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EXPERIMENTAL THERAPEUTICS

Characterization of Amikacin Drug Exposure and Nephrotoxicity in an Animal Model

Katrina Chan,[a] Kimberly R. Ledesma,[b] Weiqun Wang,[b] Vincent H. Tam[a,b]

[a]Department of Pharmacological and Pharmaceutical Sciences, University of Houston College of Pharmacy, Houston, Texas, USA
[b]Department of Pharmacy Practice and Translational Research, University of Houston College of Pharmacy, Houston, Texas, USA

ABSTRACT Despite excellent *in vitro* activity, aminoglycosides are used conservatively to treat multidrug-resistant bacterial infections due to their associated nephrotoxicity. Aminoglycosides are known to accumulate in the kidneys, but the quantitative relationship between drug exposures and nephrotoxicity is not well established. To bridge the knowledge gap, the objective of this study was to develop an animal model with clinically relevant conditions to mimic human disease progression. Single-dose pharmacokinetics were studied in Sprague-Dawley rats dosed either with 100 or 500 mg/kg of body weight of amikacin subcutaneously. Serial blood samples were collected, and serum amikacin concentrations were measured using liquid chromatography tandem mass spectrometry. Rats were also dosed with amikacin once daily for up to 10 days; blood samples were taken at baseline and daily to detect nephrotoxicity (defined as doubling of serum creatinine from baseline). Kidneys from both studies were harvested from selected rats, and amikacin concentrations in renal tissues were measured. A dose-dependent increase in systemic area under the curve (AUC) was observed, which ranged from approximately 1/3 (AUC of 53 mg·h/liter) to 3 times (AUC of 650 mg·h/liter) the expected exposure resulting from standard dosing in humans. Nephrotoxicity was significantly higher in rats given 500 mg/kg (100% versus 30%, $P = 0.003$). Kaplan-Meier analysis also showed a significant difference in nephrotoxicity onset between the two groups ($P = 0.001$). Finally, analysis of the renal tissues showed that the accumulation of amikacin could be associated with nephrotoxicity. These results are consistent with clinical observations, which support using this model in the future to investigate an intervention(s) that can be used clinically to alleviate nephrotoxicity.

KEYWORDS aminoglycosides, acute kidney injury, liquid chromatography, pharmacokinetics, high performance liquid chromatography

Infections caused by multidrug-resistant Gram-negative bacteria, such as *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, continue to occur at astounding rates worldwide (1). Many first-line antimicrobials have been rendered ineffective, and patient outcomes are usually poor (2). There have also been challenges in maintaining the antimicrobial development pipeline, further limiting effective treatment options. As a result of these hurdles, there has been a renewed interest in older, but still effective, antimicrobials, such as the aminoglycosides.

The aminoglycosides are a class of antimicrobials that remain highly effective against Gram-negative bacteria *in vitro* (3). However, these antimicrobials are limited in their clinical use because of their associated nephrotoxicity (4). A recent study showed that aminoglycoside-associated nephrotoxicity occurred in 58% of critically ill patients in intensive care units (ICU) requiring such therapy (5). In view of this undesirable adverse effect profile, aminoglycosides are mostly used in last-resort cases when first-line options have failed.

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Address correspondence to Vincent H. Tam, vtam@uh.edu.

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Clinically, aminoglycoside-associated nephrotoxicity is a significant risk factor of mortality in hospitalized patients (6). It has been shown that aminoglycoside-associated nephrotoxicity is dose dependent and correlated with the duration of therapy (7). It is also known that certain risk factors, such as concurrent treatment with vancomycin, preexisting heart disease, and preexisting kidney disease, are associated with the increased occurrence of kidney injury (6, 8). Therefore, overcoming nephrotoxicity associated with aminoglycoside therapy could significantly improve patient outcomes.

The mechanism of aminoglycoside-associated nephrotoxicity has been substantially investigated. Prior research has reported preferential uptake and accumulation of aminoglycosides in the kidneys, which then causes significant physiological changes primarily in the proximal tubule cells. This triggers multiple cellular cascades that ultimately lead to cell death (4). These cascades are believed to be a result of interactions between the very polar aminoglycosides and lipid-based cellular structures. These cascades include changes to lysosomal morphology affecting filtration efficiency, interactions with phospholipid membranes causing aggregation, shedding, and the formation of lesions, impairment of lipid enzymes and transporters, and impairment of mitochondrial functions. Collectively, these cascades are associated with cell necrosis that leads to a decline in kidney function and, in some cases, permanent kidney damage.

Relatively speaking, knowledge regarding the quantitative accumulation of aminoglycosides in the kidneys is less well known. It has been established that drug renal uptake is via a saturable transporter process, but whether there is threshold accumulation needed to trigger downstream damaging cascades is not well elucidated (9). To provide additional insights on this quantitative aspect of nephrotoxicity, an appropriate animal model that mimics the natural course of aminoglycoside-associated nephrotoxicity with clinically relevant conditions must be established. Therefore, this study aims to (i) establish a clinically relevant animal model and (ii) investigate the relationship between aminoglycoside exposure and nephrotoxicity. Clinically, gentamicin and amikacin are two of the most commonly administered aminoglycosides. Amikacin is examined in this study because it is semisynthetically derived from kanamycin and has been found to be effective against a wide range of Gram-negative bacteria (10). While there are immunoassays for gentamicin (11), these methods generally do not quantify the individual components of gentamicin (C1, C1a, and C2) and report the results in agglomerates. Consequently, amikacin was preferred in view of its single-component characteristic.

RESULTS

Single-dose pharmacokinetics study. The concentration-time profiles were reasonably characterized by the model (Fig. 1), and the best-fit parameters are shown in Table 1. To establish the human clinical relevance of amikacin dosing, the areas under the curve (AUC) observed for both dosing groups were used for correlation with the equivalent human doses. From these values, the human equivalent doses ranged from approximately 3× less than (4.6 mg/kg of body weight) to 3× more than (56.3 mg/kg) the standard human dose of amikacin (15 mg/kg daily). The spectrum of drug exposure was deemed relevant in view of the intersubject variability reported previously in neutropenic patients (12). We noted the difference in systemic AUC between the two groups of rats was not proportional to the dose. Renal tissues showed a numerically higher amikacin concentration 6 h after a single 500-mg/kg dose, but the difference was not significant (3.9 mg/g versus 2.7 mg/g, $P = 0.9$).

Nephrotoxicity study. Overall, nephrotoxicity was observed in 65% of the rats in the 2 dosing groups ($n = 10$ per group). All the rats in the 500-mg/kg group reached the endpoint, compared to only 30% in the 100-mg/kg group ($P = 0.003$). Additionally, rats in the 500-mg/kg group reached the nephrotoxicity endpoint earlier than those in the 100-mg/kg group ($P = 0.001$) (Fig. 2). Median time to reach nephrotoxicity endpoint in the 500-mg/kg group was 4 days, and that in the 100-mg/kg group was >10 days.

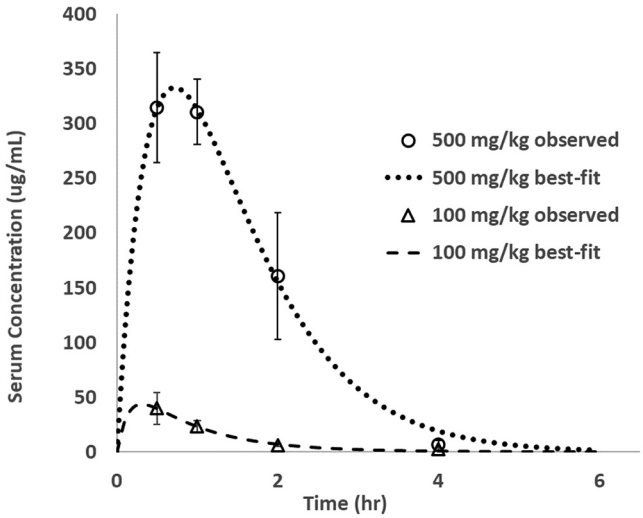

FIG 1 Pharmacokinetic profiles of a single dose of amikacin. $n = 4$ rats per group. Data are shown as means ± SD.

Moreover, a higher amikacin concentration in renal tissues was observed when nephrotoxicity was observed in the rats (30.9 mg/g versus 6.5 mg/g, $P = 0.009$) (Fig. 3).

DISCUSSION

Aminoglycosides have been used clinically and with much success against Gram-negative bacterial infections for decades (10). However, the occurrence of kidney injury was markedly high, so the use of aminoglycosides was relegated to the most desperate cases when all other options have failed, and dosing must be closely monitored (13). With the rise of multidrug-resistant infections and the limited antimicrobial pipeline, there has been a renewed interest in the aminoglycosides, with the hope that novel insights can address some of these toxicity concerns.

Numerous previous studies have attempted to elucidate the mechanism of nephrotoxicity associated with aminoglycosides. Most studies corroborate that the accumulation of aminoglycosides triggers a cascade of processes resulting in cell death and acute kidney injury (14, 15). In some cases, significant loss of kidney function is not reversible. Since many of these pathogenic pathways are better understood, research pertaining to disrupting these processes constitutes a large portion of the resurgence of interest in aminoglycosides. This is reflected in the amount of literature available on postuptake events that ultimately lead to kidney damage. Relatively speaking, fewer studies have focused on elucidating the mechanism of uptake of the aminoglycosides into the kidneys. These studies provide circumstantial evidence of an endocytic process, but anything beyond that is sparse (9). A few attempts to mitigate the uptake of

TABLE 1 Best-first parameter estimates for the single-dose pharmacokinetics study

	Best-fit estimates	
Parameter[a]	100 mg/kg	500 mg/kg
R^2	0.997	0.998
k_a (h^{-1})	1.20	1.40
k_{e1} (h^{-1})	6.77	1.39
$t_{1/2}$ (h)	0.10	0.50
V (liter/kg)	0.28	0.55
$AUC_{0\rightarrow\infty}$ (mg·h/liter)	53.0	649.7

[a]R^2, coefficient of correlation; k_a, absorption rate constant; k_{e1}, elimination rat constant; $t_{1/2}$, terminal elimination half-life; V, volume of distribution; $AUC_{0\rightarrow\infty}$, area under the concentration-time curve from 0 h to infinity.

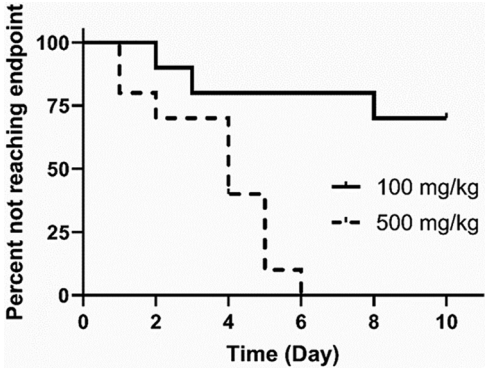

FIG 2 Time to onset of nephrotoxicity. $n = 10$ per group, $P = 0.001$.

aminoglycosides by the kidneys have also been made, but nothing tangible that can be applied clinically has been established (16).

Other models in larger mammalian species have been used in the past to study aminoglycoside nephrotoxicity (17, 18). These models are often associated with rigorous compliance regulations and higher costs. As such, they are more suitable for validating promising results (from rats) to justify downstream investigations in humans. Rats are more practical as a screening tool, since they are more phylogenically reduced. With a better understanding of how to correlate findings in the rat model to clinical observations in humans, potential interventions to reduce aminoglycoside-associated nephrotoxicity can be evaluated efficiently.

Rodents have more rapid drug clearance than humans, so in this study the doses used were selected to mimic clinical exposures when standard doses are given to humans. These ranged from approximately 1/3 (AUC of 53 mg·h/liter) to 3 times (AUC of 650 mg·h/liter) the standard clinical exposure (AUC of 173 mg·h/liter) and likely accounted for the variability seen among patients clinically. Establishing an appropriate dosing range in rats is critical for a model of acute kidney injury, as nephrotoxicity is better correlated to total systemic drug exposure rather than dose alone, particularly when the results are to be extrapolated among different mammalian species (e.g., from nonhuman data to humans). It was interesting that the pharmacokinetic study revealed a classical flip-flop phenomenon in which absorption became the rate-limiting step in the higher dose, resulting in a longer half-life and lower elimination rate. This phenomenon ultimately was reflected in an $AUC_{0\rightarrow\infty}$ increase disproportional to the dose given, as the $AUC_{0\rightarrow\infty}$ for 500 mg/kg was more than 5 times greater than the $AUC_{0\rightarrow\infty}$ for 100 mg/kg.

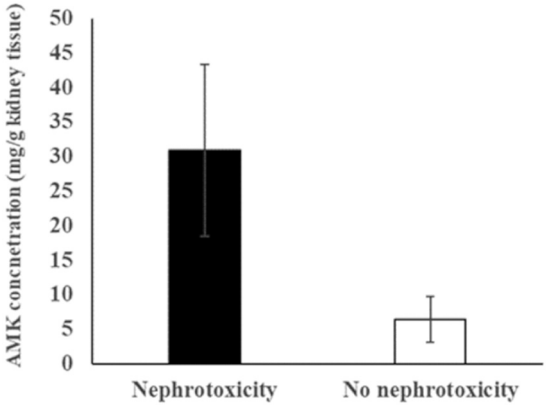

FIG 3 Amikacin (AMK) concentrations in renal tissues stratified by outcomes. $n = 4$ rats per group. Data are shown as means ± SD, $P = 0.009$.

To further establish the clinical relevance of this study, published clinical data were used for comparison (5). In this clinical study, 57% of patients developed acute kidney injury with amikacin and the patients developed acute kidney dysfunction 6.7 ± 3.1 days after the beginning of aminoglycoside therapy. In our animal model, 65% of the rats doubled their serum creatinine compared to baseline, which was indicative of substantial kidney injury. The median time to onset of kidney injury in the rats overall was 5 days. Both the prevalence of nephrotoxicity and the time to onset of nephrotoxicity were comparable to human clinical observations. Additionally, our animal findings showed the prevalence of nephrotoxicity could be predicted by daily systemic drug exposure (AUC), as seen in human clinical observations (19).

Finally, important insights were further demonstrated in the tissue accumulation study. After a single dose, there was not a significant difference in the renal accumulation of amikacin between the dosing groups. However, when stratified to outcomes, a much higher drug concentration was observed in animals with nephrotoxicity, suggesting that the duration of therapy was also a factor in drug accumulation.

There are some limitations to our study. Only amikacin was examined as a representative aminoglycoside in our animal model. Future studies could include other aminoglycosides (e.g., plazomicin and netilmicin) and compare their relative potentials for nephrotoxicity. Also, only 2 dose levels of amikacin spanning the clinical exposure range were examined. Including an intermediate dose level (e.g., 300 mg/kg, which is closer to the average drug exposure in a population) could have strengthened the robustness of the dose-response relationship. The definition of nephrotoxicity was based on serum creatinine concentration to be consistent with contemporary clinical practice. Novel urinary biomarkers (e.g., KIM-1 and NGAL) are more sensitive and allow earlier detection of the onset of acute kidney injury. Furthermore, drug concentrations from renal tissues were derived from homogenates of the whole kidneys. It is recognized that amikacin distribution between the renal cortex and medulla varies significantly (20), and more specific techniques (e.g., matrix-assisted laser desorption/ionization mass spectrometry imaging, MALDI-MSI) could be used in the future to reflect intrarenal drug distribution more accurately. Finally, to our disappointment, amikacin concentration in renal tissues after a single dose was not sufficiently informative to predict nephrotoxicity. A discriminatory threshold kidney concentration between the higher and lower dose would facilitate an even more efficient screening tool using an early surrogate biomarker. Further investigations on an intermediate time frame (e.g., after 2 or 3 doses) are warranted to ascertain if there is a significant threshold to predict nephrotoxicity during prolonged therapy.

Conclusions. Using clinically relevant conditions, an animal model for aminoglycoside-associated nephrotoxicity was established. The onset of nephrotoxicity was correlated with the daily dose, and nephrotoxicity was associated with a higher amikacin concentration in renal tissues. Ultimately, this animal model is expected to be a platform to evaluate a potential intervention(s) that could mitigate aminoglycoside-associated nephrotoxicity clinically.

MATERIALS AND METHODS

Chemicals and reagents. Heparin lock solution was obtained from SAI Chemicals (Lake Villa, IL). Amikacin sulfate (USP) for injection and saline for injection were purchased from Henry Schein (Dublin, OH). For the chromatography assay, amikacin sulfate (USP) powder was purchased from LKT Laboratories (St. Paul, MN), and tobramycin (USP) powder was purchased from Sigma-Aldrich (St. Louis, MO). PRiME MCX solid-phase extraction (SPE) cartridges (1 ml, 30 mg) were purchased from Waters (Milford, MA). Chromatography-grade methanol, isopropyl alcohol (IPA), formic acid, phosphoric acid, and ammonium hydroxide for SPE were purchased from EMD Millipore (Billerica, MA). EDTA, monopotassium phosphate, trichloroacetic acid, sodium hydroxide solution, and chromatography-grade ammonium formate were purchased from Sigma-Aldrich (St. Louis, MO). Sterile rat serum was obtained from Equitech-Bio Inc. (Kerrville, TX).

Animals. Sprague-Dawley rats (females, 200 to 250 g; males, 300 to 350 g) were purchased from Envigo (Somerset, NJ). They were given food and water *ad libitum*. The jugular vein was cannulated in selected rats to facilitate blood sampling. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Houston.

Single-dose pharmacokinetics. Female rats were given a single amikacin dose (100 mg/kg or 500 mg/kg) subcutaneously ($n = 4$ per group). Serial blood samples were obtained from each rat at 0.5, 1, 2, 4, and 6 h postdosing. All samples were allowed to clot on ice and centrifuged at 11,000 relative centrifugal force for 15 min at 4°C, and the serum was drawn off. At the conclusion of the study, kidneys were harvested from all rats and were weighed and homogenized in water. The serum and tissue homogenate samples were stored at −80°C until the drug assay was performed.

Amikacin concentrations of each sample were quantified by a validated liquid chromatography tandem mass spectrometry (LC-MS) assay (described below). The average serum concentration at each time point was calculated. The mean concentration-time profiles were characterized using a one-compartment bolus model with first-order absorption. ADAPT 5 (University of South California, Los Angeles, CA) was used to obtain best-fit parameter estimates. Unpaired *t* test was used to compare the results between the treatment groups.

LC-MS assay development. A robust LC-MS method was developed in a previous study (21). Briefly, the mobile phases were adjusted to a high pH (~11.2) to optimize elution times of the analytes. Tobramycin was used as the internal standard. The gradient and validation details were fully described previously.

Preparation of biological samples. Serum and tissue samples were prepared for assaying using solid-phase extraction (SPE). To prepare serum standards, blank rat serum was spiked with increasing concentrations of amikacin (0.25 to 80 mg/liter) and a final concentration of 20 mg/liter tobramycin (internal standard). The SPE cartridges were prepared with methanol and water, and then 100 μl of 4% phosphoric acid was mixed with each sample before being loaded onto the cartridge. This was followed by a sequential washing with ammonium formate in formic acid and methanol in water and then eluted with IPA-methanol-water-ammonium hydroxide. The samples were dried under ambient air and reconstituted in 500 μl of water prior to assaying.

For tissue standards, whole blank rat kidneys were homogenized at a ratio of 1 g tissue to 5 ml of sterile water using an IKA homogenizer (Wilmington, NC). The homogenates were spiked with increasing concentrations of amikacin (0.25 to 80 mg/liter) and a fixed concentration of tobramycin (internal standard). For each spiked homogenate, 50 μl of each was vortexed with 1 ml of extract solution (0.4 mM EDTA and 10 mM monopotassium phosphate, 2.5% trichloroacetic acid [TCA] in water solution) for 2 min. The supernatants were drawn off and adjusted with sodium hydroxide solution to a pH of 1.5 to 2 prior to being loaded onto the cartridges. The SPE cartridges were prepared as described above for the serum standards. The spiked homogenates were loaded onto the preprepared cartridges followed by methanol-formic acid and washed with reagent-grade water. The samples were then eluted with a mixture of ammonium hydroxide-water-methanol in a 25/5/70 ratio by volume. The eluents were dried under ambient air and reconstituted in 500 μl of water prior to assaying.

To prepare the kidney samples, the tissues were similarly homogenized as the standards, but only 50 μl of tissue homogenate was loaded into the cartridge after premixing with 4% phosphoric acid. After drying, the samples were reconstituted with 500 μl of water. The SPE assay for the kidney tissues was validated using FDA criterion (https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf). Previously, we found interday and intraday variations were less than 15%, recoveries were 80 to 85%, and matrix effect was less than 15%. Amikacin concentrations in renal tissue homogenates were reported after normalizing to the wet weight of kidney harvested.

Nephrotoxicity study. Rats of both genders ($n = 10$ per group) were used. Each rat was given amikacin (100 mg/kg or 500 mg/kg) once daily subcutaneously for up to 10 days. Blood samples were obtained from the tail at baseline and each day prior to dosing. The blood samples were allowed to clot and then centrifuged at 11,000 rpm for 15 min and the serum drawn off. Serum creatinine was measured for each sample (100 μl) using a clinical chemistry analyzer (Piccolo Xpress, Abaxis, Inc., Union City, CA). Nephrotoxicity was defined as a doubling of the baseline creatinine level. At the conclusion of the study, kidneys were also harvested from selected rats and stratified by outcomes (nephrotoxicity observed versus not observed, $n = 4$ each). Amikacin concentrations in these tissue samples were also assayed using LC-MS.

The proportions of nephrotoxicity observed in each treatment group were compared using Fisher's exact test, and *P* values of <0.05 were considered significant. The onset of nephrotoxicity was analyzed using Kaplan-Meier analysis and log-rank test. Right censoring was used if nephrotoxicity was not observed at the end of the experiment.

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