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EFFECT OF RIFAMPICIN ON THE BIOLOGICAL ACTIVITY OF TUBULIN

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Abstract—The effect of rifampicin on the biological properties of bovine brain tubulin was investigated. Assembly of microtubules was almost completely blocked by rifampicin, whereas the depolymerization was not affected. The drug was found to inhibit both colchicine and GTP binding to tubulin. Association of rifampicin with tubulin was confirmed by spectrophotometric method. Binding of rifampicin was found to be dependent both on temperature and time. At 0° for 1 hr 0.84 moles of rifampicin were bound per mole of tubulin.

Rifampicin, a semisynthetic antibiotic [1] is used mainly in the treatment of tuberculosis and leprosy [2]. At low concentrations it acts as a specific inhibitor of bacterial DNA directed RNA polymerase [3]. At higher doses rifampicin possesses antiviral [4, 5], anticancer [6, 7] and immunosuppressive [8] activities. It is a potent inducer of hepatic enzymes [9]. The undesirable side effects such as hepatitis, adrenal failure and hematologic side effects due to chemotherapy of rifampicin have been reported [9, 10].

Rifampicin was shown to penetrate readily into the sciatic nerves of dog and sheep [11]. It is found to have a number of effects on liver [10]. All the subcellular organelles of liver have been shown to have colchicine binding activity [12] which is a unique property of tubulin [13]. The mechanism of rifampicin action is not entirely clear and it probably has multiple effects. In the present study the effect of rifampicin on the functions of tubulin was examined. Results show rifampicin impairs effectively biological activity of the bovine brain tubulin.

MATERIALS AND METHODS

The following chemicals were obtained from Sigma Chemical Company: MES (M-8250),† EGTA (E-4398), GTP (G-5631), Tris (T-1503), glycine (G-7126) and rifampicin (R-3051). Colchicine was purchased from John Baker Inc. (Colorado, U.S.A.). [^{3}H]Colchicine and [^{3}H]GTP were supplied by New England Nuclear Company. DEAE Cellulose DE-81 filter discs were from Whatman Ltd. and nitrocellulose paper was from Millipore Corporation.

Since brain is the richest source of tubulin the experiments were conducted on bovine brain tubulin. Bovine brains were obtained from the Government abattoir, Bombay, immediately after decapitation and processed within 1 hr. Tubulin was prepared and purified by Weisenberg's method [14] modified by Bhattacharya *et al.* [15]. Pure protein was concentrated and stored as described earlier [16]. Protein was estimated by Lowry's method [17] taking bovine serum albumin as the standard. Homogeneity of tubulin was checked by urea gel electrophoresis [18]. The gels and samples were prepared as explained previously [16]. Percent purity of the protein was calculated from densitometric scanning of the gel and tubulin was found to be 90% pure.

In vitro assembly and disassembly of tubulin was observed in buffer (pH 6.5) containing 100 mM MES, 0.5 mM $MgCl_2$, 1 mM GTP, 1 mM EGTA and 4 M glycerol [19]. Turbidity was measured using a Gilford 2000 spectrophotometer at 400 nm at which rifampicin absorbs minimally. Colchicine binding was studied by the standard filter assay method using DEAE cellulose DE 81 filter discs [20] and counted as mentioned earlier [18]. GTP binding was studied using mitrocellulose membrane as explained by Arai [21]. Removal of GTP from the exchangeable site of tubulin was achieved by the method of Maccioni *et al.* [22].

Binding of rifampicin to tubulin was studied by gel filtration on Sephadex G-25. A mixture of tubulin and rifampicin was loaded on the Sephadex G-25 column (25 × 7 cm) and 1.5 ml fractions were collected. Optical density of fractions was measured at 470 nm and 280 nm. On elution with 0.01 M phosphate buffer pH 6.5 containing 0.1 mM $MgCl_2$, drug protein complex was separated clearly. Moles of rifampicin bound per mole of tubulin were calculated from the O.D. of the extensively dialysed tubulin–rifampicin complex. The concentration of the rifampicin complexed with the protein was found out from the standard graph for rifampicin absorption. The protein complexed with rifampicin was estimated and the moles of rifampicin bound per mole of tubulin were calculated. Optimum temperature for tubulin–rifampicin complex formation was detected by incubating mixtures of rifampicin and tubulin at different temperatures for 30 min and dialysing the sample extensively at 2–4° overnight. From the O.D. at 470 nm of the sample which remained

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† Abbreviations: MES, (2[*N*-Morpholino]ethane sulfonic acid, EGTA, ethyleneglycol-bis-(β-amino ethyl ether)*N*,*N*′-tetraacetic acid.

in the dialysing bag, the amount of rifampicin complexed with tubulin was found out as explained above. Optimum time for rifampicin binding to tubulin was studied by incubating mixtures of tubulin and rifampicin at 2–4° for different intervals of time and moles of rifampicin bound were determined as mentioned above. Change in absorbance of rifampicin was studied by a Perkin–Elmer double beam spectrophotometer attached with a Hitachi Recorder.

RESULTS AND DISCUSSION

The assembly–disassembly profile of tubulin in the presence of different concentrations of rifampicin is shown in Fig. 1a. Progressive decrease in the rate of polymerization was observed with the increasing concentration of the drug. The final amount of microtubules formed at 30 min period was considerably less in the presence of the drug (Fig. 1b); it is seen that the percentage of microtubule formation decreases gradually with increase in rifampicin concentration. An 8% reduction in assembly occurred when 1:2 molar ratio of tubulin: rifampicin was used. When fivefold of the drug was used, assembly was inhibited by 60%. On addition of seven-fold of rifampicin 94% inhibition of the original polymerisation was obtained.

The disassembly of microtubules shown in Fig. 1a was found to be unaffected by the presence of rifampicin. From this observation it was clear that rifampicin mainly bound to the tubulin dimer, making it incompetent to take part in the assembly process, and hence the observed polymerization was due only to drug-free tubulin dimers. If the drug had interacted with the preformed microtubules instead of tubulin dimers the effect would have been apparent in disassembly as well. It is therefore likely that there was competition between polymerisation process and rifampicin binding to tubulin dimers. It is also apparent that because rifampicin bound to the tubulin dimers preferentially the drug-free dimer concentration was reduced, leading to the decrease in the critical concentration required for the polymerization process. Similar inhibitory effect on microtubule assembly was observed in the case of other drugs such as colchicine, pentobarbital, chlorpromazine, maytansine and dapsone [13, 23–25].

That rifampicin does not bind to microtubules was proved by adding it to the preformed microtubules. From Fig. 2 it is clear that on addition of rifampicin to the preformed microtubules no disassembly took place. Microtubule formation was slowed down slightly because the drug was bound to the dimers and availability of drug-free dimers became less.

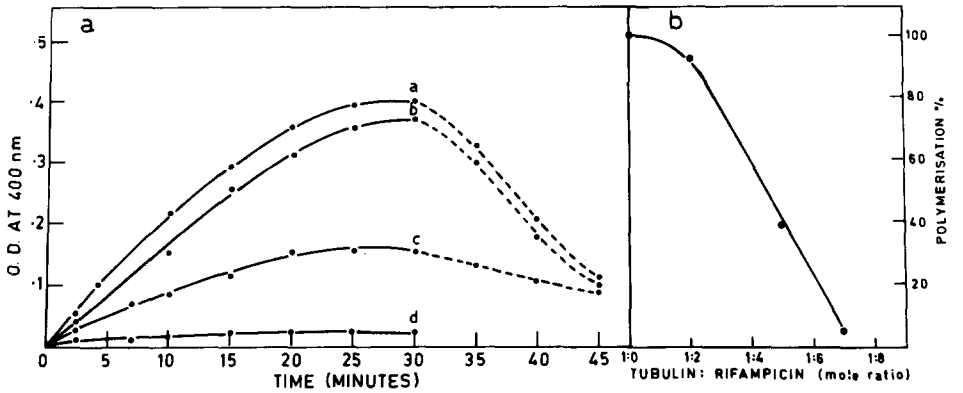

Fig. 1. (a) Assembly–disassembly profile of tubulin (3×10^{-5} M) with different ratios of tubulin: RMP. a, 1:0 b, 1:2 c, 1:5 and d, 1:7; ——, assembling at 37°; – – –, disassembly at 2–4°. (b) Percent polymerisation with different concentrations of RMP (tubulin, 3×10^{-5} M).

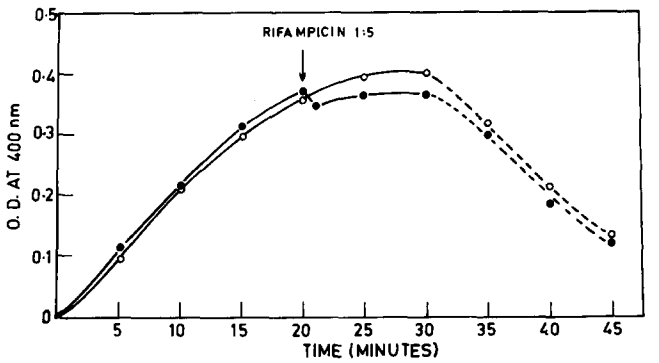

Fig. 2. Effect of RMP on preformed microtubules: O—O, tubulin (3×10^{-5} M); ●—●, tubulin with RMP (1.5×10^{-4} M); ——, assembly at 37°; – – –, disassembly at 2–4°.

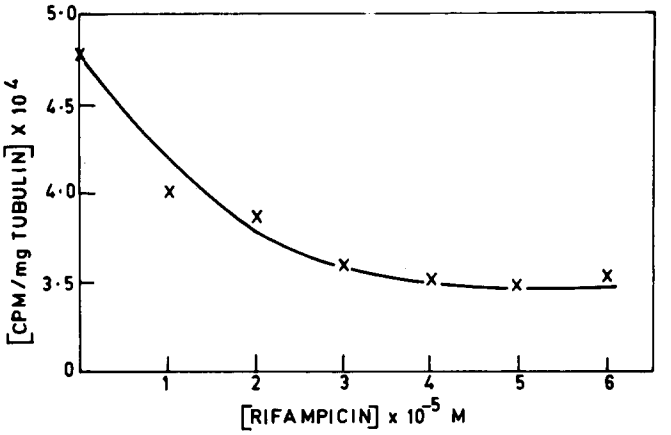

Fig. 3. Specific activity of [^{3}H]colchicine binding in presence of different concentrations of RMP.

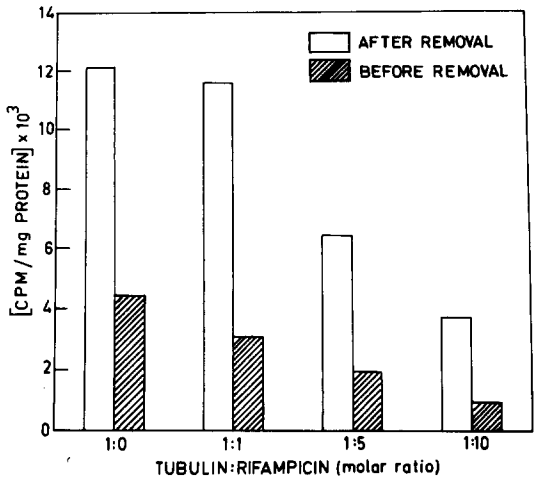

Fig. 4. Specific activity of [^{3}H]GTP binding to tubulin with varying ratios of RMP: □ after removal of "E" site GTP; ▨ before removal of "E" site GTP.

Depolymerization occurred only on cooling the sample to 0° in the same way as the drug-free control. On addition of dapsone and phenobarbital to the preformed microtubules immediate disassembly was observed [25, 26] whereas on addition of actinomycin D and pentobarbital, this phenomenon was not observed [23, 27].

Figure 3 shows the effect of rifampicin on colchicine binding property of tubulin. The downward curve indicates that the colchicine binding to tubulin was suppressed by the presence of rifampicin. There was a gradual decrease with increase in the concentration of rifampicin. A 28.4% reduction in colchicine binding was observed in the presence of sevenfold of rifampicin. Further increase in drug appeared ineffective. Other drugs such as podophyllotoxin, staganacin, nocodazole and TN-16 [28–30] inhibit colchicine binding competitively. Involvement of rifampicin in the process of GTP binding to tubulin is given in Fig. 4. Similar inhibition in GTP binding was observed before and after removal of GTP from the "E" site. It showed that rifampicin was efficient to displace "E" site GTP bound to tubulin. The possibility of rifampicin interacting with GTP was ruled out by differential spectro-

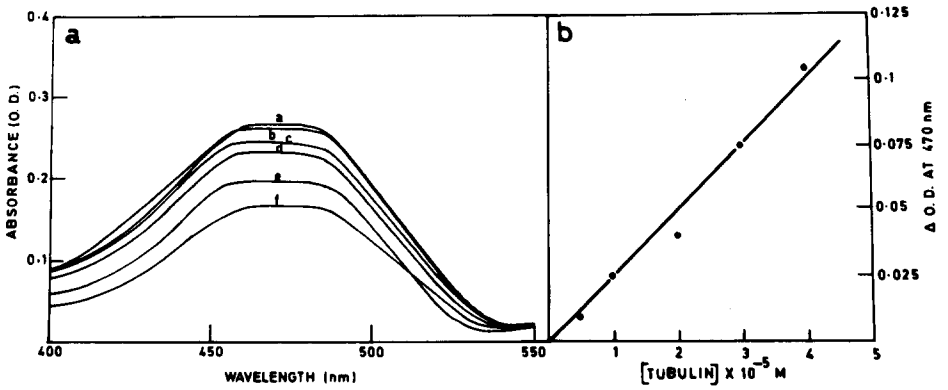

Fig. 5. (a) Absorption spectra of RMP with different concentrations of tubulin: a, RMP (2×10^{-5} M); b, RMP + tubulin (1×10^{-5} M); c, RMP + tubulin (2×10^{-5} M); d, RMP + tubulin (2×10^{-5} M); e, RMP + tubulin (3×10^{-5} M); f, RMP + tubulin (4×10^{-5} M). (b) Difference in absorbance of RMP at 470 nm, with increasing concentrations of tubulin.

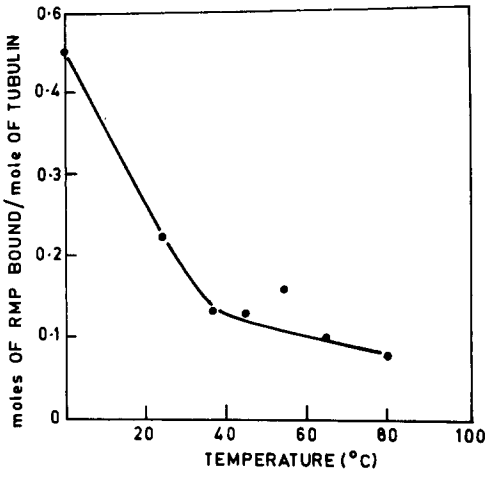

Fig. 6. Effect of temperature of RMP binding to tubulin. Tubulin (2×10^{-5} M) was incubated with RMP (2×10^{-4} M) for 30 min at varying temperatures.

photometric studies. From these experiments it was evident that rifampicin occupied either GTP binding site or colchicine site or a site other than these two sites which led to some conformational changes in these sites such that binding of both colchicine and GTP was blocked.

Absorption spectra of rifampicin in the presence of different concentrations of tubulin is shown in Fig. 5a. The absorption of rifampicin was reduced proportionately by its binding to increasing amount of tubulin. In Fig. 5b the difference in absorption at 470 nm is plotted as a function of tubulin concentration. From the upward linear graph it is clear that the decrease in absorption at 470 nm of rifampicin increases along with the increase in tubulin concentration. This data revealed that the bound drug molecules do not absorb light at this particular wavelength and the optical density measured was only due to the free drug molecules.

Moles of rifampicin bound per mole of tubulin at different temperatures is shown in Fig. 6, the curve is biphasic. Binding of rifampicin to tubulin decreased sharply from 0° to 37°. Thereafter the rate of the reaction was slow. This behaviour of rifampicin was in contrast with other drugs such as colchicine, colcemid, dapsone and actinomycin D [31, 25, 27], these drugs bind tubulin with a uniform ratio. This showed that the drug has affinity for the dimeric form of tubulin which exists at 0°. This confirmed our earlier conclusion that the drug binds tubulin in dimeric form preferentially.

Formation of rifampicin tubulin complex was further confirmed by gel filtration on Sephadex G-25. Figure 7 shows the elution profile of the rifampicin–tubulin complex and free rifampicin from the column. The sample separated into two peaks at 280 nm as well as at 470 nm. The O.D. at 280 nm of the first peak was due to the protein, and absorbance at 470 nm was due to the rifampicin complexed with it. The second peak contained mainly free rifampicin because it showed high O.D. at 470 nm. From the elution profile of the tubulin alone on a Sephadex G-25 column of the same dimensions it was proved that the second peak contained GTP which was present in the sample. So the absorption at 280 nm which was observed along with the free rifampicin peak was due to the excess GTP separated from the sample. These results leave no doubt that rifampicin was bound to tubulin efficiently. From the absorbance of the extensively dialysed tubulin–rifampicin complex, it was calculated that 0.84 mole of rifampicin was bound per mole of tubulin when the drug was incubated at 2–4° for 1 hr.

The above results clearly show that rifampicin significantly affected polymerization, colchicine and GTP binding of tubulin. From the secondary structure of tubulin it was found that the tubulin molecule exists in α belix, β pleats and random coil at 0° [32]. On raising the temperature to 37° α helical content vanishes and β pleats increase. From our studies of rifampicin binding to tubulin it appears that the presence of α helix in the molecule enhances rifampicin binding to tubulin. But it is noticed that even in the absence of α helix, i.e. above 37°, the drug binding did take place. But the amount of drug bound above 37° was much less when compared to that bound at lower temperatures.

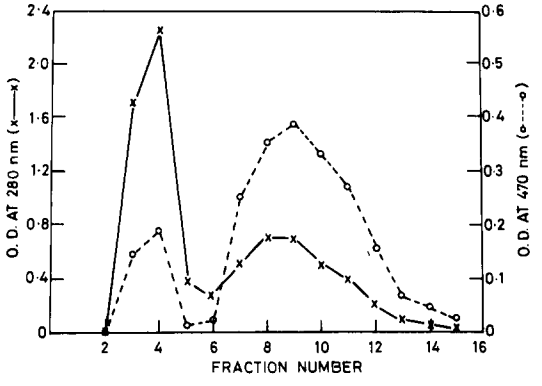

Fig. 7. Elution pattern of a mixture of RMP and tubulin from sephadex G-25 column (0.7 × 25 cm). Tubulin 4.5×10^{-5} M was incubated at 2° with 10-fold RMP for 1 hr, loaded on the column and eluted with PM buffer. 1.5 ml fractions were collected.

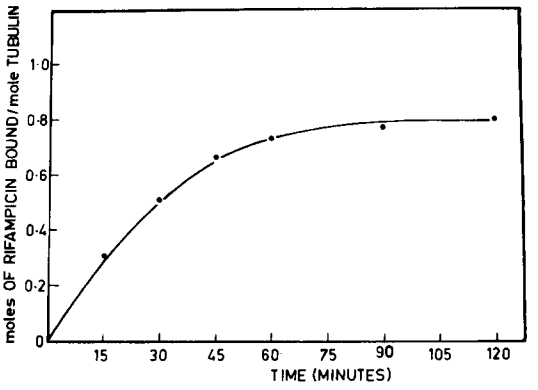

Fig. 8. Time dependence of RMP binding to tubulin. Tubulin was treated with RMP (2×10^{-4} M) at 0° for specified periods of time.

Figure 8 gives the time of equilibration for rifampicin binding to tubulin. It was seen that at 0° the reaction was almost complete within 1 hr and thereafter rifampicin binding slowed down, and the reaction was complete within 90 min. Tubulin–rifampicin complex formation was slower when compared to colcemid, actinomycin-D, and DDS binding to tubulin [25, 27, 31].

Since microtubules are involved in a number of cellular processes in addition to mitosis, secretion, cellular motion, membrane regulation and nutrient transport, it is important to learn how various drugs, which cause adverse effects on either short or long term therapy, or interfere with the function of microtubules behave at the molecular level. These results therefore have implication in explaining at least some of the adverse effects of this drug. It is likely that the adverse effects observed on liver could be partly due to rifampicin binding to tubulin, inactivating it from its normal function. On the other hand its anticancer effect may also be due to the same effect as is immobilization of the membrane and tubulin subcellular organelles inhibiting cellular functions and cell division.

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