INTERACTION OF VIOMYCIN AND AMINOGLYCOSIDE ANTIBIOTICS WITH TUBULIN AND MICROTUBULES

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The aminoglycoside and viomycin groups of antibiotics are known to cause nephrotoxicity and ototoxicity, but the mechanism of action of these antibacterial antibiotics against mammalian cells has not been well elucidated. There have been some investigations on codon misreading by aminoglycoside antibiotics with mammalian ribosomes. Streptomycin has been reported to promote miscoding on mitochondrial ribosomes but not on cytoplasmic ribosomes. Paromomycin and lividomycin B stimulate translation errors in a system derived from cultured human cells[1~4].

In the course of screening for tubulin-binding antibiotics, we found that several aminoglycosides and viomycin interact with tubulin and microtubules *in vitro*. Addition of the antibiotics to purified porcine brain tubulin was observed to cause an increase in turbidity, and the negatively stained image of the turbid solution showed ordered tubulin assembly with double walls. When the antibiotics were added to microtubules polymerized *in vitro* at 37°C, microtubules were reconstituted into duplexes, accompanied by the formation of rigid bundles of a crystalline nature. This note presents the results of optical density analysis and electron-microscopic observation of tubulin assembly in the presence of aminoglycosides and viomycin.

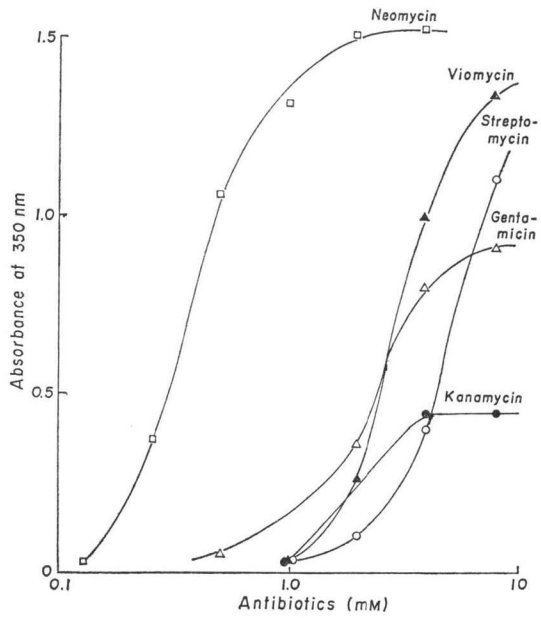
Purified tubulin was prepared from porcine brain by the method of SCHELANSKI *et al.*[5] When streptomycin, kanamycin, neomycin, gentamicin or viomycin was mixed with purified tubulin in buffer, as described in the legend of Fig. 1, there was an immediate increase of turbidity at 18°C, that reached a maximum in 5 minutes. The products were collected by centrifugation, and analyzed by SDS-polyacrylamide gel electrophoresis, showing that they consisted of tubulin (data are not shown). Natural microtubule assembly did not occur in the absence of the antibiotics under the same conditions. The final turbidity attained was dependent upon antibiotic concentration at a constant concentration of tubulin. Neomycin was more active in inducing the formation of the insoluble complex than the other agents (Fig. 1).

Electron-microscopic analysis revealed a new type of tubulin assembly, formed by the interaction with neomycin, streptomycin, kanamycin, gentamicin or viomycin, that showed characteristic ordered structure. The structure was dif-

Fig. 1. Formation of insoluble antibiotic-tubulin complex.

Porcine brain tubulin, in a buffer of 100 mM MES (2-(N-morpholino)-ethanesulfonic acid), 0.5 mM $MgCl_2$, 50 mM KCl and 1 mM GTP, pH 6.85, was mixed with various concentrations of streptomycin, kanamycin, neomycin, gentamicin or viomycin in the same buffer without GTP; and the mixture was incubated at 18°C for 7 minutes.

The final concentration of tubulin was 0.9 mg/ml. The turbidity was monitored by optical density at 350 nm. The maximal absorbance attained at each antibiotic concentration was plotted against the logarithm of final drug concentration.



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ferent from natural microtubules or vinblastine-induced microtubular crystals[6,7], but resembled the tubulin assembly induced by polycations[8,9]. It possessed double walls with a diameter of 25 nm at the outer edge of the inner wall and 38 nm of the outer wall. Both walls showed the same axial periodicity of 5 nm each (Fig. 2). Its optical diffraction pattern exhibited relatively strong 5 nm meridional and off-meridional reflections. Off-meridional reflections of 4 nm, obtained in natural microtubule assembly as described later, could not be recognized (Fig. 3a).

Addition of neomycin to microtubules, normally assembled *in vitro* at 37°C, induced reassembly of microtubules and laterally attached bundle formation. An immediate increase of turbidity occurred. Electron-microscopic observation at an early stage of neomycin addition showed the microtubules with 4-nm axial periodicity were surrounded by an outer wall with a diameter of 38 nm (Fig. 5). Finally, the newly assembled structure possessed double walls, accompanied by lateral association or bundle formation, as presented in Fig. 4b. The optical diffraction pattern showed clear 5 nm meridional reflections, and both 4 and 5 nm off-meridional reflections (Fig. 3b).

For the purpose of elucidating the nature of microtubular crystal formation by neomycin, streptomycin, kanamycin, gentamicin or viomycin, we have investigated the effects of various ag nts which were reported to block natural microtubule assembly. Low temperature (4°C), 1 ~ 10 mM colchicine and 5 mM N-ethylmaleimide, which prevented natural microtubule assembly, did not significantly affect the formation of the antibiotic-tubulin complex. Other inhibitors of natural microtubule assembly, 4 mM $CaCl_2$ and 3.2 mM EDTA (ethylenedinitrilotetraacetic acid), exhibited weak inhibitory effects on the antibiotic-tubulin interaction, and the antibiotic-tubulin complex was not degraded on exposure to these reagents. The effect of EDTA was not reversed by Mg^{2+}. The results suggested that the mechanism of tubulin-antibiotic complex formation was different from that of natural microtubule assembly.

KCl at a concentration of 600 mM completely blocked antibiotic-tubulin complex formation, and the constituted complex was immediately degraded by the addition of 600 mM KCl. The same effect was observed with natural microtubule assembly.

Since tubulin is an acidic protein and the antibiotics used are basic substances, the complex formation may be due to ionic interaction. Polycations (DEAE-dextran, protamine, polylysine and spermine) and basic proteins (histone and RNase A) have been reported to induce or facilitate new types of tubulin assembly[8,9], and the antibiotic-induced assembly of tubulin and microtubules resembles the structure formed by polycations and basic proteins, with double walls or duplex. In this experiment, we were able to explain the mechanism of the genesis of two different assembly forms induced by the same drugs. Starting from tubulin, the double wall type with the same periodicity of 5 nm was obtained, as in the case of ERICKSON and VOTER[9] and the spermine-induced tubules by JACOBS *et al.*[8] When microtubules were the starting material, the assembled tubulin-complex had two different periodical walls, called the "duplex tubule" by JACOBS *et al.*[8] Tubulin, remaining in a soluble state or partially decomposed from microtubules, attached and overlapped to microtubules with 4-nm periodicity, and constituted the outer wall with 5-nm periodicity. Subsequently, the outer wall to outer wall interaction caused lateral association, accompanied by the formation of crystalline-like structure.

The complex formation with tubulin or microtubules seems to be characteristic to the aminoglycoside and viomycin groups of antibiotics. Addition of some other basic antibiotics, such as bleomycin and anthracycline antibiotics, to tubulin solution did not cause an increase in turbidity under the current conditions, although daunorubicin was reported to block normal microtubular assembly[13].

It remains to be determined whether the interaction of viomycin and aminoglycosides with tubulin and microtubules actually occurrs in animals or man. Aminoglycoside antibiotics are not generally active against intracellular bacteria, indicating that the antibiotics do not easily penetrate into mammalian cells. However, in some cases aminoglycosides may inhibit the growth of intracellular bacteria and penetrate slowly into cultured macrophages, BHK cells and rat embryo fibroblasts[10,11].

The biological significance of the interaction of viomycin and aminoglycosides with tubulin and microtubules is not known at this time. It

Fig. 2. Electron micrographs of negatively stained images of neomycin-induced tubulin assembly.

Samples were stained negatively with 4% uranyl acetate. Inserted scales were 50 nm. a. Lateral views. b. End-on views.

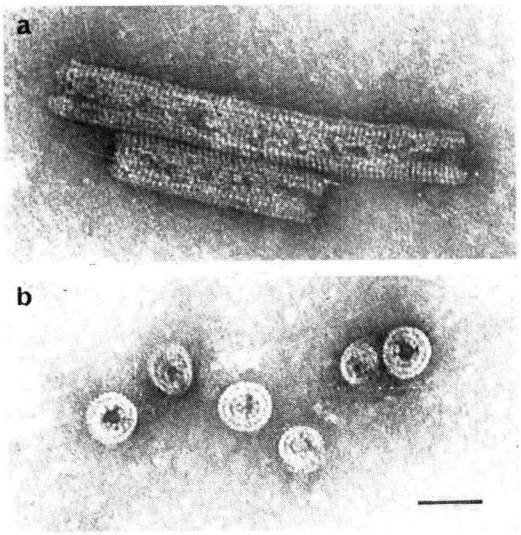


Fig. 3. Optical diffraction patterns of tubulin and microtubule assembly. (a: left, b: right)

Optical diffraction patterns were obtained by an optical diffractometer with He-Ne laser similar to KLUG & BERGER's lens system[12].

a. The pattern obtained from the negative of electron micrograph of neomycin-induced tubulin assembly with two walls of 5-nm periodicity.

b. The pattern obtained from the negative of electron micrograph of a part of neomycin-induced microtubule assembly with duplex crystalline form.

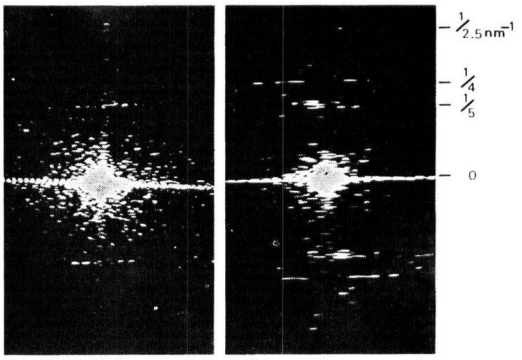


Fig. 4. Electron micrographs of negatively stained image of natural tubulin assembly and neomycin-induced microtubule assembly.

Negatively stained by the same procedure as Fig. 2. Inserted scales were 50 nm. a. Natural microtubules assembled at 37°C. b. Crystalline-like structure of neomycin-induced microtubule assembly.

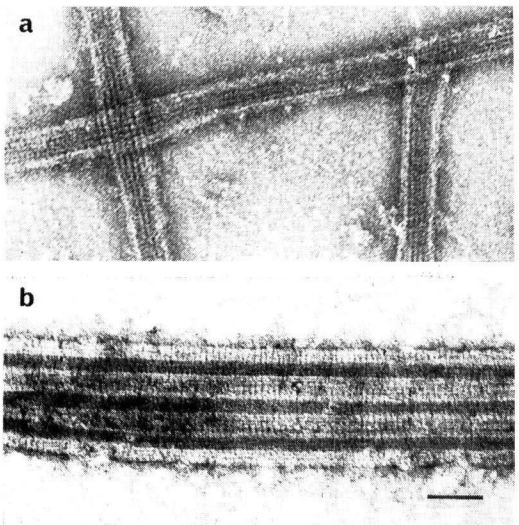
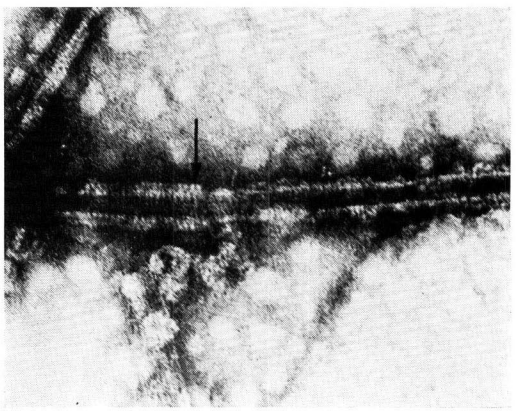


Fig. 5. Electron micrograph of negatively stained image of natural microtubules, partly surrounded by a neomycin-induced outer wall.

Arrow shows a growing point of the duplex outer wall.



might be related to the side effects of the drugs: ototoxicity and nephrotoxicity. The antibiotics might penetrate into cochlear sensory cells (hair cells) and into the renal cells, and might interact with tubulin and microtubules in these cells.

In addition, the formation of insoluble complexes with tubulin suggests that the antibiotics may be convenient for the preparation or purification of tubulin from crude cell extracts.

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