STUDIES ON MACROCYCLIC LACTONE ANTIBIOTICS

XI.† ANTI-MITOTIC AND ANTI-TUBULIN ACTIVITY OF NEW ANTITUMOR ANTIBIOTICS, RHIZOXIN AND ITS HOMOLOGUES

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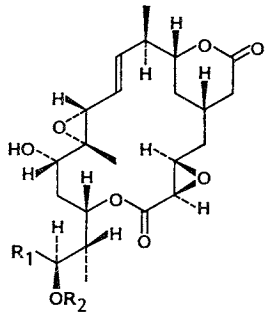
The mode of action of rhizoxin (**1a**), a new antitumor macrolide, was investigated. Rhizoxin inhibited fusion of the male and the female pronuclei in fertilized sea urchin eggs and inhibited cilia formation in the deciliated sea urchin embryos. *In vitro*, polymerization of tubulin isolated from porcine brains was completely inhibited at a 1×10^{-5} M concentration of rhizoxin, and tubulin which had been polymerized by incubation at 37°C for 30 minutes was depolymerized by addition of 1×10^{-5} M of the drug. Activity of rhizoxin against tubulin polymerization was compared with those of other anti-tubulin drugs such as colchicine, vinblastine and ansamitocin P-3. The homologues of rhizoxin, **1b**~**3b**, also inhibited polymerization of the purified microtuble protein at almost the same extent as rhizoxin.

Rhizoxin (**1a**) is a 16 membered macrolide isolated from *Rhizopus chinensis* Rh-2, the pathogen of the rice seedling blight.[2,3] The fungus produced also the homologues of rhizoxin, **1b**~**3b**,[4] **4**[3] and **5**††. Rhizoxin showed similar chemotherapeutic effects to those of vincristine against L1210 and P388 leukemia-bearing mice. The drug was also effective against B16 melanoma, inoculated ip or sc.[5] Rhizoxin inhibited the mitosis of the tumor cells in a manner similar to that of *Vinca* alkaloids as revealed by morphological study and flow cytometry analysis suggesting that rhizoxin inhibits microtubule polymerization.[5] We have, therefore, studied the effects of rhizoxin on cell division in sea urchin egg to survey the mode of action of the drug and on porcine brain tubulin polymerization to confirm the results obtained using sea urchin embryos. Inhibitory activity on tubulin polymerization by rhizoxin was compared with that by colchicine, vinblastine and ansamitocin P-3[6] (a maytansinoid compound). Anti-tubulin activities of the rhizoxin homologues (**1b**~**3b**, **4** and **5**††) and of the rhizoxin derivatives (**6**~**8**††) were also studied (Figs. 1 and 2).

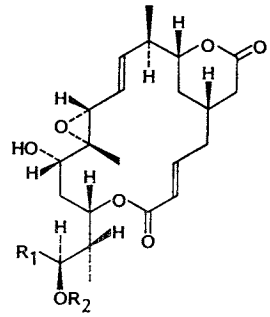
† See ref 1.

†† Structural assignments of compounds **5**, **6** and **8** are based on unpublished data.

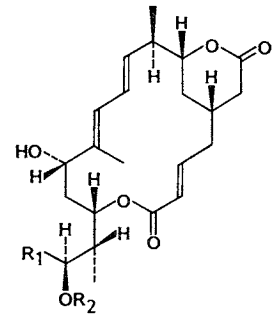
Fig. 1. Structures of naturally occurring rhizoxin homologues.



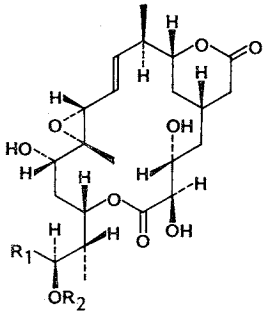
1a, 1b



2a, 2b



3a, 3b



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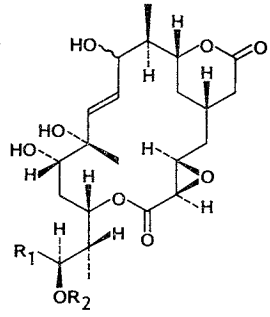

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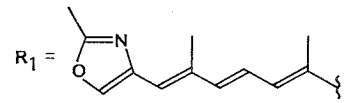
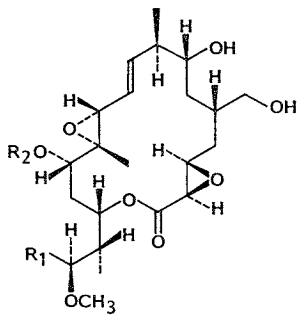
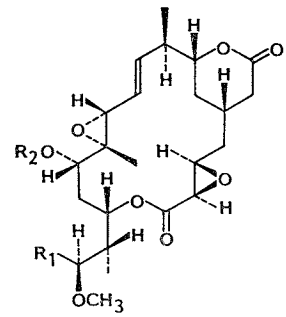


1a $R_2 = CH_3$
1b $R_2 = H$
2a $R_2 = CH_3$
2b $R_2 = H$
3a $R_2 = CH_3$
3b $R_2 = H$
4 $R_2 = CH_3$
5 $R_2 = CH_3$

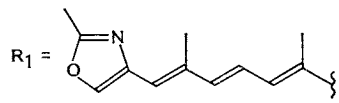
Fig. 2. Structures of rhizoxin derivatives.



6



7, 8



6 $R_2 = H$
7 $R_2 = Ac$
8 $R_2 = COCH_2CH_2COOH$

Materials and Methods

Chemicals

Colchicine (CLC) and vinblastine (VLB) were purchased from Sigma Chemical Company. Ansamitocin P-3 (P-3) was offered by Takeda Chemical Industries, Ltd. Rhizoxin (RZX, **1a**) and its homologues (**1b**~**3b**, **4** and **5**) were isolated from the culture broth of *Rhizopus chinensis* Rh-2 and the derivatives **6**~**8** were synthesized in our laboratory. All other chemicals were reagent grade.

Morphological Observation of Sea Urchin Embryos

The sea urchin, *Hemicentrotus pulcherrimus*, was used. Eggs and sperm were obtained by injecting 0.6 M KCl into the body cavity. Eggs were washed three times with artificial sea water and inseminated. Fertilized eggs were developed at 20°C. Just before and after insemination, RZX was added at the indicated final concentration to the egg or embryo suspension. RZX was dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO was 1%, which did not affect early development. Morphological observation was carried out by staining with a lactoorcein solution.[7]

Inhibition of cilia formation in sea urchin embryos was tested using gastrula stage embryos deciliated by treatment with sea water containing 0.5 M NaCl for 2 minutes. Then deciliated embryos were transferred to the normal sea water containing various concentrations of RZX.

Purification of Microtubule Protein

Microtubule protein was prepared from porcine brains using the polymerization-depolymerization method of SHELANSKI with slight modifications.[8] A typical run is as follows: Fresh brains cooled on ice and washed with an aqueous solution containing 100 mM 4-morpholinoethanesulfonic acid (MES), 1 mM ethyleneglycol bis(2-aminoethylether)tetraacetic acid (EGTA), 1 mM 2-mercaptoethanol (2-ME), 0.5 mM $MgCl_2$, pH 6.5, were homogenized in 0.5 ml/g of MES buffer (100 mM MES, 1 mM EGTA, 0.5 mM 2-ME, 1 mM GTP, pH 6.5) in Waring Blender under ice cooling. After centrifugation at 50,000×*g* for 30 minutes at 4°C, the supernatant was mixed with an equal volume of glycerol buffer (100 mM MES, 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM 2-ME, 1 mM GTP, 8 M glycerol, at pH 6.5) and warmed at 37°C for 40 minutes to polymerize tubulin. The polymerized tubulin in the solution was collected as a pellet by centrifugation at 100,000×*g* for 45 minutes at 25°C. The pellet was resuspended in 100 ml of MES buffer and was, after homogenation in teflon/glass homogenizer, chilled at 0°C for 30 minutes to depolymerize. After centrifugation of the suspension at 100,000×*g* for 60 minutes at 4°C, equal volume of glycerol buffer was added to the supernatant solution, and the solution was stored at −70°C.

The protein concentration of the solution was determined by the method of LOWRY *et al.*[9] with bovine serum albumin as the standard.

The microtubule protein solution thus prepared consists of *ca.* 75% of tubulin and *ca.* 25% of microtubule associated proteins (MAPs) (MAP 1, MAP 2, tau).

Immediately before use, the protein was further purified from the stock solution by a repeated cycle of the polymerization-depolymerization step.

Polymerization and Depolymerization Assay

Polymerization of tubulin and depolymerization of microtubules were followed by turbidity measurement[10] at 37°C in MES buffer solution containing 2 mg of protein per ml of solution. The turbidity was measured at 400 nm on a Shimadzu UV-300 Photospectrometer. The drugs were added as DMSO solution.

Electron Microscopy

The samples of microtubule protein were put on 100 mesh Formvar grid and negatively stained with 2% uranyl acetate. The samples were examined with a Jeol 200CX electron microscope.

Results

Effect of RZX on Cleavage of Fertilized Sea Urchin Eggs

RZX was added to the sea urchin embryo suspension 5 minutes after fertilization. At 0.8×

Fig. 3. Effect of rhizoxin on pronuclear fusion of fertilized sea urchin eggs.

Fertilized eggs were allowed to develop in the presence of 1.6×10^{-8} M rhizoxin for 140 minutes during which period control embryos reached the 4-cell stage. Two clusters of pycnotic chromosomes (arrow) are apparent.

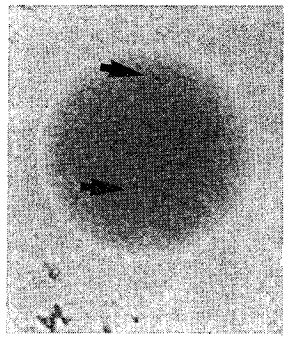


Fig. 4. Effect of rhizoxin on microtubule polymerization.

Microtubule protein (2 mg/ml) was mixed with rhizoxin at 0°C and was incubated at 37°C. Final rhizoxin concentrations are: 0 (●), 2 μM (○), 5 μM (■) and 10 μM (□).

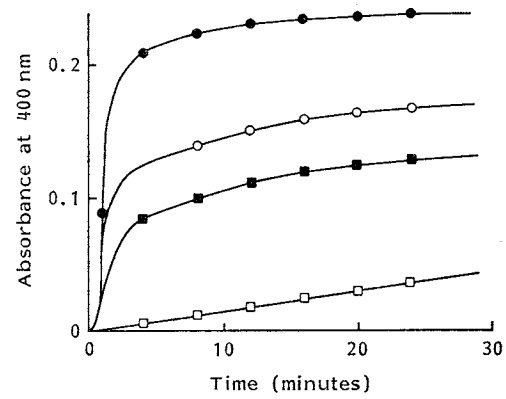
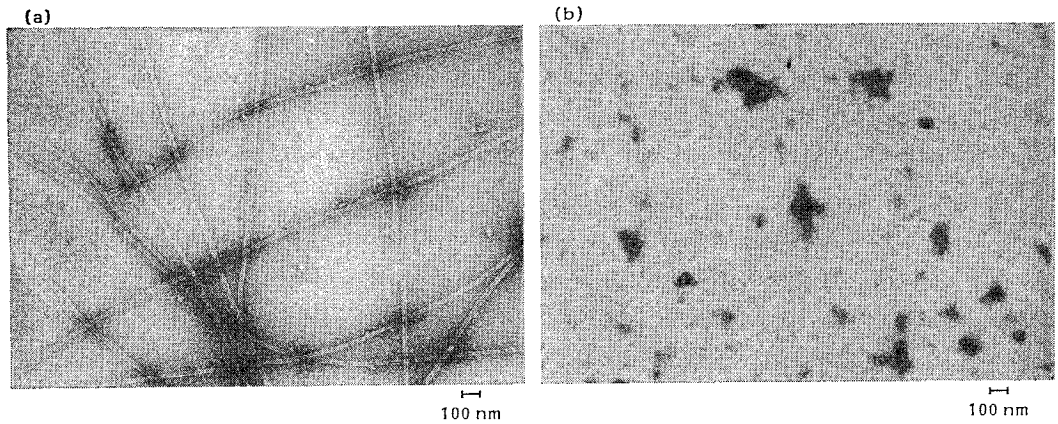


Fig. 5. Electron micrograph.

a) Microtubules formed by incubation of microtubule protein at 37°C for 30 minutes.
b) Microtubule protein incubated at 37°C for 30 minutes in the presence of 10 μM rhizoxin.



10^{-8} M RZX, fertilized eggs did not cleave and remained at the 1-cell stage, and, at 0.16×10^{-8} M, development of some embryos progressed normally or slowly and some did not. When sperms were added to the egg suspension after addition of RZX, fertilized membrane formed and the subsequent development was the same as above.

In sea urchin eggs treated with 1.6×10^{-8} M RZX, fusion of the male and the female pronuclei never occurred during the 140 minutes observation time while control embryos attained the 4-cell stage (Fig. 3). The result shown in Fig. 3 indicated that rupture of the pronuclear membrane and chromosomal condensation occurred in the presence of the drug but the progression from prometaphase to metaphase did not occur. In the deciliated sea urchin embryos at the gastrula stage treated with 16×10^{-8} M RZX, cilia formation was inhibited. These observations suggested that RZX may interfere with microtubule formation.

Effect of RZX on DNA, RNA and Protein Synthesis

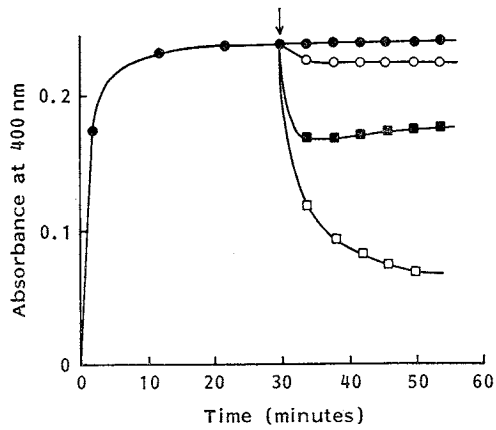
Embryos at the gastrula stage were incubated for 60 minutes with [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine. DNA, RNA and protein synthesis were detected by incorporation of these tritiated precursors into acid insoluble fraction. RNA and protein synthesis were not inhibited by 1.6×10^{-5} M RZX, whereas 50% inhibition of DNA synthesis was observed at *ca.* 1.6×10^{-5} M. This inhibition may be a secondary effect of inhibition of cell cycle progression.

Effect of RZX on the Polymerization of Tubulin

The time course of the polymerization reaction of 2 mg of porcine microtubule protein in 1 ml of MES buffer and the inhibitory effects of the drug added at various concentrations are shown in Fig. 4. Polymerization of tubulin occurred rapidly and leveled off at *ca.* 30 minutes incubation at 37°C.

Fig. 6. Disruptive effect of rhizoxin on microtubules.

Microtubule proteins were incubated at 37°C and the assembly was monitored by turbidity at 400 nm. Thirty minutes after the incubation, rhizoxin solutions were added. The final rhizoxin concentrations were: 0 (●), 2 μM (○), 5 μM (■) and 10 μM (□).



By adding RZX at various concentrations, the polymerization of tubulin was inhibited in a concentration dependent manner and, at 5×10^{-6} and 10×10^{-6} M, 50% and almost 100% inhibition were observed, respectively. In the electron microscope, no polymerized microtubule was observed when 10×10^{-6} M of the drug was added (Fig. 5).

Effect of RZX on Polymerized Microtubule Protein

The time courses of the depolymerization of polymerized microtubules by addition of various concentrations of RZX were shown in the Fig. 6. The drug was added after 30 minutes incubation of the microtubule protein at 37°C.

By addition of 5×10^{-6} M and 10×10^{-6} M of the drug, *ca.* 30 and 70% depolymerization was attained, respectively.

Comparison of the Effect of RZX, VLB, P-3 and CLC on the Tubulin Polymerization

The anti-tubulin activities of RZX, VLB and P-3 were compared by adding various concentrations of drugs to the brain microtubule protein solution (Fig. 7). RZX and P-3 inhibited polymerization of brain tubulin in similar manner. The activity of VLB is as about twice as strong as those of RZX and P-3, and the activity of CLC was weaker than that of RZX. IC_{50} values of RZX, P-3, VLB and CLC were determined as 5×10^{-6}, 5×10^{-6}, 2×10^{-6} and 10×10^{-6} M, respectively, under the same assay conditions.

Aggregation, observed as increasing relative absorption, occurred with VLB at a concentration of 1×10^{-5} M or more, whereas no such aggregation was caused by RZX and P-3 even at a concentration as high as 1×10^{-4} M (Fig. 7).

Fig. 7. Comparative effect of anti-mitotic drugs on microtubule assembly.

Microtubule protein (2 mg/ml) was mixed with various concentrations of drugs and were incubated at 37°C. Relative absorbance after 20 minutes were given *vs.* drug concentrations. The drugs compared are: Rhizoxin (●), ansamitocin P-3 (○) and vinblastine (□).

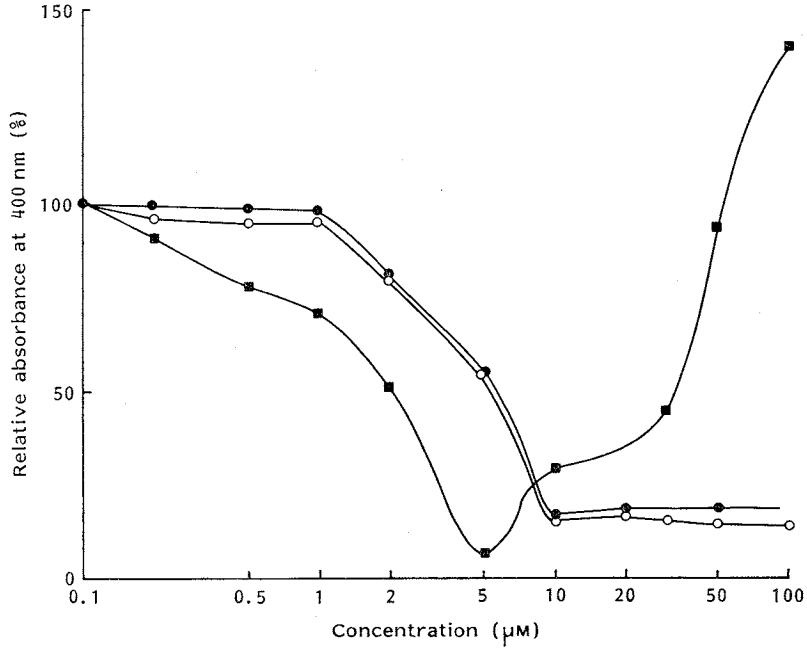


Table 1. Inhibitory activity of rhizoxin, and its homologues against microtubule polymerization.

	1a	1b	2a	2b	3a	3b	4	5	6	7	8
IC_{50} (μM)	5	7	5	7	7	7	50	>100	20	>20	>100

Microtubule protein (2 mg/ml) was mixed with various concentrations of compounds and incubated for 30 minutes at 37°C. IC_{50} values were determined by measuring turbidity at 400 nm.

Effect of RZX Homologues and Derivatives on the Polymerization of Tubulin

Different concentrations of RZX homologues (**1b**~**3b**, **4** and **5**) isolated as the metabolites of *Rhizopus chinensis* Rh-2 and of RZX derivatives (**6**~**8**) were added to microtubule protein and their ability to inhibit tubulin polymerization was measured. IC_{50} values of these compounds are shown in Table 1. The homologues **1b**~**3b** had high activities as compared to that of RZX, and compounds **4** and **6** showed lower but still notably high activities, whereas compounds **5** and **8** were inactive even at a concentration of 1×10^{-4} M or more. The activity of compound **7** could not be examined because of insolubility of this compound in the protein solution at higher concentration.

Discussion

RZX has been known to inhibit the growth of rice seedling roots, a variety of fungi and a variety of tumor cells *in vitro*, whereas growth of all bacteria tested was not affected.[2,3,5] This fact indicated that the mode of action of RZX is specific for eukaryotic cells.

The present study, and that reported earlier,[5] demonstrated that RZX inhibits pronuclear fusion and cleavage in sea urchin eggs; prevents the polymerization of tubulin; depolymerizes microtubules

and causes morphological changes of tumor cells. These activities are, in general, similar to those of spindle poisons such as colchicine, podophyllotoxin, steganacin, *Vinca* alkaloids and maytansinoids, suggesting that this drug, like other spindle poisons, exerts its anti-mitotic activity through an effect on spindle microtubules, which do not exist in the prokaryotic cells. RZX shows the highest activity of all the naturally occurring mitosis inhibitors.

In the electron microscope, however, RZX-treated tubulin and preformed microtubules showed only small granules even with the sample treated at high concentration (1×10^{-4} M), whereas tubulin treated with the same concentration of VLB showed spiral structure attributed to aggregation. This means that RZX and VLB have a somewhat dissimilar effect on tubulin.

Since RZX and its homologues **1b**~**3b** have similarly high inhibitory effects on the brain tubulin polymerization, the two epoxy groups present in the RZX molecule are not essential for the activity. This may indicate that fixation of the molecular geometry either by epoxy groups or by olefinic linkages should be important for the interaction with the protein. Inactivity of compounds **7** and **8** against microtubule polymerization would suggest a significant role of the hydroxy group at C-13 in RZX binding to the tubulin.

Studies on the binding affinity and the binding site of RZX on tubulin molecule will be reported elsewhere.

Acknowledgment

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