Studies on the Nephrotoxicity of Aminoglycoside Antibiotics and Protection from These Effects. (5). Interaction of Tobramycin with Latamoxef in Vitro

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The interaction of tobramycin (TOB) with latamoxef (LMOX) was studied in vitro. Solutions containing TOB alone, LMOX alone or both of these compounds in varying molar ratios (TOB:LMOX = 1:1, 1:2, 1:4 and 2:1) were incubated at 37°C for 0.5, 1, 3 and 5 h after adjusting to pH 7.4. Aliquots sampled at a suitable time were subjected to paper electrophoresis (PE) and thin layer chromatography (TLC). In PE, the spots of TOB and LMOX were observed as single spots on the cathode and anode sides, respectively. However, the spot associated with TOB overlapped with that associated with LMOX on the cathode side when aliquots of the solution containing both TOB and LMOX were analyzed. It seemed that the degree of overlapping became stronger with an increase in incubation time, and there were no spots corresponding to TOB alone in the mixture of TOB-LMOX (1:4). TLC analysis showed that the spot of LMOX radiated fluorescence with Rf value 0.38. On the other hand, in the mixtures, there was a definite decrease in fluorescence of LMOX at the position of Rf value 0.38, compared with that of LMOX alone. Furthermore, the spot associated with LMOX, which overlapped with that associated with TOB, also appeared at the origin on the TLC plate. The ultraviolet spectrum of the mixture of TOB-LMOX (1:2) showed a decrease in the intensity of absorption of LMOX at 268 nm. These interactions between TOB and LMOX were also observed in rat serum and its filtrate in vitro. In addition, we used infrared (IR) spectrophotometry to obtain some information on the manner of the interaction of TOB with LMOX. The IR spectrum of the reactive product of TOB with LMOX indicated disappearance of the β-lactam (1750 cm⁻¹) ring of LMOX. These results indicate that TOB chemically reacts with LMOX in vitro.

Keywords — aminoglycoside; nephrotoxicity; tobramycin; latamoxef; interaction

Introduction

Sastrasinh et al. 1) reported that aminoglycoside antibiotics (AGs), including gentamicin (GM), bound to acidic phospholipids such as phosphatidylinositol, which was contained in brush border membranes (BBMs) of renal proximal tubular cells in rats. It has been proposed that the binding of AGs to acidic phospholipids is due to the electrostatic interaction between the aminogroups in the AGs molecule and phosphate groups in acidic phospholipids,2-4) and that AGs are transported into tubular cells by an energy-dependent transport system,5) probably pinocytosis, and sequestered in lysosomes,6,7) after binding to BBMs.

We have previously reported that the protective effect of latamoxef (LMOX), an oxacephem antibiotic, against tobramycin (TOB)-induced nephrotoxicity in rats is partially due to the suppression of intrarenal TOB accumulation by combination with LMOX. 8) LMOX has two carboxyl groups in the molecule and, thereby, has a negative charge derived from the groups at physiological pH, whereas TOB is positively charged because of its amino groups. Therefore, to clarify the mechanism by which LMOX suppresses the intrarenal TOB accumulation in vivo, we have hypothesized that positively charged TOB may be able to interact with negatively charged LMOX by ionic binding in vivo, that the interaction may inhibit the binding of TOB to acidic phospholipids forming BBMs, and that the inhibition of binding of TOB to BBMs may contribute to the decrease in intrarenal TOB accumulation.

In the present study, we investigated an interaction of TOB with LMOX in vitro by the following methods: paper electrophoresis (PE), thin layer chromatography (TLC), and ultraviolet (UV) and infrared (IR) spectrophotometries.
Materials and Methods

**Materials**—TOB and LMOX were provided by Shionogi Co., Ltd. in the forms of free and disodium salt, respectively. The chemical structures of TOB and LMOX are illustrated in Fig. 1. Blood was obtained from the jugular veins of male Sprague-Dawley rats weighing about 230 g (Shizuoka Laboratory Animal Center), and the serum was separated by centrifugation at 3000 rpm for 10 min. The filtrate of serum was prepared by centrifugation at 3300 rpm for 15 min using MPS-1 with YMT membrane (Amicon).

**PE Analysis**—TOB alone, LMOX alone and a mixture of both of these drugs (molar ratio; TOB:LMOX = 1:1, 1:2, 1:4 and 2:1) were dissolved in H₂O and the pH was adjusted to 7.4 by the addition of HCl or NaOH. After adjusting pH, each solution containing drugs was incubated for 0.5, 1, 3 and 5 h at 37 °C. Aliquots sampled at a suitable time were subjected to PE. PE was carried out for 1 h using 70 mM Tris-HCl buffer (pH 7.4) and then the papers (2.5 × 15 cm) were dried. The spots associated with TOB on the paper were visualized by ninhydrin (0.2 w/v% acetone solution) reaction, and UV irradiation (λ = 254 nm) was used for detecting the spots associated with LMOX.

**TLC Analysis**—Aliquots of each solution containing the drugs as described above were subjected to TLC using Kieselgel 60 F₂₅₄ (Merck). After development of aliquots of drug solutions with the solvent consisting of CHCl₃, CH₃OH, H₂O and CH₃COOH (13:13:5:1, v:v), the spots associated with TOB and LMOX were detected by the same methods as that used in PE. In addition, TOB alone, LMOX alone, and the mixture of both (molar ratio; TOB:LMOX = 2:1) were dissolved in a filtrate of rat serum prepared with MPS-1 and in rat serum alone, and incubated at 37 °C; aliquots of samples were subjected to TLC.

**UV Spectrophotometry**—The mixture of TOB and LMOX (molar ratio; TOB:LMOX = 1:2) was dissolved in H₂O, and the pH was adjusted to 7.4, followed by incubation at 37 °C for 3 h. The UV absorption of the solution was recorded with a Uvidec-510 spectrophotometer (Nihonbunko, Japan) and was compared with that of the solution containing LMOX alone.

**IR Spectrophotometry**—Incubation of solution containing TOB and LMOX (molar ratio; TOB:LMOX = 1:2) produced precipitates whitish yellow in color, which seemed to be the reactive product of TOB with LMOX. The reactive product filtrated through a filter paper was dried, and IR spectrum was obtained in a pressed KBr discus using an IR-810 spectrometer (Nihonbunko, Japan).

**Results**

**PE Analysis**

Figure 2 shows the interaction of TOB with
Fig. 2. Interaction of TOB with LMOX in H₂O Analyzed by Paper Electrophoresis

Black spots and circle lines on the paper indicate the spots corresponding with TOB and LMOX, respectively. Broken circles indicate traces of LMOX. The broken circles with arrowhead indicate new spot which has negative charge and is visualized by UV irradiation. O, origin; T, TOB alone; L, LMOX alone; T+L, reaction mixture of TOB and LMOX; #T+L, TOB and LMOX (the drugs were separately spotted at the origin).
Fig. 3. Interaction of TOB with LMOX in H₂O Analyzed by TLC

UV, ultraviolet irradiation; Ni, ninhydrin reaction; O., origin; T, TOB; L, LMOX; TL, reaction mixture of TOB and LMOX. Arrowheads indicate a new spot which has low polarity and is visualized by UV irradiation.
LMOX analyzed by PE. Spots of TOB and LMOX were observed as single spots on the cathode and anode side, respectively. When the solutions containing TOB and LMOX were incubated at 37 °C for various periods of incubation time, the spot associated with TOB overlapped with that associated with LMOX on the cathode side. The degree of overlap seemed to become stronger as incubation time increased. Incubation of the solution containing TOB and LMOX (molar ratio; TOB:LMOX = 1:4) resulted in disappearance of the spot corresponding to TOB alone. In contrast, no spot corresponding to LMOX alone was observed on the paper in the mixture of TOB-LMOX (2:1) after 5 h incubation. Additionally, in the mixture of TOB and LMOX a new spot which absorbed UV seemed to appear in the anode side after incubation. When aliquots of TOB and LMOX were separately spotted at the point of origin on the paper and analyzed by PE, there was no spot representing the overlapping of both compounds on the cathode side.

**TLC Analysis**

As shown in Fig. 3, the spots of TOB and LMOX were found at the origin and at the position of *Rf* value 0.38, respectively, on the TLC plate. When TOB and LMOX were incubated in various molar ratios, the spot associated with LMOX, which overlapped with that associated with TOB, newly appeared at the origin on the TLC plate. In the mixture of TOB-LMOX (2:1), the spot corresponding to LMOX alone at the position of *Rf* value 0.38 completely disappeared after 5 h incubation, as observed in PE analysis. In addition, we detected a new spot, which was visualized by UV irradiation but not by ninhydrin reaction, at the position of a higher *Rf* value.

**UV Spectrophotometry**

Figure 4 shows the UV spectra of LMOX and the mixture of TOB and LMOX (molar ratio; TOB:LMOX = 1:2). The peaks of absorption of LMOX were revealed at 268 and 225 nm, which would be derived from the coupling of electrons involved with double bond, carbonyl, and aromatic groups, including phenol and tetrasol. However, in the mixture, the intensity of the peak at 268 nm was strikingly lower.

**Interaction of Tobramycin with Latamoxef in Filtrate of Rat Serum and Serum Alone**

We further examined the interaction of TOB with LMOX in the filtrate of rat serum prepared with MPS-1, and in rat serum freshly prepared. As shown in Fig. 5 (A), the spot associated with LMOX overlapped with that associated with TOB at the point of origin on the plate, and the spot corresponding to LMOX alone disappeared with the course of incubation time. In addition, a new spot was detected at the position of higher *Rf* value, as indicated in Fig. 3.

Figure 5 (B) shows an interaction of TOB with LMOX in rat serum. TLC analysis indicated
Fig. 5. Interaction of TOB with LMOX (A) in the Filtrate of Rat Serum Analyzed by TLC and (B) in Rat Serum Analyzed by TLC.

(A) TOB and LMOX (molar ratio; TOB:LMOX = 2:1) were dissolved in the filtrate of rat serum and incubated at 37 °C for 0.5, 1, 2 and 3 h. UV, UV irradiation; Ni, ninhydrin reaction; T, TOB; L, LMOX; TL, reaction mixture of TOB and LMOX; F, filtrate of rat serum. Arrowheads indicate new spots which have low polarity and are visualized by UV irradiation.

(B) TOB and LMOX (molar ratio; TOB:LMOX = 2:1) were dissolved in rat serum and incubated at 37 °C for 3 h. L, latamoxef; T+L, reaction mixture of TOB and LMOX; S, rat serum.
Fig. 6. IR Spectra of Chemical Compounds

TOB and LMOX (molar ratio; TOB:LMOX = 1:2) were incubated at 37°C, and the resultant reactive product of both drugs was filtrated and dried. A, TOB alone; B, LMOX alone; C, TOB and LMOX; D, reactive products of TOB with LMOX.

that TOB interacted with LMOX even in serum including high molecular weight proteins such as albumin.

**IR Spectrophotometry**

Figures 6-A and B represent the IR spectra of TOB and LMOX, respectively. The IR spectra of TOB and LMOX demonstrated the existence of N-H and O-H stretching (3200—3400 cm⁻¹), C-H stretching (3000 cm⁻¹), N-H bending (1580—1600 cm⁻¹) and C-O stretching (1030 cm⁻¹), and C=O stretching (1750 cm⁻¹) of β-lactam, C=O stretching (1670 cm⁻¹) of amide, and N-H bending (1510 cm⁻¹) of amide, respectively, as typical bands. As shown in Fig. 6-C, no significant changes were observed in the mixture of the two drugs, and the spectrum showed only the combination of the spectra of TOB and LMOX. The IR spectrum of the reactive product of TOB with LMOX showed no absorption at 1750 cm⁻¹, but there was an increase in intensity of the amide (1670 cm⁻¹) bond, implying that TOB reacted with LMOX with degradation of the β-lactam ring in the LMOX molecule (Fig. 6-D).

**Discussion**

We have reported that intrarenal TOB accumulation is suppressed by combination with LMOX in vivo. In the present study, we focused our attention on a possible direct interaction of TOB with LMOX in order to clarify the mechanism by which LMOX suppresses the intrarenal TOB accumulation in rats. To determine whether TOB interacts with LMOX in vitro, we used the PE method. The results with PE showed that the spots corresponding to TOB and LMOX were found on the cathode and anode side, respectively, indicating that TOB has a positive charge and LMOX has a negative
charge at physiological pH. It is very likely that the positive charge of TOB is formed by protonation of amino groups in the molecule and that the negative charge of LMOX is derived from carboxyl groups. Moreover, we detected that the spot associated with TOB overlapped that associated with LMOX on the cathode side, and that the spot corresponding to TOB disappeared with the course of incubation time in the mixture of TOB–LMOX (1:4), while in the mixture of TOB–LMOX (2:1) the spot corresponding to LMOX was not observed at all after 5 h incubation. When TOB and LMOX were separately spotted at the origin, the overlapping of both compounds was not detected on the cathode side. This indicates that the overlapping of the two compounds is due to the interaction of TOB with LMOX but not due to an artifact such as the tailing of spots. Besides this, a new spot, which had a negative charge and was visualized by UV irradiation but not by ninhydrin reaction, was found at a site different from the site of LMOX alone, suggesting that a degradative product resulted from the interaction of TOB with LMOX (Fig. 2).

To examine the interaction of TOB with LMOX and to determine whether a degradative product resulted from the interaction, we used TLC analysis as well as PE. In a preliminary experiment, we studied the effect of each solvent used for developing TOB and LMOX by TLC on the stabilities of TOB and LMOX. The results showed that LMOX was markedly degraded by the solvent, including 28% NH₄OH used for developing TOB, whereas TOB was stable for the solvent used for the development of LMOX. Consequently, we chose the solvent containing CHCl₃, CH₃OH, H₂O and CH₃COOH (13:13:5:1, v:v:v) used for the development of LMOX in order to study the interaction of TOB with LMOX. The spot associated with TOB overlapped with that associated with LMOX at the origin on the TLC plate. Since TOB was ionized in the solvent used in this study and, consequently, fixed at the origin on the silica-gel plate, the overlapping of the two drugs was detected at the origin. In the mixture of TOB–LMOX (2:1), the spot of LMOX disappeared with the course of incubation time, as was also demonstrated with PE. In addition, as TLC analysis clearly showed a new spot which had low polarity and was visualized by UV irradiation but not by ninhydrin reaction, it is very likely that a degradative product results from the interaction of TOB with LMOX (Fig. 3). We demonstrated that the interaction of TOB with LMOX not only in H₂O but also in Krebs–Ringer solution (data not shown). UV spectrum of the mixture of TOB–LMOX (1:2) also showed a striking decrease in the intensity of the absorption at 268 nm (Fig. 4).

These results obtained by PE, TLC and UV showed that TOB interacted with LMOX in vitro, accompanied by degradation of a part of TOB or LMOX molecule.

Furthermore, to examine the possibility that TOB can interact with LMOX in vivo, we used a rat serum filtrate and rat serum alone as solvents instead of H₂O and the Krebs–Ringer solution.

When TOB and LMOX were dissolved in the filtrate of rat serum, TLC analysis of the TOB–LMOX mixture (2:1) showed that TOB interacted with LMOX with the course of incubation time (Fig. 5), and this was almost the same result as that observed in H₂O. Since the filtrate of serum, which includes electrolytes and low molecular proteins, seems to be almost identical in composition to the tubular luminal fluid filtered through the glomerular basement membrane (GBM) of renal glomeruli, and TOB and LMOX are filtered into tubular lumen through GBM as a common excretion pathway, it is possible that TOB interacts with LMOX within tubular lumen close to proximal tubular cells in vivo. Additionally, we confirmed that TOB interacted with LMOX even in rat serum, which includes high molecular weight proteins such as albumin and globulins (Fig. 5). This finding also raises the possibility that TOB interacts with LMOX in blood regardless of the existence of many proteins. If the interaction of TOB with LMOX observed in the present study was induced in vivo, the interaction would contribute to the suppression of intrarenal TOB accumulation by combination with LMOX by inhibiting the binding of TOB to BBMs.

Furthermore, as we could obtain a precipitate which seemed to be a reactive product of TOB
and LMOX, we analyzed the structure of the precipitate using an IR spectrophotometer to obtain information on the molecular mechanism of the interaction of TOB with LMOX.

The IR spectrum of LMOX showed a sharp peak which identifies C=O stretching of β-lactam at 1750 cm⁻¹ (Fig. 6-B). When the mixture of TOB and LMOX was subjected to IR spectrophotometry, the spectrum indicated only the combination of the spectra of TOB and LMOX, and no marked changes were detected (Fig. 6-C). Although the IR spectrum of the precipitate obtained by incubating the TOB-LMOX mixture (1:2) was similar to that of Fig. 6-C but not sharp, it indicated the obvious disappearance of C=O stretching (1750 cm⁻¹) of β-lactam of LMOX. Besides this, there was an increase in the intensity of C=O stretching (1670 cm⁻¹) of amide in the precipitate (Fig. 6-D). These results with IR spectrophotometry suggest that TOB may chemically react with LMOX and form a complex with a degradation of the β-lactam ring. Although the molecular mechanism of degradation of the β-lactam ring is unknown, it would involve the nucleophilic reaction of the nitrogen atom in the TOB molecule with the carbon atom of carbonyl of β-lactam in LMOX. Additionally, the time dependency of the interaction between TOB and LMOX in PE also may suggest that TOB slowly reacts with LMOX with a degradation of the β-lactam ring of LMOX.

PE analysis used to detect the interaction of TOB with LMOX demonstrated that TOB bound to LMOX with a stronger binding force than ionic force, such as covalent bonds and hydrogen bonds. If TOB bound to LMOX with an ionic force like that of NaCl, the overlapping of TOB and LMOX could not be detected in PE analysis. However, the possibility cannot be excluded that electrostatic binding between TOB and LMOX, which would be induced in very early stage of the interaction, is involved in the interaction. Therefore, further studies are needed to clarify the precise molecular mechanism of the interaction and the chemical structure of the complex of TOB and LMOX, and analysis of the molecular mechanism of the interaction of TOB and LMOX may provide a way to avoid the nephrotoxicity induced by AGs.

In conclusion, the results in the present study indicate that TOB chemically reacts with LMOX in H₂O, in the filtrate of rat serum, and in rat serum alone in vitro. Although the precise molecular mechanism of the interaction of TOB with LMOX remains unknown, covalent binding with a degradation of β-lactam ring of LMOX may be, at least in part, involved in the interaction.

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References

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