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Interaction of Rhizoxin with Bovine Brain Tubulin[1]

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ABSTRACT

Rhizoxin is an antitumor drug prepared from the fungus *Rhizopus chinensis*. It is an inhibitor of microtubule assembly and a potent competitive inhibitor of the binding of tubulin of ansamitocin P-3, a maytansine analogue. Rhizoxin also weakly inhibits vinblastine binding to tubulin. We have previously found that maytansine and vinblastine differ strikingly from each other in many ways, including their effects on tubulin sulfhydryl groups and on tubulin decay. Since the structure of rhizoxin is very different from that of vinblastine and only slightly resembles that of maytansine, we decided to compare its interaction with tubulin with those of the other two drugs, using systems which discriminate between the effects of the latter two drugs. We found that rhizoxin acts like maytansine in that it completely prevents formation of an intrachain cross-link in β-tubulin by *N,N'*-ethylenebis(iodoacetamide), whereas vinblastine only partially inhibits this. Half-maximal inhibition of formation of this cross-link was observed at 2.5 μM rhizoxin. We found previously that the rate of binding of tubulin to the fluorescent probe bis(8-anilinonaphthalene 1-sulfonate) is a good indicator of tubulin decay and that vinblastine strongly inhibits this, whereas maytansine has no effect. We here report that rhizoxin acts like maytansine in that it has no effect on decay. Thus, despite the fact that its resemblance to maytansine is small, rhizoxin appears to interact with tubulin in very much the same way as does maytansine.

INTRODUCTION

Rhizoxin (Fig. 1) is an antimitotic drug purified from the fungus *Rhizopus chinensis*; it has been shown to be toxic to a variety of tumor cell lines and to be effective *in vitro* and *in vivo* against several tumors, including vincristine- and Adriamycin-resistant ones (1). In some tumor cell lines, rhizoxin has been found to be more cytotoxic than vincristine (1). Rhizoxin is also an inhibitor of microtubule assembly *in vitro* and *in vivo* and a potent competitive inhibitor of the binding of ansamitocin P-3 to tubulin; it is also a weak inhibitor of the binding of vinblastine to tubulin (2). Ansamitocin P-3 is a close structural analogue to maytansine (Fig. 2). Rhizoxin resembles maytansine in that it inhibits vinblastine-induced tubulin aggregation (2). Since vinblastine and maytansine are competitive inhibitors of each other's binding to tubulin (3, 4), it is interesting that rhizoxin seems to selectively inhibit the binding of a maytansine analogue and yet has a much weaker effect on the binding of vinblastine (2).

Although both vinblastine and maytansine inhibit microtubule assembly (5), the two drugs differ greatly from each other in their effects on the tubulin molecule. One of these differences is that vinblastine inhibits the alkylation of tubulin by iodo[^{14}C]-acetamide whereas maytansine has little effect on this process (6). Another significant difference is that maytansine completely inhibits formation of a covalent intrachain cross-link in the β subunit of tubulin induced by EBI;[3] vinblastine only partly inhibits formation of the cross-link (7). This particular cross-link, designated β^s, is between cysteines 12 and either 201 or 211 (8). The β^s cross-link is a useful discriminator among the effects of tubulin ligands since one set of ligands, namely, colchicine, podophyllotoxin, and nocodazole, all enhance its formation (7). By contrast, a second EBI-induced intrachain cross-link in β-tubulin, designated β*, between cysteines 239 and 354, has its formation enhanced by vinblastine and maytansine and inhibited by colchicine, podophyllotoxin, and nocodazole (9, 10). Another difference between vinblastine and maytansine is that vinblastine strongly inhibits the time-dependent increase in the binding of tubulin to the fluorescent probe bis(8-anilinonaphthalene 1-sulfonate (BisANS), whereas maytansine has no effect on this process (11).

Since rhizoxin bears no structural resemblance to vinblastine and has a fairly slight similarity to maytansine, we were interested in examining the effects of rhizoxin on the interaction of tubulin with EBI and on the decay of tubulin. We find that rhizoxin resembles maytansine in that it completely inhibits formation of the β^s cross-link and that it has no effect on BisANS binding to tubulin.

MATERIALS AND METHODS

Materials. Rhizoxin was purified from the fungus *R. chinensis* as described by Iwasaki *et al.* (12). Vinblastine was a kind gift of the Eli Lilly Corporation (Indianapolis, IN). All other materials were obtained or prepared as previously described (13).

Tubulin. Microtubule protein was purified from bovine brain by the method of Fellous *et al.* (14). For cross-linking experiments, tubulin was purified from the microtubule protein pellets on a double column consisting of G-25 and phosphocellulose, as described previously (7). The buffer for these experiments consisted of 100 mM 2-(*N*-morpholino)ethanesulfonic acid (pH 6.4), 0.1 mM EDTA, and 1 mM ethyleneglycol bis(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid. Tubulin used in fluorescence experiments was purified from the microtubule protein pellets by chromatography on phosphocellulose (14) in the same buffer, but also containing 1 mM GTP, 0.5 mM $MgCl_2$, and 1 mM β-mercaptoethanol.

Cross-Linking. Freshly prepared tubulin was incubated with EBI as previously described (7). Samples were then reduced, carboxymethylated, and subjected to polyacrylamide gel electrophoresis (17). For quantitation of the yields of the β^s and β* cross-linked species, the gels were stained with fast green and scanned at 640 nm in a Gilford 250 spectrophotometer equipped with a linear transport. Reduced and carboxymethylated conalbumin was used as an internal standard for calculating the yields of the two cross-links (7).

Fluorescence. Tubulin was incubated at 37°C with the drug to be tested and then an aliquot was removed and mixed with BisANS. The resulting fluorescence was measured in a SLM/Aminco SPF-500C spectrofluorometer set in the ratio mode and connected to a temperature-controlled water bath. Excitation and emission were at 385 and 490 nm, respectively.

Other Methods. Protein concentration was measured by the method of Lowry *et al.* (15), as modified by Schacterle and Pollack (16), using bovine serum albumin as a standard. Samples were subjected to poly-

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[3] The abbreviations used are: EBI, *N,N'*-ethylenebis(iodoacetamide); BisANS, bis(8-anilinonaphthalene 1-sulfonate).

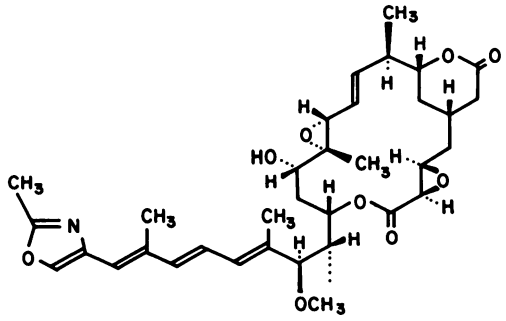

Fig. 1. Structure of rhizoxin.

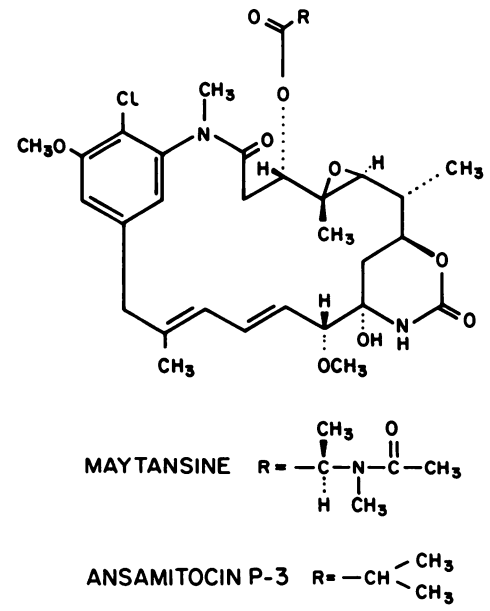

Fig. 2. Structures of maytansine and ansamitocin P-3.

acrylamide gel electrophoresis on gels containing 5.5% polyacrylamide using the system of Laemmli (17). In certain experiments, the modified Laemmli system of Banerjee *et al.* (18) was used, in which the pH of the resolving gel is 9.1 and the Tris concentration is 0.74 M.

RESULTS

Effects of Rhizoxin on the Interaction of Tubulin with EBI. The tubulin molecule is M_r 100,000 heterodimer consisting of two M_r 50,000 subunits called α and β (19). When chordate tubulin is reduced and carboxymethylated and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, using the system of Laemmli (17), the tubulin runs as 3 distinct bands; in order of increasing mobility, these bands are designated α (corresponding to the α subunit), β_2, and β_1 (13). The β_1 and β_2 bands correspond to isotypically distinct forms of β-tubulin, any of which can pair with α to form an α/β heterodimer (13).

When tubulin is incubated with EBI in the absence of any ligand, two intrachain cross-links form in the β subunit, resulting in the formation of three discrete electrophoretic species, each of which has an electrophoretic mobility higher than that of the non-cross-linked β bands. These species are designated as follows: (*a*) β^s, containing the β^s cross-link; (*b*) β^*, containing the β^* cross-link; and (*c*) β^{s*}, containing both the β^s and the β^* cross-links. In order of increasing electrophoretic mobility, they are: β^s, β^*, β^{s*} (Fig. 3, *Lanes 3* and *4*). The β_2 form of tubulin does not participate in this cross-link formation (7, 13); hence the β^s, β^*, and β^{s*} bands all arise from β_1. In the Laemmli system, the resolution between the β^s band and the β_1 band is poor, so that it is difficult in Fig. 3 to see the β^s band in some of the lanes. As shown in Fig. 3 (*Lanes 5* and *6*), in the presence of rhizoxin, formation of the β^{s*} band is completely inhibited, while that of the β^* band is enhanced. This indicates that rhizoxin is suppressing formation of the β^s cross-link. The effect of GTP is very similar to that of rhizoxin (Fig. 3, *Lane 10*). We have found previously that maytansine and GTP can each strongly inhibit formation of the β^s cross-link (7). The effect of vinblastine is similar but not identical to that of rhizoxin in that it inhibits β^{s*} formation, but only partially (Fig. 3, *Lane 7*), while enhancing that of β^*, indicating that vinblastine is partly inhibiting formation of the β^s cross-link. Rhizoxin, GTP, maytansine, and vinblastine all are similar in that they enhance formation of the β^* cross-link (Fig. 3, *Lanes 5–7, 10*). This contrasts with the effect of podophyllotoxin, which acts like colchicine and nocodazole and inhibits formation of the β^* cross-link, while enhancing that of β^s (Fig. 3, *Lane 13*). In order to observe more directly the effect of rhizoxin on the formation of the β^s cross-link, the experiment shown in Fig. 4 was performed in which tubulin was reacted with EBI in the presence of both rhizoxin and podophyllotoxin and the results were analyzed on the modified Laemmli system of Banerjee *et al* (18). In this system, there is much more resolution between the β^s and β_1 bands than is obtained in the unmodified Laemmli system. On this system, however, the β^* and β^s bands comigrate, necessitating the presence of podophyllotoxin in the incubation reaction with EBI, to prevent β^* formation. As can be seen in Fig. 4, rhizoxin-induced inhibition of β^s formation was very notable at 2 μM rhizoxin.

In order to determine the concentration dependence of the effect of rhizoxin on the formation of the β^s cross-link, an experiment was performed in which tubulin was cross-linked with EBI in the presence of a series of rhizoxin concentrations and also in the presence of 50 μM podophyllotoxin. With podophyllotoxin inhibiting formation of the β^* cross-link, only the β^s cross-link could form. The results were analyzed on the modified system of Banerjee *et al* (18). The results of this experiment (Fig. 5) show that half-maximal inhibition of β^s formation is obtained at a rhizoxin concentration of 2.5 μM. In summary, the results shown in Figs. 3–5 represent three separate experiments on three preparations of tubulin; in each experiment, rhizoxin caused complete inhibition of β^s formation, as we have previously reported to be the case for maytansine (7). The results shown in Figs. 4 and 5 indicate that very low concentrations (2–2.5 μM) of rhizoxin are sufficient to accomplish this.

Effect of Rhizoxin on the Interaction of Tubulin with BisANS. An unusual property of tubulin is that it exhibits a time-dependent increase in the binding to the fluorescent probe BisANS (11). This is assumed to reflect tubulin decay (11). Vinblastine, which is a strong inhibitor of tubulin decay, strongly inhibits the time-dependent increase in binding to BisANS; in contrast, maytansine has no effect at all on BisANS binding (11). In three separate experiments, each on a different preparation of tubulin, the effects of rhizoxin and vinblastine on the binding of BisANS to tubulin were compared. All three experiments gave the same result, namely, that rhizoxin resembles maytansine in that it does not affect the rate of BisANS binding to tubulin. In each of these experiments, one of which is shown in Fig. 6, vinblastine strongly inhibited BisANS binding, as we have shown previously (11).

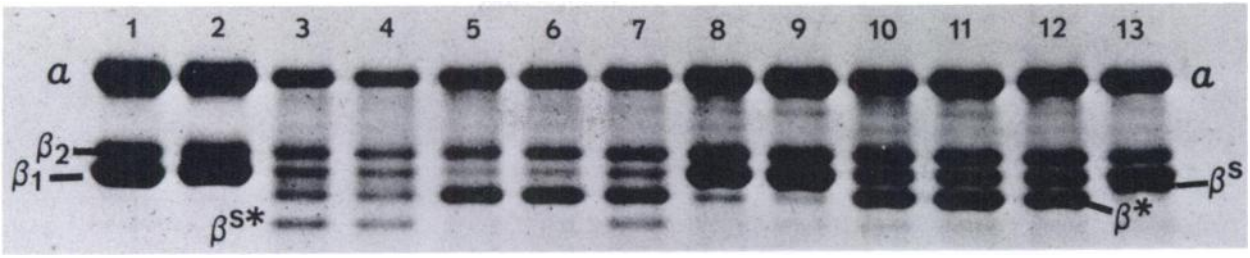

Fig. 3. Effect of rhizoxin and other drugs on the formation of the β^s and β^* cross-links by EBI. Aliquots (250 μl) of tubulin (0.66 mg/ml), containing reduced and carboxymethylated conalbumin (0.20 mg/ml), were incubated for 1 h at 30°C in the absence (*Lanes 1* and *2*) or presence (*Lanes 3–13*) of 0.91 mM EBI and of the following drugs, as indicated: *Lanes 3 and 4*, no addition; *Lanes 5* and *6*, 50 μM rhizoxin; *Lane 7*, 50 μM vinblastine; *Lane 8*, 50 μM rhizoxin + 50 μM podophyllotoxin; *Lane 9*, 50 μM vinblastine + 50 μM podophyllotoxin; *Lane 10*, 1 mM GTP; *Lane 11*, 1 mM GTP + 50 μM vinblastine; *Lane 12*, 1 mM GTP + 50 μM rhizoxin; *Lane 13*, 50 μM podophyllotoxin. After the incubation samples were reduced and carboxymethylated and analyzed on the system of Laemmli (17). Note: On this particular gel, it is difficult to resolve the β^* band from the β_1 band. However, the presence of the β^{s*} band indicates that the β^s cross-link is being formed. Rhizoxin completely inhibits formation of the β^{s*} band. GTP is almost as effective as rhizoxin, but vinblastine only partially blocks formation of the β^{s*} band. Rhizoxin, as well as GTP and vinblastine, enhances formation of the β^* cross-link, whereas podophyllotoxin inhibits β^* formation.

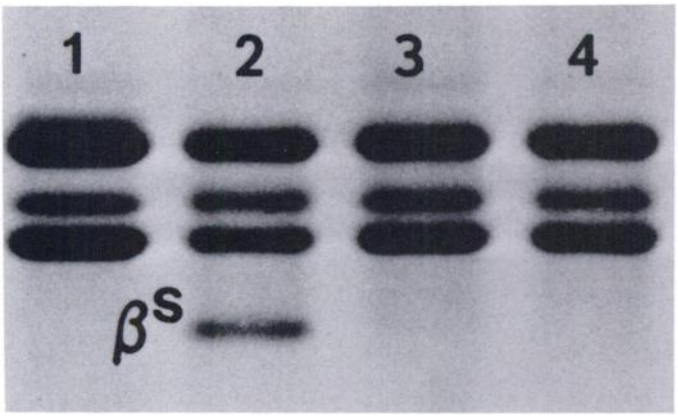

Fig. 4. Effect of rhizoxin on formation of the β^s cross-link. Aliquots (250 μl) of tubulin (0.66 mg/ml), containing reduced and carboxymethylated conalbumin (0.20 mg/ml), were incubated for 1 h at 30°C in the absence (*Lane 1*) or presence (*Lanes 2–4*) of EBI. The sample in *Lane 3* also contained 1 μM rhizoxin, while that in *Lane 4* contained 2 μM rhizoxin. All samples contained 50 μM podophyllotoxin to inhibit formation of the β^* cross-link. The samples were reduced and carboxymethylated and analyzed on the modified Laemmli system (2).

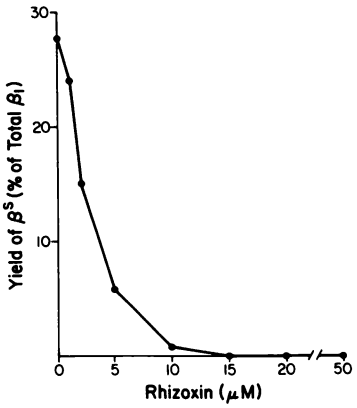

Fig. 5. Effect of rhizoxin concentration on the formation of the β^s cross-link. Aliquots (250 μl) of tubulin (0.66 mg/ml), containing reduced and carboxymethylated conalbumin (0.20 mg/ml), were incubated with EBI as in Fig. 4. The indicated concentrations of rhizoxin were also present. All samples contained 50 μM podophyllotoxin to prevent β^* formation. Samples were reduced and carboxymethylated and analyzed on the modified Laemmli system (2) and the yield of the β^s cross-link was determined. The modified Laemmli system gives much better resolution between β_1 and β^s than does the regular Laemmli system and allows the yield of β^s to be measured accurately, provided that β^* formation is inhibited, as can be done if podophyllotoxin is present in the samples.

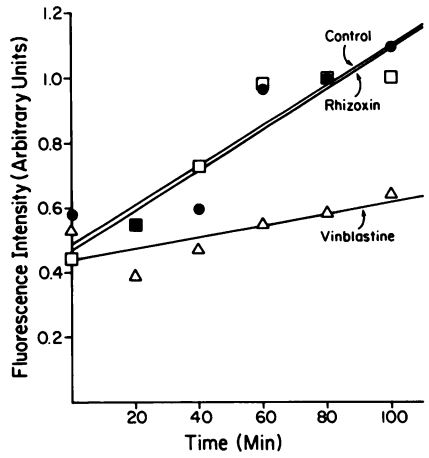

Fig. 6. Effects of rhizoxin and vinblastine on the binding of BisANS to tubulin. Aliquots (10 ml) of tubulin (0.2 mg/ml) were incubated at 37°C in either the absence of any drug (●) or in the presence of either 20 μM rhizoxin (□) or 20 μM vinblastine (△). At the indicated times, 1.0-ml aliquots were removed, placed in a fluorescence cuvet in a spectrophotofluorimeter, and made 10 μM in BisANS. Excitation and emission were at 385 and 490 nm, respectively.

Table 1 *Summary of effects of the drugs which bind to tubulin at the vinblastine/maytansine site, contrasted to the effects of colchicine and podophyllotoxin*

Drug	Effect on β^s formation	Effect on β^* formation	Effect on BisANS binding	Ref.
Vinblastine	↓	↑	↓↓↓	6, 10
Maytansine	↓↓	↑		6, 10
Rhizoxin	↓↓	↑		
Phomopsin A	↓↓	↑	↓↓↓↓	18, 26
Colchicine	↑	↓↓	↓↓	6, 9, 10
Podophyllotoxin	↑	↓↓	↓	6, 9, 10

DISCUSSION

The sulfhydryl groups of tubulin have been very useful probes for the interaction of the tubulin molecule with ligands. They may be used to make very fine discriminations among the effects of specific ligands on the tubulin molecule. The bifunctional alkylating agent, EBI, makes two covalent cross-links in the β-tubulin subunit. One of these cross-links, designated β^*, connects cysteinyl residues 239 and 354; the other cross-link, designated β^s, connects cysteinyl residues 12 and either 201 or 211 (8, 9). Colchicine and other ligands which bind at the colchicine-binding site inhibit β^* formation and enhance that of β^s (7, 10). Vinblastine, maytansine, phomopsin A, and, as shown in the present paper, rhizoxin have precisely the opposite effect, namely, they inhibit β^s formation and enhance that of β^* (7). This observation is consistent with the idea that rhizoxin binds at or near the vinblastine site on tubulin (2). However, EBI is able to discriminate among the ligands which bind at this site; vinblastine itself causes only partial inhibition of β^s

formation (7). In contrast, maytansine and phomopsin A can completely block β^s formation (20). In this respect, rhizoxin more closely resembles maytansine and phomopsin A, since it also completely blocks β^s formation. It has been suggested that the difference in the effects on β^s formation among these drugs could indicate that maytansine and vinblastine have overlapping binding sites with one of the β^s sulfhydryls being located in that region of the binding site where maytansine, but not vinblastine, binds (21). If that model is true, then rhizoxin would bind mostly in the maytansine region of the binding site.

Rhizoxin also resembles maytansine in that it has no effect on the binding of BisANS to tubulin. The fluorescent probe BisANS binds to apolar regions on proteins (22–25). Tubulin is unusual in that it binds to BisANS in a time-dependent fashion which may reflect the decay of the functional properties of tubulin (11). Drugs, such as vinblastine, which inhibit tubulin decay, greatly diminish the rate of BisANS binding to tubulin. Phomopsin A, which is an even more potent inhibitor of tubulin decay than is vinblastine, appears to completely eliminate the time-dependent binding of BisANS to tubulin (26). Colchicine and podophyllotoxin have effects on BisANS binding that are significant but smaller than that of vinblastine (11). In contrast, maytansine and rhizoxin have no effect on BisANS binding. The contrasting effects of these various drugs on the tubulin molecule are compared with those of rhizoxin in Table 1.

Overall, the effects of rhizoxin on tubulin resemble those of maytansine more than they do any of the other drugs which bind at the vinblastine/maytansine site. The two drugs are structurally similar in that they both contain macrocycles which have some specific structural features which are similar or identical (Figs. 1 or 2). For example, the cyclic carbamate of maytansine is structurally analogous to the lactone of rhizoxin. Our results suggest that these relatively minor structural features are sufficient to give rhizoxin and maytansine very similar mechanisms of action in their binding to tubulin.

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