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SELECTIVE MEMBRANE TOXICITY OF AMINOGLYCOSIDE ANTIBIOTICS IN MEMBRANE VESICLES ISOLATED FROM PROXIMAL RENAL TUBULES OF THE RAT

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Abstract—A considerable body of evidence suggests that the nephrotoxic potential of aminoglycoside antibiotics may be associated with the degree of membrane binding and subsequent membrane damage in the renal tubules. In this study, we isolated functional basolateral and luminal membrane vesicles from rat renal cortex, incubated each membrane type in the presence of 1 mM concentrations of either neomycin, netilmicin, gentamicin, hydroxygentamicin, or amikacin, and monitored the activities of the marker enzymes alkaline phosphatase (ALP) and λ-glutamyltransferase (GGT) (luminal) or ouabain-sensitive Na^+,K^+-ATPase (basolateral) to determine if there were any selective drug-related alterations of enzyme activities. While none of the five aminoglycosides had any substantive effect upon enzyme activities of luminal vesicles, all five drugs inhibited the basolateral marker enzyme. Neomycin produced the greatest inhibition, hydroxygentamicin and amikacin the least, and gentamicin and netilmicin were intermediate in the inhibition of the enzyme. These results are in accordance with the known relative nephrotoxicity of these same drugs and indicate the usefulness of isolated renal membrane vesicles for *in vitro* toxicological studies of novel aminoglycosides.

Several important antibiotics, in particular the aminoglycosides, in high doses or after prolonged therapy, can produce symptoms of nephrotoxicity typical of altered renal tubule function. This nephrotoxic potential limits the therapeutic usefulness of the aminoglycosides [1]. At the cellular level, there is evidence that the renal membrane binding of aminoglycosides plays a direct role in the renal pathogenesis [2]. The cellular or molecular mechanism for this organ-specific toxicity is not known with certainty, but could involve lysosomal dysfunction [3]. However, recent studies have suggested that the role of lysosomal involvement is minimal [4]. Alternatively, toxicity could involve specific damage to the tubule plasma membrane [5], either the luminal (LM) or basolateral (BLM) membranes or both. Selective binding and subsequent damage to the BLM is of particular interest in the search for a toxicological mechanism because this membrane is a major site of the Na^+,K^+-ATPase transport system that is essential to normal renal function.

In this study, we isolated both LM and BLM as functional vesicles and then exposed them to equimolar concentrations of either neomycin, netilmicin, hydroxygentamicin, gentamicin, or amikacin. Alkaline phosphatase (ALP) and λ-glutamyltransferase (GGT) were monitored as representative marker enzymes for membrane function in LM vesicles. Na^+,K^+-ATPase served as a similar marker for membrane function in BLM vesicles. The effects of each of the five aminoglycosides upon these enzyme activities in the two membrane types were compared in this study. The results of this study contribute to the understanding of the relative nephrotoxilogical potencies of these compounds and reveal some advantages to using this *in vitro* system as a mechanistically-based screen to predict the relative *in vivo* nephrotoxicity of novel aminoglycoside antibiotics.

MATERIALS AND METHODS

Animals. Crl:COBS CD (SR) BR male rats were obtained from the Charles River Breeding Laboratories (Wilmington, MA). Animals were monitored for murine viruses, acclimated for >1 week, provided filtered tap water and Agway RMH 3000 pelleted food *at lib.*, and housed under environmentally controlled conditions of 70 ± 2°; 50% ± 5% relative humidity; and a 12-hr light/dark cycle. The rats used in this study weighed approximately 275 g each.

Chemicals and reagents. Phenyl methylsulfonyl fluoride (PMSF), adenosine-5′-triphosphate (disodium salt), reduced nicotinamide-adenine-dinucleotide, D-sorbitol, D-mannitol, Trizma HCl and base, sodium succinate, 2,6-dichlorophenol-indophenol, D-glucose-6-phosphate, cacodylic acid (sodium salt), 4-aminoantipyrine, horseradish peroxidase, ouabain, ouabain-sensitive rabbit kidney Na^+,K^+-ATPase, bovine kidney alkaline phosphatase, bovine kidney λ-glutamyltransferase and pyruvate kinase-lactic acid dehydrogenase suspension were purchased from Sigma Chemical Co. (St Louis, MO).

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Percoll and density marker beads were from Pharmacia (Piscataway, NJ); *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) sodium salt and free acid were from Behring Diagnostics (La Jolla, CA); and glucose oxidase was purchased from Boehringer Mannheim (Indianapolis, IN). Bicinchoninic acid (BCA) and protein assay reagents were obtained from Pierce Chemical (Rockford, IL).

Gentamicin and amikacin (free bases) and neomycin sulfate were purchased from Sigma. Hydroxygentamicin sulfate was a gift from the Sterling-Winthrop Research Institute (Rensselaer, NY), and netilmicin sulfate was a gift from the Schering Corp. (Bloomfield, NJ).

Enzyme assays of marker enzymes. All enzyme assays and protein determinations were completed on the Cobas-Bio centrifugal analyzer (Roche Diagnostics). Some enzyme activities were determined according to autoanalyzer procedures provided by the reagent manufacturer and based upon published procedures. Alkaline phosphatase (ALP; EC 3.1.3.1) was assayed with Baker reagents [6], λ-glutamyltransferase (GGT; EC 2.3.2.2) was assayed with Boehringer Mannheim Diagnostics reagents [7], and acid phosphatase (ACP, EC 3.1.3.2) was assayed with Sigma Diagnostic reagents [8].

Other enzyme activities were determined using procedures described in the literature that were adapted in our laboratory for use in the Cobas-Bio analyzer. Succinate dehydrogenase (SDH, EC 1.3.99.1) was assayed by the reduction of indolphenol monitored at 600 nm by the procedure of Green *et al.* [9] as modified by Earl and Korner [10] and Sheikh and Moller [11]. Glucose-6-phosphatase (G6Pase, EC 3.1.3.9) was assayed by the spectrophotometric methods of Gierow and Gergil [12, 13], a glucose oxidase-peroxidase coupled assay with the quantitation of quinoneimine at 510 nm. Na^+,K^+-ATPase (EC 3.6.1.4) was determined by the three-step linked enzymatic method [14] which involves the formation of ADP and subsequent oxidation of NADH and decrease in absorption at 340 nm in the presence or absence of 1 mM ouabain. We increased the final concentrations of the combined pyruvate kinase-lactic dehydrogenase suspension 5-fold. The inhibitory effects of all five drugs were also measured with GGT, ALP, or ouabain-sensitive NA^+,K^+-ATPase enzymes purchased from Sigma and prepared in medium D. Enzyme activities were determined at 37° except for GGT and ALP which were assayed at 30°.

Because the presence of mannitol or Tris can interfere with protein determination by the Lowry method [15], the BCA procedure described by Redinbaugh and Turley [16] adapted for use on the Cobas-Bio analyzer was used to monitor the protein content of the individual membrane fractions.

Isolation of membrane vesicles. Solutions used for the isolation of membrane vesicles were prepared as follows: medium B contained 0.30 M sucrose in deionized water buffered with 25 mM Hepes and adjusted to pH 7.1 (23°) with NaOH. Medium C consisted of medium B containing 0.2 mM PMSF, a proteinase inhibitor. Medium D contained 0.30 M D-mannitol and 25 mM Hepes in deionized water adjusted to pH 7.1 (23°) with NaOH. Medium E was medium D which contained 5 mM EDTA and 0.2 mM PMSF. Medium F was medium D to which was added 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$. Because of limited PMSF stability in dilute solutions, 100 mM PMSF stock was prepared in 2-propanol and added to the various media to achieve the desired final concentration just prior to use.

With modifications, the general procedure of Sheikh and Moller [11] for the isolation of renal membrane vesicles from rabbit kidney was used for isolation of basolateral (BLM) and luminal (LM) membranes from the rat renal cortex. Centrifugation steps were completed in either a Dupont Sorvall RC-5B or a Beckman L-2 refrigerated centrifuge. Membrane vesicles were prepared on four different occasions from the kidneys of four to five rats. Rats were killed by CO_2, and the kidneys were removed, placed in 4° saline and then perfused with medium B. Kidneys were sectioned, and the pars recta region was dissected, minced, and homogenized over ice in medium C, 5 ml/g tissue, in a Potter–Elvehjem homogenizer, 5–10 strokes at a medium setting of a Tri-R Stir-R motor. The homogenate was centrifuged at 900 *g* for 10 min and the pellet was discarded. The supernatant (fraction A) was centrifuged twice at 9200 *g*, for 10 min each time. The lower dark sediment was discarded and the supernatant and fluffy layer (upper 25% of the sediment) were retained as fraction B. This was re-homogenized by hand with the Potter–Elvehjem homogenizer and then centrifuged at 48,000 *g* for 30 min. The lower dark part of the pellet and the red supernatant were discarded. The fluffy layer or fraction C was suspended in 50 ml of medium F, homogenized again, gently stirred at 4° for 1 hr, and then centrifuged at 1500 *g* for 15 min. Both the supernatant and the pellet were retained and processed as follows for the preparation of LM and BLM vesicles. The supernatant was centrifuged at 48,000 *g* for 20 min, and the pellet was washed in 8 ml of medium E, homogenized again, and then centrifuged at 48,000 *g* for 20 min. The resulting pellet or fraction D was resuspended in medium D + PMSF using a Dounce homogenizer, 1.5 ml/5 g cortex, and was considered the LM vesicle fraction. The pellet from the 1500 *g* centrifugation step was suspended in 25 ml of medium E, homogenized by hand, held for 15 min, and then centrifuged at 48,000 *g* for 20 min. This step was repeated using medium D + PMSF instead of medium E. The pellet or fraction E was suspended in 3 ml of medium D + PMSF, layered onto a 16% Percoll gradient (6.04 ml Percoll in 28 ml of medium D + 3 ml of the fraction E suspension), and then centrifuged at 38,000 *g* for 10 min. The upper half of the cloudy layer that formed in the gradient region with densities between 1.032 and 1.049 was removed and again centrifuged at 100,000 *g* for 1 hr. Fraction F (BLM membranes), the layer over the glassy Percoll pellet, was removed and then resuspended in medium D + PMSF, 1.5 ml/5 g cortex, with a Dounce homogenizer. Fractions D and F were frozen in liquid N_2 and stored at −80°.

Processing for electron microscopy. LM (fraction D) and BLM (fraction F) samples were suspended

in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 hr at 4°. These suspensions were centrifuged in an Eppendorf Microfuge at 10,000 *g* for 1 min. Fresh fixative was added to the pellets and the tubes were stored overnight at 4°. Membrane pellets were washed three times with cacodylate buffer, then post-fixed in 2% osmium tetroxide for 2 hr at 4°. The pellets were washed once in 0.1 M cacodylate buffer, three times in distilled water, and then stained in 2% uranyl acetate for 2 hr at room temperature. They were next washed in distilled water, dehydrated through a graded ethanol series, cleared 2× in propylene oxide, infiltrated first with 1:2 propylene oxide: Spurr's resin and then with pure Spurr's resin. Finally, the specimens were embedded, cured at 70° overnight, sectioned, stained with uranyl acetate/lead citrate, and examined with a Zeiss EM 109 microscope.

Testing of aminoglycosides. All five aminoglycosides were prepared as 10 mM stocks in deionized water so that the final test concentration for each drug was 1 mM. Initially, either the LM or BLM vesicles were incubated with one of the five drugs for either 15, 30, or 60 min at 37°, and then ALP, GGT, and ATPase activities were assayed. Additional experiments with 1, 3, and 5 mM concentrations of each aminoglycoside incubated with either BLM or LM vesicles were also completed. For comparison, these drugs were also tested under equivalent conditions with either ALP, GGT, or ouabain-sensitive ATPase enzymes purchased from Sigma. Control enzyme activities were determined concurrently in similar membrane vesicle preparations in the absence of test drugs. Na^+,K^+-ATPase was determined as the ouabain-sensitive fraction of total ATPase activity. All five aminoglycosides were also incubated with ouabain-sensitive ATPase (Sigma) or BLM Na^+,K^+-ATPase in the absence of ATP and with ATP in the absence of any Na^+,K^+-ATPase. These experiments demonstrated no drug-associated effect on the linked enzyme assay for ATPase. The type of Na^+,K^+-ATPase inhibition was determined for each of the five aminoglycosides by testing each at five different concentrations of the substrate, ATP, and then constructing Lineweaver–Burk plots.

Statistics. Marker enzyme activity from replicate experiments for the various treatment and control conditions for each membrane marker enzyme were tested for differences among sample means by one-way analysis of variance. When significant differences were found, sample means for each of the various drug treatment conditions were also individually compared to corresponding control means using the pooled variance *t*-test, where applicable. The data were also analyzed using Duncan's multiple range test. The criterion of statistical significance was set at $P < 0.05$.

RESULTS

A summary of the marker enzyme data following three independent attempts to isolate either luminal (fraction D) or basolateral (fraction F) membrane vesicles from rat renal cortex is presented in Table 1. The luminal membrane of the renal tubule is especially rich in GGT and ALP, whereas the basolateral membrane is rich in Na^+,K^+-ATPase. AP is a marker for lysosomes, G6Pase for endoplasmic reticulum, and SDH for mitochondria. Fraction D analyses indicated an 8- to 9-fold enrichment in luminal membrane content on the basis of the two specific enzyme markers compared to the original tissue homogenate. On the basis of Na^+,K^+-ATPase activity, the basolateral fraction isolation technique produced a 13-fold enrichment in the content of this membrane in fraction F.

These LM and BLM enrichment factors were similar to the results of Aramaki *et al.* [17] who used different procedures for the isolation of brush border (9-fold) and basolateral (17-fold) membranes from rat kidney. Some contamination of fraction D with lysosomes and fraction F with endoplasmic reticulum was noted, as reported previously by Sheikh and Moller [11]. Representative electron micrographs of the two membrane preparations (not shown) demonstrated the physical integrity of these membrane vesicles and the absence of membrane sheets.

A summary of the effects of each of the five aminoglycosides upon specific marker enzyme activities of each of the two membrane vesicle types is presented in Fig. 1. These data indicate no effect by any of the five drugs upon the luminal membrane vesicles when both the GGT and ALP results are considered (not significant at $P < 0.05$). For comparison, when the five aminoglycosides were tested with GGT type II from bovine kidney, the mean activities (mean ± 1 SD, N = 3) were: 101 ± 9, 105 ± 4, 104 ± 6, 98 ± 6, and 104 ± 5% of control values for neomycin, netilmicin, hydroxygentamicin, gentamicin, and amikacin respectively. In contrast, all five aminoglycosides caused reproducible, significant ($P < 0.05$) inhibition of the basolateral marker, Na^+,K^+ATPase, compared to controls, with the greatest effect noted for neomycin and the least noted for amikacin. Similar inhibitions of ouabain-sensitive Na^+,K^+-ATPase from rabbit kidney were noted for 1 mM concentrations of neomycin, netilimicin, hydroxygentamicin, gentamicin, and amikacin (22 ± 3, 25 ± 0, 19 ± 5, 22 ± 3, and 17 ± 4% respectively; mean ± 1 SD, N = 3). Results from the Duncan's multiple range test for variance in the BLM ATPase inhibition data indicated significant differences ($P < 0.05$) between neomycin and either hydroxygentamicin or amikacin.

When 1, 3 or 5 mM concentrations of each aminoglycoside were incubated with each membrane type, the slopes of the inhibition curves for GGT and ALP in the LM vesicles varied from −0.25 to 1.25%; i.e. there were no dose-related increases in enzyme inhibition. In similar experiments with BLM vesicles and 1, 3 or 5 mM concentrations of each drug, the slopes of curves were: −6.42, −6.15, −7.78, −1.35, and +5.25, for neomycin, netilmicin, hydroxygentamicin, gentamicin, and amikacin, respectively, where Na^+,K^+-ATPase activity was measured as μmol/mg/min. This is the same rank order of potency as shown in Fig. 1 except for hydroxygentamicin.

The Lineweaver–Burk plots for the individual aminoglycosides tested for the inhibition of BLM

Table 1. Marker enzyme activities in isolated basolateral or luminal membrane preparations

Fraction	ALP (units/mg)	GGT (units/mg)	AP (units/mg)	G6Pase (units/mg)	SDH (units/mg)	NA^+,K^+-ATPase (μmol/mg/min)
A	0.80 ± 0.13	2.37 ± 0.35	0.013 ± 0.001	0.043 ± 0.006	0.045 ± 0.009	9.07 ± 4.12
D	6.17 ± 0.91	20.40 ± 1.15	0.037 ± 0.006	0.041 ± 0.005	0.020 ± 0.013	6.03 ± 3.20
F	0.99 ± 0.01	5.17 ± 0.81	0.013 ± 0.001	0.240 ± 0.047	0.027 ± 0.010	116 ± 14.5

The mean activities (±1 SD) for all three isolates are shown. Fraction A is the first supernatant from the tissue homogenate. Fractions D and F correspond to the luminal and basolateral vesicle fractions respectively.

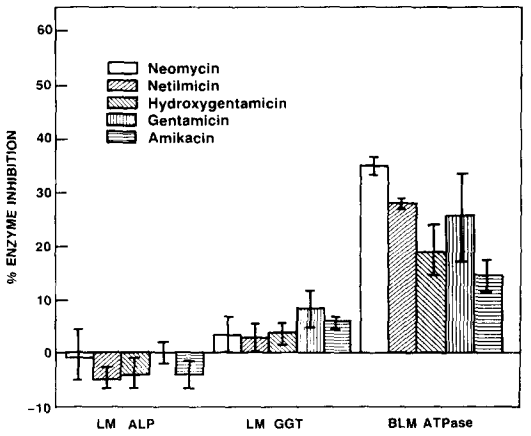

Fig. 1. Summary of enzyme inhibition data when membrane vesicles were incubated in the presence of 1 mM concentrations of the indicated aminoglycoside for 30 min. Individual values were calculated as a percent of corresponding enzyme activities in concurrent solvent control preparations where no inhibition = 0%. Values shown are sample means ±1 SE for N = 4 or N = 6 (duplicate assays of three independent samples). Compared to controls, all five aminoglycosides caused a significant inhibition of BLM Na^+,K^+-ATPase ($P < 0.05$). Significant differences between inhibition by neomycin and inhibition by either hydroxygentamin or amikacin were also found ($P < 0.05$).

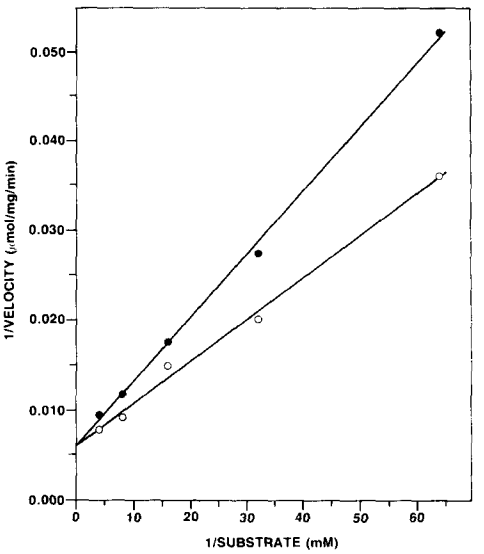

Fig. 2. Lineweaver–Burk plot of the inhibition of Na^+,K^+-ATPase in the presence of 1 mM netilmicin. Key: (○) no inhibitor; and (●) with inhibitor.

ATPase at varied concentrations of ATP demonstrated that the plots crossed on the ordinate for netilmicin, gentamicin, and hydroxygentamicin; however, the corresponding plots for neomycin and amikacin did not follow Michaelis–Menten kinetics. The plot for netilmicin is shown in Fig. 2. Similar enzyme inhibition studies were conducted for the five aminoglycosides in the absence of membranes by using ouabain-sensitive Na^+,K^+-ATPase from rabbit kidney. These data (not shown) indicated that inhibition by neomycin was not described by Michaelis–Menten kinetics; netilmicin, gentamicin, and hydroxygentamicin were all competitive inhibitors of Na^+,K^+-ATPase; and inhibition by amikacin was non-competitive. Except for amikacin, these results were the same as those obtained with BLM Na^+,K^+-ATPase.

DISCUSSION

Previous workers have shown the inhibition of Na^+,K^+-ATPase by genatamicin in isolated perfused rat kidney [18], the inhibition of canine or rat renal Na^+,K^+-ATPase by neomycin [19, 20], the inhibition of Na^+,K^+-ATPase and acid phosphatase in renal cortical homogenates from gentamicin-dosed rats [21], and the inhibition of Na^+,K^+-ATPase by amakacin, dideoxykanamycin B, and gentamicin entrapped within basolateral membrane vesicles isolated from the rat, but not when externally added to those same vesicles [17]. The present study represents the first extensive study of the effects of five aminoglycosides upon three membrane marker enzymes when externally added to either BLM and LM vesicles isolated by a single technique from the same kidneys. The vesicles used in these studies were morphologically intact when viewed electron microscopically and functionally active in terms of either GGT and ALP for the luminal membranes or Na^+,K^+-ATPase for the basolateral membranes. Marker enzyme data further indicated an 8- to 9-fold enrichment of the LM content and a 13-fold enrichment of the BLM content of the respective vesicle preparations. The BLM vesicle preparations were estimated to be 70% leaky using 0.03% Triton X-100 and the latency of ATPase procedure of Boumendil-Podevin and Podevin [22].

When incubated in the presence of equimolar concentrations of any one of the five aminoglycosides, no effect was noted when the marker enzymes GGT and ALP were assayed in the luminal membrane

vesicle preparations. In contrast, neomycin, netilmicin, and to a lesser extent, hydroxygentamicin, gentamicin, and amikacin all inhibited ($P < 0.05$) the functional Na^+,K^+-ATPase in the basolateral membrane vesicles. Under these conditions, the inhibition of membrane ATPase was competitive for netilmicin, gentamicin, and hydroxygentamicin. The inhibition of neomycin was greater for the BLM vesicle Na^+,K^+-ATPase than the Sigma preparation, and the effects of increasing concentrations of amikacin on the BLM enzyme were clearly different than the other four aminoglycosides which suggests the importance of monitoring Na^+,K^+-ATPase *in situ*. Aminoglycosides, in general, are not metabolized [23] and, therefore, would reach the kidney unchanged. Thus, accumulation of any of these five, particularly neomycin and netilmicin, within the basolateral membranes of the rat renal tubules could result in the competitive (netilmicin, hydroxygentamicin, and gentamicin) or complex inhibition (neomycin and amikacin) of membrane Na^+,K^+-ATPase. Such inhibition could be mechanistically important in the drug-associated nephrotoxicity of these compounds.

Our data for the inhibitory effects of gentamicin and amikacin differ from the results of Aramaki *et al.* [17] who noted little inhibitory effect upon basolateral Na^+,K^+-ATPase when vesicles were incubated for 6 hr at 4° in the presence of 1 mM concentrations of either aminoglycoside added externally to the medium. They concluded that no enzyme inhibition occurred because the aminoglycosides could not be transported into the basolateral membrane vesicles even though 59% of their vesicles were leaky and another 28% were sealed inside out. Using isolated membrane vesicles, Williams *et al.* [18, 26] have shown that gentamicin binds to the basolateral membrane, but is not translocated and that increasing the temperature from 4° to 37° causes a small but significantly increased uptake. Lipsky and Leitman [19] have also shown that the degree of inhibition of canine renal Na^+,K^+-ATPase by neomycin is temperature dependent with nearly maximal inhibition occurring above 19°. Using solubilized Na^+,K^+-ATPase from rat kidney, Darrow [20] has demonstrated a pronounced inactivation by neomycin that can be prevented by lowering the temperature to 2–4° during exposure of the enzyme to antibiotic. The explanation for the differences between our data and the previous work of Aramaki *et al.* [17] is most likely due to the differences in incubation temperature for the drug and enzyme combinations.

The known relative binding affinities of various polycationic aminoglycosides to renal tubule membranes are in general concordance with the observed inhibition of membrane Na^+,K^+-ATPase. Sastrasinh *et al.* [24] have reported the binding affinity for a series of aminoglycosides in isolated rat renal brush-border membranes to be as follows: neomycin > netilmicin > tobramycin > gentamicin > amikacin. Similar studies by other workers using rat renal brush-border membranes indicated that the order of binding was neomycin > tobramycin > gentamicin [23] and neomycin > tobramycin ~ gentamicin ~ netilmicin > amikcain [25]. Similar figures are not available for basolateral membrane binding; however, for at least two of these aminoglycosides, binding is greater in the rat BLM than the LM [25, 26], and a similar binding order is assumed.

The relative nephrotoxicities of aminoglycosides in the rat have also been described by several investigators. Eosinophilic granulation and vacuolization of renal proximal tubular epithelium, interstitial inflammation, and tubular dilation were observed microscopically with three of these drugs in the following order of severity: gentamicin > netilmicin > hydroxygentamicin [27]. Other studies have indicated that neomycin is more nephrotoxic than gentamicin [1] and that gentamicin is more nephrotoxic than amikacin [28, 29]. Williams *et al.* [25] have concluded that neomycin is consistently the most nephrotoxic aminoglycoside while amikacin possesses low nephrotoxic potential–two observations that clearly correspond with our data for the *in situ* inhibition of Na^+,K^+-ATPase in isolated BLM vesicles.

Taken together, our data support the notion that inhibition of BLM ATPase, subsequent to specific binding, is associated with the known nephrotoxicity of these drugs. Moreover, isolated renal membrane vesicles from the species of interest may provide a simple *in vitro* screening mode for the assessment of nephrotoxicity of novel aminoglycosides.

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