly distributed to cancerous lesions,²¹ to our knowledge, the IMS study that revealed a correlation between damaged kidney regions and drug accumulation was limited.^{24,25} The high concentration/accumulation of a drug and/or its metabolites in tissues after repeated administration has been shown by MALDI-TOF MS analysis to be involved in the increasing responses to such drugs.²⁶ Furthermore, MALDI-TOF IMS is a powerful drug discovery tool for the study of correlations between test compounds and histopathological changes in *in vivo* toxicologic studies.

In the MALDI IMS study, TOF detectors are extremely sensitive and, therefore, well suited to MALDI IMS, where small numbers of ions are produced for each pixel. The popularity of this instrument has led to several commercial

Figure 6 Identification of phosphatidylinositol (PI; 18:0/20:4), phosphatidylethanolamine (PE; 18:0/20:4), and PE (16:0/20:4) in rat kidney samples (A–D) and the effects of 800 mg/kg azithromycin (AZM) treatment on phospholipid levels (E and F). Renal extracts from AZM-treated rats were subjected to liquid chromatography–high-resolution mass spectrometry (LC-HRMS) to obtain mass chromatograms (B) and MS^2 spectra (D) of the target phospholipids. The high-performance LC retention times of the authentic standards (A) and the product ion mass spectrum (C) are shown. The effects of AZM treatment on the renal (E) and systemic (F) phospholipid levels were investigated by measuring the HRMS chromatographic peak intensities of the renal and plasma phospholipids of interest. Data are shown as means $\pm$ SD (E and F). $n = 5$ (E and F). * $P < 0.05$, *** $P < 0.001$ versus control (t -test). AA, automatic integrated area of a detected peak; MS/MS, tandem MS; RT, retention time.

companies developing and producing TOF-based IMS systems, including hardware and software. But limitations of this technology for small molecule imaging are in the limited MS/MS capability relative to other platform [eg, Fourier transform ion cyclotron resonance (FTICR)].²⁷ FTICR and orbital trapping provide the highest mass resolution and accuracy of all mass analyzers.²⁸ In fact, with the superior mass resolving power of the FTICR, it was easily possible to baseline separate three compounds and show that each of these chemical species had distinct spatial

distribution in tissue; this was not possible in the TOF experiment.²⁹ Since then, MALDI-FTICR IMS has become increasingly popular in the pharmaceutical industry for the identification and localization of drugs and metabolites in tissues.³⁰ Although some advantages of FTICR compared with TOF have been reported, the highest resolution and mass accuracy of FTICR are not required in the present IMS study because AZM, three related metabolites, and some phospholipids can easily be separated by m/z , and accumulated in inner stripes of outer medulla. However, the

spatial distribution of AZM in renal cortex could not be resolved in the present TOF study; the linear or radial distributions of AZM were consistent with the vacuolated collecting duct demonstrated by classic microscopic analysis.

The results of our study show that 24 hours after the last dose of a 7-day oral administration, AZM accumulated in the tubular epithelium of the inner stripe of the outer medulla and cortical collecting duct. However, it was previously reported that a strong fluorescent signal was detected in the kidney with a patchy distribution in the proximal tubule (glomeruli were negative) 2 hours after mice were subjected to a single i.p. administration of fluorescently labeled AZM.³¹ After single or repeated administration, AZM was observed to be localized primarily in renal proximal tubules within the well-developed endocytic/lysosomal complex,¹¹ and undigested AZM accumulated in lysosomes within the inner stripe of the outer medulla and collecting duct, but was not observed within these well-developed endocytic complexes. The inner stripe of the outer medulla is composed of the collecting duct, thin limb, ascending loop component, and ascending straight distal tubules; it does not contain proximal tubules.³² Most intracellular AZM has been shown to be located in acidic organelles, such as lysosomes³³; this finding is consistent with results of the present study, because these regions are strongly positive for LAMP-2, a lysosomal marker protein. Intracellular AZM was also found in vacuolated collecting ducts. In addition, LAMP-2 immunohistochemistry could be used to evaluate DIPL.^{34,35} It was also reported that AZM not only accumulates in macrophages, which have a high endocytic ability, but also in lysosomal lamellar bodies in human meibomian epithelial cells³⁶ and leukocytes,³⁷ which do not have well-developed endocytic/lysosomal complexes. Long-term treatment with aminoglycosides has been shown to induce DIPL in distal tubules and collecting ducts, locations where such drugs are taken up by endocytosis.³⁸ These studies suggested the reason why AZM was accumulated in the inner stripe of kidney outer medulla and collecting duct of cortex, which do not have well-developed endocytic complex, after repeated administrations.

Telithromycin, an antibiotic belonging to the macrolide group, has a potential renal DIPL, but these lesions were not described in detail.³⁹ Antibiotic gentamicin and some compounds have potential to DIPL in the convoluted proximal tubules, but others may induce it in the straight tubules.⁴⁰ The present study showed that AZM induced DIPL mainly in collecting duct, and tilorine, an interferon inducer, causes DIPL in the distal convoluted tubules as a limited example of a primary toxic response for the distal nephron.⁴¹ In tissues, although common primary targets in DIPL are lung, liver, and peripheral blood cells, numerous other targets, which are lens and cornea of eyes, spleen, and brain, have been reported.^{1,42} The kidney appears to be less sensitive to DIPL than other tissues. Multiple factors affect

the ability to produce renal DIPL, including species, strain, tissue affinity, and pharmacokinetic properties.⁴³ Therefore, it is appropriate that the lesions that showed DIPL were different with each compound in kidney, which has structural and functional heterogeneity.³²

The use of MALDI-TOF IMS allowed the visualization of the accumulation of AZM and its metabolites (descladinose AZM, descladinose dehydroxy-2-ene AZM, and desmethyl AZM) within the inner stripe of the outer medulla. AZM has been shown to be metabolized into descladinose AZM and desmethyl AZM in rats after its oral administration.⁴⁴ Desmethyl AZM was reportedly identified as two different metabolites: 3'-N-desmethyl AZM and 6-N-desmethyl AZM.⁴⁴ Because only 3'-N-desmethyl AZM has been found in rat urine after oral AZM administration,⁴⁴ the m/z 735.4986 ion found in rat kidneys, corresponding to the $[M+H]^+$ ion of desmethyl AZM, might be derived from 3'-N-desmethyl AZM. Descladinose dehydroxy-2-ene AZM has only been previously identified in ball python but not in mammals.²² Interestingly, our study showed that m/z 573.411, which corresponds to the $[M+H]^+$ ion of descladinose dehydroxy-2-ene AZM, was detected in AZM-treated rats.

The present study showed that AZM and several phospholipids were colocalized in the inner stripe of the outer medulla. In addition, rat renal extracts were subjected to LC-HRMS analysis, resulting in the identification of PI (18:0/20:4), PE (18:0/20:4), and PE (16:0/20:4). These three phospholipids were reportedly increased in rat urine after treatment with gentamycin, which can cause DIPL in the kidney.¹ DIPL involves the trapping of drugs within late endosomal/lysosomal compartments, followed by the gradual accumulation of drug-phospholipid complexes within the internal lysosomal membrane.¹⁰ AZM and its colocalized phospholipids are believed to accumulate in these complexes within the inner stripe of the outer medulla. In a recent report, high-resolution subcellular imaging using Nano Secondary Ion Mass Spectrometer and electron microscopy showed that ¹²⁷I from labeled amiodarone and ³¹P from labeled phospholipids accumulated in multilamellar lysosomes in lung macrophages.⁴⁵ In addition, the present study revealed that the m/z 865.48 ion, which is thought to be the $[M-H]^-$ ion of 22:6 BMP, a putative biomarker of DIPL,¹⁰ colocalized with AZM. The repeated oral administration of AZM to rats not only induces DIPL in the kidney^{1,46} but also increases 22:6 BMP levels in rat urine. Subsequently, 22:6 BMP was specifically localized in intravesicular vesicles of late endosomes/lysosomes, where it is able to participate in protein and cholesterol sorting.⁴⁷ Moreover, the phospholipid at m/z 865.48 was shown to accumulate in cerebella of mice with Sandhoff disease, a lysosomal storage disorder similar to DIPL; these authors identified the phospholipid as 22:6 BMP using IMS.⁴⁸ These results support the validity of our structural interpretation of the compound at m/z 865.48. Although 22:6 BMP is regarded as a reliable biomarker of tissue DIPL,¹⁰

its instability makes it difficult to measure: The ester bonds between the fatty acid and glycerol are easily cleaved in the collision cell of the spectrometer during LC-MS/MS.⁴⁹ IMS is a simple and highly sensitive method and requires no extraction step for the analysis of 22:6 BMP.

The compounds at m/z 760.580 were found to be distributed similarly in kidneys of both AZM- and vehicle-treated rats and colocalized with AZM. A previous report identified the compound at m/z 760.580 as phosphatidylcholine (34:1), which was observed within the inner stripe of the mouse outer medulla.²³ Therefore, it appears that the compound at m/z 760.580 is derived from phosphatidylcholine (34:1), and the accumulated region of AZM was the inner stripe of the outer medulla.

PI (18:0/20:4) is considered a housekeeping lipid that is uniformly distributed in both healthy and polycystic rat kidneys⁵⁰; the present study revealed that this phospholipid accumulated in the inner stripe of the outer medulla and increased in the kidney after repeated AZM treatment. In addition, PE (18:0/20:4) and PE (16:0/20:4) accumulated in the inner stripe of the outer medulla after AZM administration, but their levels did not change in whole kidney samples.

In conclusion, a correlation was found between damaged kidney regions and the accumulation of AZM using MALDI-TOF IMS. These observations are useful for analyzing the formation of drug-phospholipid complexes in lipid storage disorders, such as DIPL. Vacuolated areas (confirmed by histopathology) and areas containing high levels of AZM (observed by IMS) were confined to similar regions, including the inner stripe of the outer medulla. Moreover, a MALDI-TOF IMS analysis of the kidney showed that AZM and several phospholipids, including 22:6 BMP, colocalized in the outer medulla. These results suggest that such analytical tools may help reveal the toxicity mechanism and identify putative biomarkers of DIPL.

Acknowledgments

We thank Yuko Yoneyama for histopathological experiments, Yu Kajiyama for blood chemistry, Aya Izaki and Satoshi Tsuji for urine chemistry, and Kohnosuke Kinoshita for critical reading of the manuscript.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2018.05.018>.

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