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Effects of Aminoglycoside Antibiotics on Lysosomal Enzymes of Rat Kidney

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The binding of three aminoglycoside antibiotics, gentamicin, amikacin, and 3′,4′-dideoxykanamycin B, to various lysosomal enzymes, *N*-acetyl-β-D-glucosaminidase, acid phosphatase, and lysozyme, was examined *in vitro* by using an affinity column of 3′,4′-dideoxykanamycin B-conjugated Sepharose 4B in order to investigate the mechanism of the accumulation of aminoglycoside antibiotics in the kidney. *N*-Acetyl-β-D-glucosaminidase and lysozyme were bound to the affinity column, but acid phosphatase was not adsorbed on the column. The activity of lysozyme was strongly inhibited by the addition of the three aminoglycosides at the concentration of 1 mM. In the case of *N*-acetyl-β-D-glucosaminidase, the activity was significantly inhibited in the presence of amikacin, but, in contrast, the activity was slightly activated by the addition of gentamicin or 3′,4′-dideoxykanamycin B. Acid phosphatase activity was not affected by the addition of any of the aminoglycosides. Lineweaver–Burk plots indicate that the inhibition of *N*-acetyl-β-D-glucosaminidase by amikacin is competitive. In the case of lysozyme, competitive inhibition by all three aminoglycosides was observed with *Micrococcus luteus* cells as the substrate. These findings indicate that the lysosomal enzyme activities may be influenced by the binding of aminoglycosides, and this binding may represent one of the accumulation mechanisms of aminoglycosides in the kidney.

Keywords——aminoglycoside antibiotic; lysosomal enzyme; *N*-acetyl-β-D-glucosaminidase; lysozyme; acid phosphatase; 3′,4′-dideoxykanamycin B; gentamicin; amikacin

The major side effect of aminoglycoside antibiotics is considered to be nephrotoxicity as a result of their accumulation at the proximal tubular cells.[1] However, the biological mechanism of the accumulation or the toxicity of these drugs is still unclear. Silverblatt and Kuehn[1] reported that gentamicin, an aminoglycoside, was accumulated in lysosomes within the rat proximal tubular cells at a much higher concentration than in other organelles. Furthermore, it was reported that aminoglycosides affected the urinary excretion of lysosomal acid hydrolases[2] and the intracellular distribution of *N*-acetyl-β-D-glucosaminidase, a lysosomal enzyme.[3] Thus, it is considered that the nephrotoxicity of aminoglycosides is closely related to the lysosomes of proximal tubular cells. It was previously reported that 3′,4′-dideoxykanamycin B was bound to *N*-acetyl-β-D-glucosaminidase obtained from rat kidney cortex, and the enzyme activity was significantly increased.[4]

In this report, we studied the binding of three kinds of aminoglycoside antibiotics, 3′,4′-dideoxykanamycin B, amikacin, and gentamicin, to the lysosomal enzymes, *N*-acetyl-β-D-glucosaminidase, acid phosphatase, and lysozyme, by using an affinity column of 3′,4′-dideoxykanamycin B-conjugated Sepharose 4B. The binding of aminoglycoside antibiotics to the lysosomal enzymes could represent one of the mechanisms of accumulation of these antibiotics in the kidney, and the resulting effect on the enzymatic activity may be involved in the onset of the renal toxicity caused by these antibiotics.

Materials and Methods

Materials——3′,4′-Dideoxykanamycin B (DKB) was kindly donated by Meiji Seika Kaisha Ltd. (Tokyo), amikacin by Banyu Pharmaceutical Co., Ltd. (Tokyo), and gentamicin by Shionogi Pharmaceutical Co., Ltd. (Osaka). Spray-dried cell powder of *Micrococcus luteus* ATCC 4698 was obtained from Miles Laboratory Inc. (U.S.A.). The other reagents were of the best available grade. Male Wistar rats (200—250 g) were purchased from Tokyo Jikken Dobutsu (Tokyo).

Preparation of Enzyme Solution——The kidney cortex obtained from 20 h-fasted rats was minced and homogenized with a Potter–Elvehjem type homogenizer (clearance 0.18 mm), and 20% (w/v) homogenate was prepared with 10 mM phosphate buffer (pH 6.5). The homogenate was ultrasonicated at 25 μm (amplitude of oscillation) for 5 min, and then the enzyme solution was obtained as the supernatant by ultracentrifugation at $100000 \times g$ for 30 min.

Assay of Enzyme Activity——With *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide as the substrate, the *N*-acetyl-β-D-glucosaminidase activity was determined according to the method of Loomis.[5] With disodium phenylphosphate as the substrate, the acid phosphatase activity was determined according to the improved Kind–King method.[6] Lysozyme activity was determined as the decrease of the turbidity (absorbance at 560 nm) according to Junowicz and Charm,[7] with *M. luteus* cells as the substrate. The protein concentration was determined by the method of Lowry *et al.*[8] and by measuring the absorbance at 280 nm.

Preparation of the immobilized 3′,4′-dideoxykanamycin B column was described in the previous report.[4]

Results and Discussion

Binding of DKB to Lysosomal Enzymes

Enzyme solution, which was prepared as described in Materials and Methods, was applied to the affinity column of DKB-conjugated Sepharose 4B in order to investigate the binding of the aminoglycoside to lysosomal enzymes. As shown in Fig. 1, only acid phosphatase activity was detected in the eluate when the affinity column was eluted with 10 mM phosphate buffer (pH 6.5). When the elution buffer was changed from 10 mM

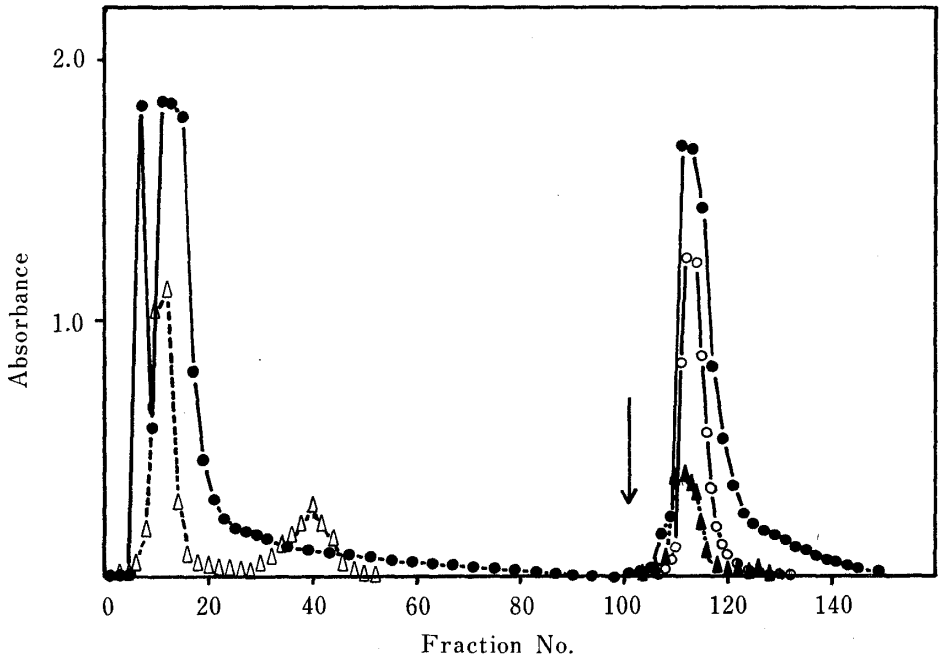

Fig. 1. Affinity Chromatography of the Enzyme Solution Obtained from Rat Kidney Cortex

The column size was 1.3 × 15 cm (1 fraction = 1.2 ml). The arrow indicates the change of the elution buffer from 10 mM phosphate buffer (pH 6.5) to 10 mM phosphate buffer containing 1.0 M NaCl.

—●—, protein (absorbance at 280 nm); —○—, *N*-acetyl-β-D-glucosaminidase activity (absorbance at 400 nm); ---▲---, lysozyme activity (absorbance at 560 nm); ---△---, acid phosphatase activity (absorbance at 500 nm).

phosphate buffer (pH 6.5) to 10 mM phosphate buffer (pH 6.5) containing 1.0 M NaCl, *N*-acetyl-β-D-glucosaminidase and lysozyme were eluted from the affinity column overlapping with the protein peak, but no acid phosphatase activity could be detected. The yields of acid phosphatase, *N*-acetyl-β-D-glucosaminidase, and lysozyme were 88, 85, and 79%, respectively. As non-specific adsorption of *N*-acetyl-β-D-glucosaminidase and lysozyme on Sepharose 4B could not be observed (data not shown), it was considered that the adsorption of *N*-acetyl-β-D-glucosaminidase and lysozyme on the affinity column was the result of the binding of DKB to these enzyme molecules.

Previously, it was reported that *N*-acetyl-β-D-glucosaminidase was a glycoprotein containing mannose-6-phosphate at the non-reducing terminal of the sugar chain.[9] Then, Sastrasinh *et al.*[10] and Feldman *et al.*[11] reported that aminoglycoside antibiotics bound to phospholipid, especially to phosphatidilinositol or diphosphatidilinositol, mediated by the ionic interaction between the phosphate moiety of phospholipids and the amino group of aminoglycosides. Thus, the adsorption of *N*-acetyl-β-D-glycosaminidase on DKB-conjugated Sepharose 4B may occur as the result of the binding of amino groups of the DKB to the mannose-6-phosphate moiety of *N*-acetyl-β-D-glucosaminidase. In fact, about 20% of *N*-acetyl-β-D-glucosaminidase bound to DKB-conjugated Sepharose 4B was eluted from the affinity column with 20 mM mannose-6-phosphate solution, and the remaining *N*-acetyl-β-D-glucosaminidase was detected in the eluate after a change of elution buffer from mannose-6-phosphate solution to 1.0 M NaCl solution, as shown in Fig. 2. Since DKB contains 3-deoxy-3-amino-D-glucose and/or 6-deoxy-6-amino-D-glucosamine in its molecule, *N*-acetyl-β-D-glucosaminidase may recognize DKB as a substrate, so that specific adsorption of this enzyme to the affinity column may occur.

Similarly, lysozyme may recognize DKB as a substrate (since it hydrolyzes cell wall peptidoglycan, which consists of *N*-acetyl-β-D-glucosamine and *N*-acetylmuramic acid), and thus be bound to the affinity column. The affinity of lysozyme to DKB-conjugated Sepharose 4B was drastically changed when a different pH of elution buffer was used for the affinity chromatography (Fig. 3). When the affinity chromatography was carried out with pH 5.0

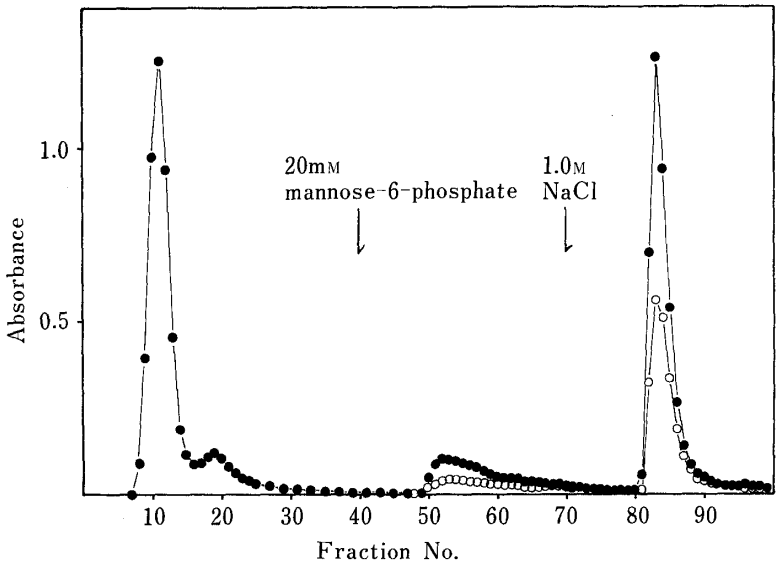

Fig. 2. Affinity Chromatography of the Enzyme Solution Obtained from Rat Kidney Cortex.

The column size was 1.5 × 16 cm (1 fraction = 1.2 ml). Arrows indicate the change of the elution buffer from 10 mM phosphate buffer (pH 6.5) to 10 mM phosphate buffer containing 20 mM mannose-6-phosphate or 1.0 M NaCl.
—●—, protein (absorbance at 280 nm); —○—, *N*-acetyl-β-D-glucosaminidase activity (absorbance at 400 nm).

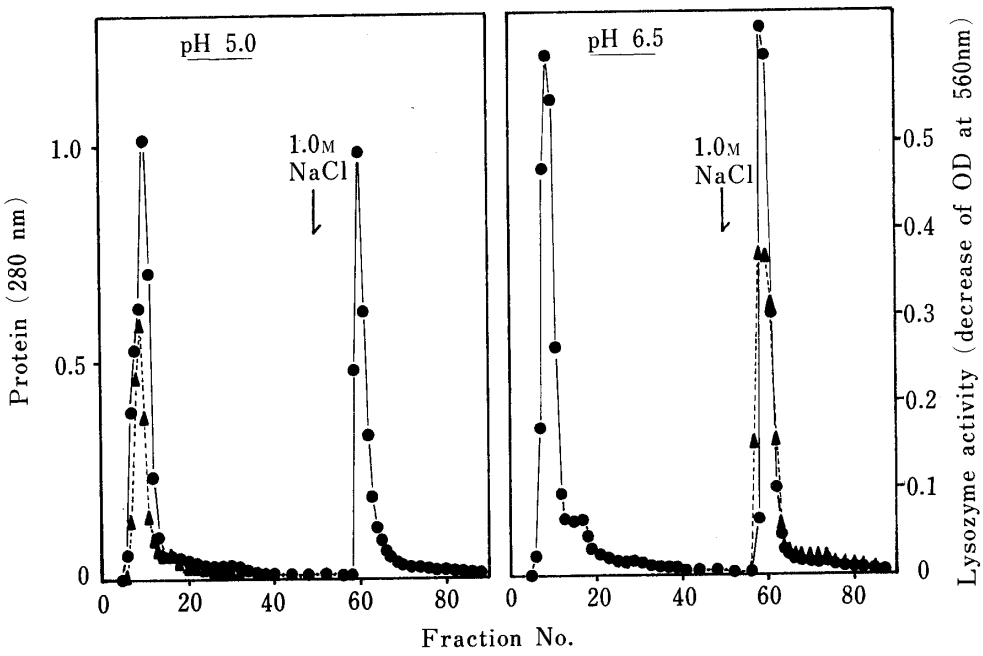

Fig. 3. Affinity Chromatography of the Enzyme Solution Using 10 mM Acetate Buffer pH 5.0 (left) and 10 mM Phosphate Buffer pH 6.5 (right)

Arrows indicate the change to the above buffers containing 1.0 M NaCl.
—●—, protein (absorbance at 280 nm); ---▲---, lysozyme activity (absorbance at 560 nm).

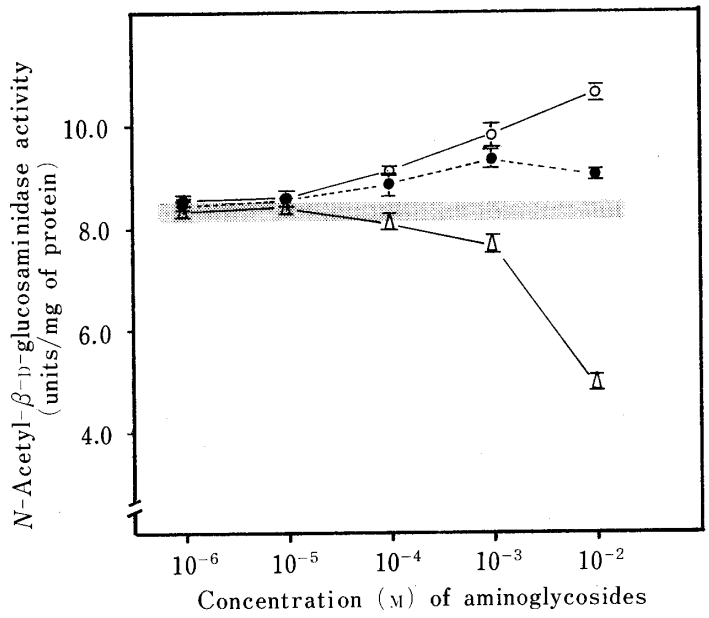

Fig. 4. Effect of Aminoglycosides on *N*-Acetyl-β-D-glucosaminidase Activity

The enzyme solution was obtained from rat kidney cortex. Points are the mean ± S.D. of at least triplicate determinations. The dotted bar indicates the control (without aminoglycoside) value.
---●---, DKB; —○—, gentamicin; —△—, amikacin.

acetate buffer (10 mM), all of the lysozyme activity was detected in the flow-through fraction, and none was bound to the column. Furthermore, when the affinity chromatography was carried out with pH 8.0 phosphate buffer (10 mM), lysozyme activity could not be detected in the flow-through fraction or the bound fraction because of enzyme denaturation at this

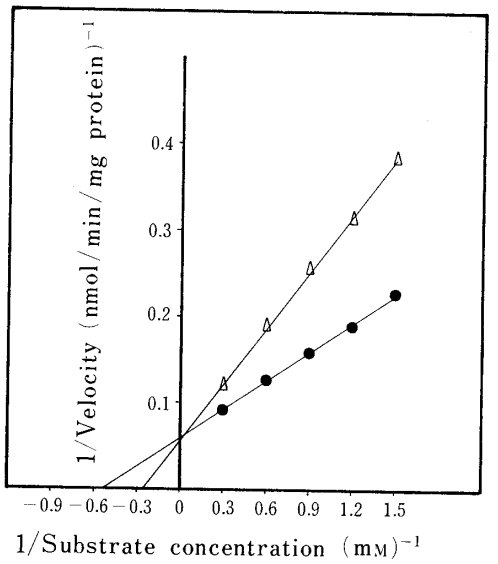

Fig. 5. Lineweaver–Burk Plot of Amikacin Inhibition of *N*-Acetyl-β-D-glucosaminidase

The enzyme solution was obtained from rat kidney cortex. Experiments were performed in triplicate.
●, without amikacin; △, 10 mM amikacin.

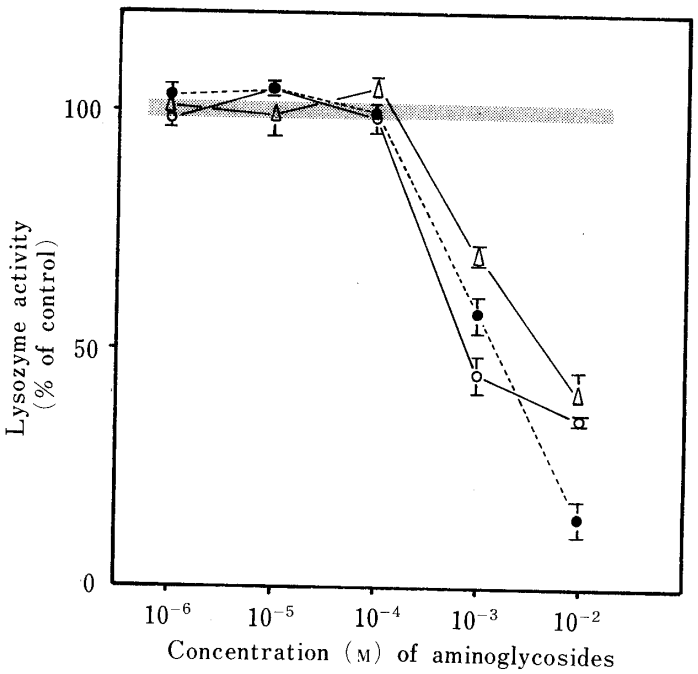

Fig. 6. Effect of Aminoglycosides on Lysozyme Activity

The enzyme solution was obtained from rat kidney cortex. Points are the means±S.D. of at least triplicate determinations. The dotted bar indicates the control (without aminoglycoside) value.
---●---, DKB; —○—, gentamicin; —△—, amikacin.

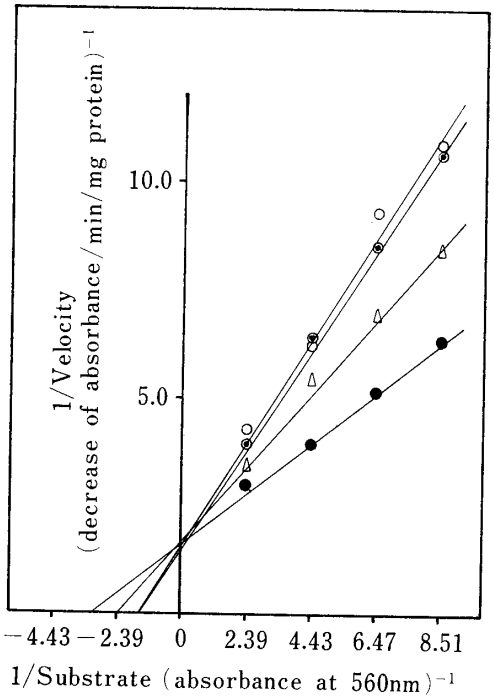

Fig. 7. Lineweaver–Burk Plot of Aminoglycoside Inhibitions of Lysozyme Activity

Experiments were performed in triplicate.
●, control; ⊙, 0.5 mM DKB; △, 0.5 mM amikacin; ○, 0.5 mM gentamicin.

TABLE I. K_m and V_{max} Values of Lysozyme in the Presence of DKB, Gentamicin, and Amikacin

Drug	K_m [a]	V_{max} [b]
Control	0.344	0.625
0.5 mM DKB	0.768	0.704
0.5 mM Gentamicin	0.706	0.633
0.5 mM Amikacin	0.476	0.598

a) Absorbance at 560 nm.
b) Decrease of absorbance at 560 nm/min/mg of protein.

alkaline pH. These results indicate that the lysozyme may recognize DKB as a substrate and thus bind to the affinity column.

Effects of Aminoglycosides on the Enzyme Activities

Next, the effects of DKB, gentamicin, and amikacin on the activities of the three lysosomal enzymes were examined. The enzyme solution obtained from rat kidney cortex was preincubated with various concentrations of aminoglycosides at 37 °C for 10 min, and after the addition of substrate solution, the mixture was incubated at 37 °C for 30 min. As shown in Fig. 4, *N*-acetyl-*β*-D-glucosaminidase activity was influenced by the addition of aminoglycosides. In the case of DKB and gentamicin, this enzyme was activated slightly at concentrations of 10^{-3} and 10^{-2} M; however, amikacin strongly inhibited the activity at 10^{-2} M.

The nature of the inhibition of *N*-acetyl-*β*-D-glucosaminidase was investigated by means of Lineweaver–Burk plots (Fig. 5). The plots were linear and they intersected the ordinate at almost equivalent values. The K_m value of this enzyme in the absence of amikacin was 1.92 mM, and V_{max} was 17.1 nmol of *p*-nitrophenyl-*N*-acetyl-*β*-D-glucosaminide cleaved per min per mg of protein. In the presence of amikacin, K_m was 4.23 mM and V_{max} was 18.5 nmol. These V_{max} values are almost equal, so the type of inhibition of *N*-acetyl-*β*-D-glucosaminidase by amikacin is competitive.

In contrast, DKB and gentamicin had almost no effect on the *N*-acetyl-*β*-D-glucosaminidase activity. This difference may be caused by the differences of their molecular structures, *e.g.* the presence of side chain, the number of amino groups and/or the position of amino groups in the aminoglycosides.

In the case of lysozyme, the enzyme activity was strongly inhibited by the three aminoglycosides at the concentrations of 10^{-3} and 10^{-2} M, as shown in Fig. 6.

The nature of the inhibition was investigated by measuring the ability of three aminoglycosides to inhibit the lysozyme activity with *M. luteus* cells as the substrate. In this case, rat renal cortical lysosomal fraction prepared as described by Meisner[3] was used as the enzyme solution. The double reciprocal plots were linear and intersected the ordinate at almost the same points (Fig. 7). The K_m and V_{max} values obtained from the Lineweaver–Burk plots are summarized in Table I; the inhibition of lysozyme by all three aminoglycosides was competitive. Tulkens and Trouet[12] reported that intracellular bacteria became less sensitive to aminoglycosides when they entered into the cells. From the above results, it may be considered that the reason for this phenomenon is not a decrease of bacterial sensitivity to aminoglycosides but the inhibition of lysozyme activity by the aminoglycosides.

On ther other hand, acid phosphatase could not be adsorbed on the DKB-conjugated Sepharose 4B affinity column, and the activity of this enzyme was not influenced by the addition of aminoglycosides at concentrations from 10^{-6} to 10^{-2} M (data not shown).

The nephrotoxicity of aminoglycosides is a result of their accumulation in the kidney proximal tubular cells, especially in the lysosomal fraction.[1] The above results suggest that aminoglycoside antibiotics bind to the lysosomal enzymes, *N*-acetyl-*β*-D-glucosaminidase and lysozyme, and thus accumulate in the lysosomes of the kidney proximal tubular cells.

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