Inhibition of microtubule assembly in vitro by TN-16, a synthetic antitumor drug

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An antitumor drug, 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione (TN-16) inhibited the assembly of porcine brain microtubules in vitro. The assembly induced by taxol was also suppressed by the drug. However, the latter required much higher concentration of TN-16 than the former. Binding studies by means of the fluorometric method and the spun-column procedure indicate that the inhibition was caused by the reversible binding of the drug to the colchicine-sensitive site of tubulin. The affinity of TN-16 to tubulin was almost equal to that of nocodazole.

Antitumor drug *TN-16* *Nocodazole* *Taxol* *Tubulin* *Microtubule assembly*

1. INTRODUCTION

TN-16 (fig.1) is a synthetic compound which arrests mitosis of tumor cells at metaphase. The metaphase-arrested cells immediately divide when the drug is removed from the culture medium [1,2].

Here I report that the drug inhibits the assembly of brain microtubules in vitro by binding to the colchicine-sensitive site of tubulin.

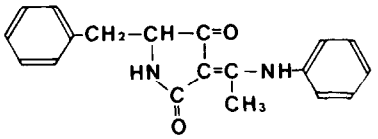

Fig.1. Structural formula of TN-16.

2. MATERIALS AND METHODS

2.1. *Materials*

Nitrocellulose membrane filters (0.45 μm) and Sephadex G-50 were purchased from Sartorius and Pharmacia, respectively. TN-16 was obtained from Wako Pure Chemical, taxol from the US National Cancer Institute and nocodazole from Aldrich Chemical. These 3 drugs were dissolved in DMSO. [^{3}H]GTP and [^{3}H]colchicine were purchased from Amersham, and phosphoenolpyruvate (PEP) and pyruvate kinase (PK) from Boehringer.

Abbreviations: TN-16, 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione; MES, 2-(*N*-morpholino)ethane sulfonic acid; EGTA, ethyleneglycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acids; DMSO, dimethylsulfoxide

2.2. *Preparation of microtubule proteins*

Porcine brain microtubules were prepared by two cycles of temperature-dependent assembly–disassembly as in [3] with some modifications. To minimize contamination of free guanine nucleotides into the microtubule preparation, second-cycle assembly was carried out with $<50\,\mu$M GTP and a GTP regenerating system (1 mM PEP and 10 μg PK/ml). Microtubule proteins were finally dissolved in buffer A (0.1 M MES, pH 6.5, 1 mM EGTA and 0.5 mM magnesium acetate) containing 10 μM GTP and 25% (v/v) glycerol and stored at -80°C until use.

2.3. *Assay methods for microtubule assembly*

Microtubule assembly was carried out in buffer A containing 10% (v/v) glycerol, 10 μM [^{3}H]GTP,

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1 mM PEP and 10 μg PK/ml. Time-course of the assembly was monitored by measurement of increase in turbidity at 350 nm [4] and its extent was assayed by incorporation of labeled guanine nucleotides into microtubules. The incorporation was measured by use of the nitrocellulose membrane-filter procedure [5]. Microtubules were assembled with 30 min incubation at 30°C, followed by addition of non-labeled GPT at 0.5–1.0 mM final conc. After further incubation for 5 min at the same temperature, radioactive guanine nucleotides still bound to tubulin were collected on a nitrocellulose membrane filter.

2.4. *Measurement of drug-binding to tubulin*

Binding experiments were performed in buffer B (20 mM MES, pH 6.5, 5 mM magnesium acetate, 0.4 M potassium acetate and 10% glycerol). Time-course of colchicine-binding was followed by increase in fluorescence intensity at 430 nm [6,7]. To assay extent of drug-binding, the spun-column procedure [8] was used. Amount of bound drug was determined from radioactivity recovered in the fraction of the tubulin–drug complex or from absorbance of the fraction. For determination of colchicine and TN-16, absorbance at 350 nm and 315 nm, respectively.

3. RESULTS

3.1. *Inhibition of microtubule assembly by TN-16*

Time-course of microtubule assembly was monitored by increase in turbidity at 350 nm. The assembly was inhibited when an anti-tumor drug, TN-16, was present in the medium. A stoichiometric amount of the drug showed >50% inhibition (fig.2A).

To study requirement of the drug for inhibition, its effect on the extent of the assembly was assayed by the filtration procedure as in section 2. Under this condition, [^{3}H]GTP bound to free tubulin was almost completely replaced by non-labeled GTP in the medium whereas [^{3}H]GDP incorporated into microtubules was not replaced [9,10]. Therefore, radioactivity recovered on the filter reflects the amount of polymerized tubulin. As shown in fig.2B, 20 and 190 pmol tubulin were incorporated into microtubules when reaction mixtures contained 2.5 μM (125 pmol/50 μl) and 5.7 μM (285 pmol/50 μl) tubulin, respectively. The critical

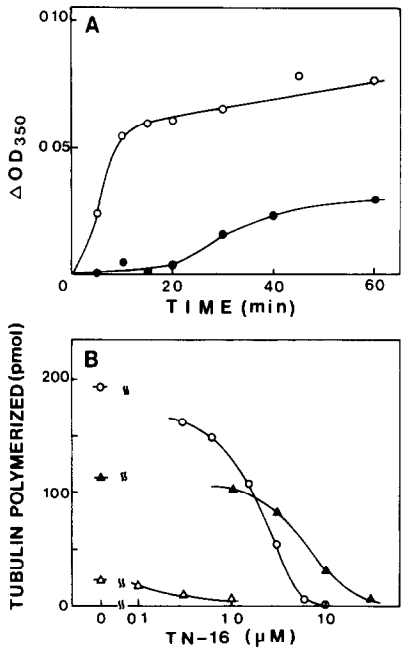

Fig.2. Effect of TN-16 on the assembly of porcine brain microtubules. (A) Microtubule proteins (~1.5 mg/ml) were incubated at 30°C with and without TN-16. At the time indicated, turbidity at 350 nm was measured: (—○—) without TN-16; (—●—) with 10 μM TN-16. (B) Microtubule proteins were finally dissolved in 50 μl buffer A containing 10% (v/v) glycerol, 10 μM [^{3}H]GTP, 1 mM phosphoenolpyruvate, 10 μg pyruvate kinase/ml and the indicated concentration of TN-16. After addition of 1 μl DMSO or 125 μM taxol, microtubules were assembled after 30 min incubation at 30°C. The amount of tubulin polymerized was assayed by the filtration procedure in section 2: (—○—) 285 pmol tubulin without taxol; (—△—) 125 pmol tubulin without taxol; (—▲—) 125 pmol tubulin with 2.5 μM taxol.

concentration obtained from the above results was 2.0 μM (~0.2 mg/ml). The value corresponds well with that in [4].

Fig.2B shows that the extent of inhibition by TN-16 depended on tubulin concentration. For 50% inhibition of the assembly of 2.5 μM tubulin, 0.4 μM TN-16 was required, while 1.7 μM TN-16 was required when tubulin was 5.7 μM. The drug also inhibited assembly induced by taxol which is known to promote microtubule assembly [11]. However, a much higher [TN-16] was required for inhibition. About 6 μM TN-16 was required for 50% inhibition of the taxol-induced assembly of

2.5 μM tubulin. This fact might suggest that taxol-binding affects affinity of TN-16 to tubulin.

3.2. *Inhibition of colchicine binding to tubulin by TN-16*

Formation of the tubulin–colchicine complex was monitored by an increase in fluorescence intensity at 430 nm. The increase in the fluorescence was suppressed when TN-16 was present (fig.3A). The drug has an absorption maximum at 315 nm. The absorbance of its 30 μM solution at the excitation wavelength is 0.17 and there is no overlap between the absorption spectrum of TN-16 and the fluorescence one of tubulin–colchicine complex. From these facts, it can be concluded that the presence of TN-16 has no significant effect on the fluorescence intensity under this condition. Therefore, the above result might be due to the fact that TN-16 inhibited the binding of colchicine to tubulin. The fact was confirmed by the direct measurement of the tubulin–[^{3}H]colchicine complex formed. The complex was isolated by the spun-column procedure [8]. TN-16 inhibited the complex formation in a dose-dependent manner (fig.3B). The figure also shows that its ability for inhibition is almost the same as that of nocodazole.

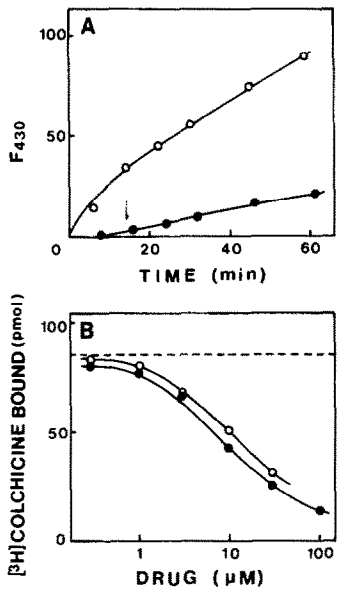

Fig.3. Effect of TN-16 on formation of the tubulin–colchicine complex. (A) Tubulin (10 μM) was dissolved in 2.5 ml ice-cold buffer B containing 20 μM GTP and 20 μM colchicine. After addition of 50 μl DMSO or 1.5 mM TN-16, the solutions were incubated at 30°C and the fluorescence intensity at 430 nm was measured at the time indicated. Excitation wavelength was 350 nm: (—○—) without TN-16; (—●—) with 30 μM TN-16. (B) Tubulin was incubated for 30 min at 30°C in buffer B containing 20 μM GTP, 15 μM [^{3}H]colchicine and the indicated concentration of TN-16 or nocodazole. The amount of [^{3}H]colchicine bound was assayed by the spun-column procedure [8]: (– – –) amount of [^{3}H]colchicine bound in the absence of TN-16 and nocodazole; (—●—) with TN-16; (—○—) with nocodazole.

In the presence of TN-16, ~5 min time lag was observed in the formation of the tubulin–colchicine complex (fig.3A). The time lag can be explained when we assume that TN-16 rapidly binds to the colchicine-binding site of tubulin and the bound drug was gradually replaced by colchicine. The above assumption was confirmed in table 1. Binding of TN-16 was completed within 10 min even at 0°C and the bound drug was replaced by the colchicine added. Low recovery of bound TN-16 might be due to dissociation of the tubulin–TN-16 complex during gel filtration and the dissociation rate of the complex at 30°C is probably faster than at 0°C.

Nocodazole is a synthetic inhibitor of

Table 1

Reversibility of TN-16 binding to tubulin

Tube no.	Additions	TN-16 bound (nmol)	Colchicine-bound (nmol)
1	TN-16 (4 nmol)	0.44	–
2	TN-16 (4 nmol)	0.32	–
3	TN-16 (4 nmol) + colchicine (30 nmol)	0.08	0.88
4	Colchicine (30 nmol)	–	1.01

Each of the 4 tubes contained 0.4 ml final vol.; buffer B, 20 μM GTP, 1.2 nmol tubulin and 4 nmol TN-16 (tubes 1–3) or 30 nmol colchicine (tube 4). Tube 1 was incubated for 10 min at 0°C and tube 4 for 60 min at 30°C. Then the amount of TN-16 or colchicine bound was assayed by the spun-column procedure [8]. After incubation for 10 min at 30°C, 2 μl distilled water and 15 mM colchicine were added to 2 and 3, respectively. The 2 tubes were incubated for a further 60 min at 30°C, then the amount of bound drugs was assayed

microtubule assembly which reversibly and rapidly binds to the colchicine-sensitive site of tubulin [12,13]. These results indicate that both TN-16 and nocodazole bind to tubulin in the same manner. Furthermore, they have almost equal affinity to porcine brain tubulin. However, affinity of yeast tubulin to TN-16 is very different from that to nocodazole (not shown). Nocodazole and other benzimidazole carbamate derivatives inhibit growth and microtubule assembly of yeast [14,15], while growth of yeast, *Saccharomyces cerevisiae*, was not inhibited by TN-16. The result might reflect the fact that there is no structural relationship between the two drugs.

3.3. *Concluding remarks*

The results reported here can be summarized as follows:

(i) TN-16 inhibited the microtubule assembly in vitro;

(ii) The drug reversibly binds to the colchicine-binding site of tubulin.

They indicate that the antitumor activity of TN-16 is due to its direct inhibition of the microtubule assembly process. Furthermore, the reversible binding explains the fact that tumor cells arrested at the metaphase begin to divide immediately after removal of the drug [1,2].

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