Vol. 129, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1985 Pages 200-205

THE EFFECT OF TN-16 ON THE ALKYLATION OF TUBULIN

Mary Carmen Roach and Richard F. Luduena

Department of Biochemistry
The University of Texas Health Science Center
San Antonio, TX 78284

Received April 12, 1985

The synthetic anti-tumor drug 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione (TN-16) is known to block microtubule assembly and colchicine binding to tubulin, although its structure does not resemble those of either colchicine, podophyllotoxin, or nocodazole (Arai, FEBS Lett. 155:273-276 (1983)). We have found that TN-16 affects the intra-chain cross-linking of β-tubulin by N,N'-ethylene-bis(iodoacetamide) in a manner identical to that of colchicine, podophyllotoxin, and nocodazole, but different from that of vinblastine or maytansine. TN-16 also inhibits alkylation of tubulin by iodo[^{14}C]acetamide, as do colchicine and its congeners. TN-16 appears to bind to tubulin at the colchicine binding site and one of its phenyl groups is likely to bind at the site on tubulin where colchicine's A ring binds.

TN-16 (3-(1-anilinoethylidene)-5-benzylpyrrolidine)[1] (Figure 1) is a synthetic drug which binds to tubulin with high affinity, blocks microtubule assembly and inhibits colchicine binding (1). It is possible that TN-16 binds to the colchicine binding site, but it is structurally dissimilar, not only to colchicine, but also to nocodazole and podophyllotoxin, two drugs whose structures are quite different from colchicine's but which are known to be competitive inhibitors of colchicine binding (2,3). The only structural feature common to all four of these compounds is the phenyl group of TN-16 and nocodazole which becomes a trimethoxyphenyl group in colchicine and podophyllotoxin. It is thus not clear exactly where on the tubulin molecule TN-16 binds, nor if its conformational effects on tubulin are similar to those of colchicine, podophyllotoxin, and nocodazole.

We have developed a system which is capable of giving detailed information about the events occurring at the ligand-binding sites of tubulin and even about specific portions of the colchicine binding site. We have found that when tubulin

[1] Abbreviations used are: TN-16, 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione; EBI, N,N'-ethylene-bis(iodoacetamide).

0006-291X/85 $1.50

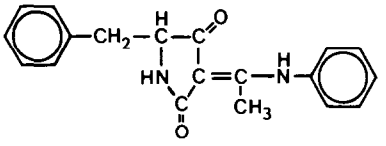

Figure 1: Structure of TN-16.

is reacted with N,N'-ethylene-bis(iodoacetamide)(EBI), it can generate two intra-chain cross-links in the β-subunit of tubulin, designated β* and β^s (5,6). Formation of β* is completely blocked by colchicine, podophyllotoxin and nocodazole, each of which enhances formation of β^s. Conversely, formation of β^s is inhibited by vinblastine, maytansine and GTP, each of which enhances formation of β* (5). Formation of β* is also inhibited by a series of single-ring analogues of colchicine's trimethoxyphenyl group (A-ring), whereas tropolone methyl ether, an analogue of colchicine's tropolone (C) ring, enhances β* formation (7). It thus seems that β* formation is inhibited by binding of ligands to that portion of the colchicine binding site where the A-ring binds. We have also found that colchicine, podophyllotoxin and nocodazole inhibit the alkylation of tubulin by iodo[^{14}C]acetamide, an effect that appears to be conformational in nature (6,7).

When the interactions of TN-16 with tubulin were examined using the systems just described, it was found to completely block β* formation but to enhance β^s formation. It also inhibited alkylation with iodo[^{14}C]acetamide. The effects were dose-dependent and suggest that TN-16 binds to tubulin at a site that overlaps the binding site of colchicine's A ring and that its conformational effects on tubulin as reflected by the accessibility of sulfhydryl groups are similar to those of colchicine.

MATERIALS AND METHODS

TN-16 was purchased from the Wako Chemical Co., Dallas, TX. All other materials and methods were as previously described (5). In experiments where tubulin was alkylated with EBI in the absence of GTP, three electrophoretically distinct derivatives of β_1-tubulin were generated, as has already been reported (5). The three bands are designated β_1^s, β_1*, and β_1^s*. The first and third contain the β^s cross-links. In all cases, the total amount of β_1 applied to the gel was calculated using conalbumin as an internal standard (4,5). The % β^s represents the percentage of the total β_1 applied to the gel which consists of β_1^s and β_1^s*, while the % β* represents the percentage of the total β_1 applied to the gel which consists of β_1* and β_1^s*. The % $\beta_1$agg represents the percentage of β_1 which has formed a high molecular weight cross-linked aggregate and cannot enter the gel.

All experiments were done using bovine brain tubulin purified on phosphocellulose (9) and, unless otherwise indicated, were performed in 100 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.4, containing 1 mM ethylene glycolbis(β-aminoethyl ether)-N,N',N,N'-tetraacetic acid, 0.1 mM ethylenediaminetetraacetic acid, 0.5 mM $MgCl_2$, 1 mM β-mercaptoethanol and 1 mM GTP. The GTP was omitted from the buffer in certain experiments.

RESULTS AND DISCUSSION

Table I shows the effects of TN-16 and other anti-mitotic drugs on the alkylation of tubulin by iodo[^{14}C]acetamide. As can be seen, TN-16 significantly suppresses alkylation to an extent that is significantly less than that caused by vinblastine but not significantly different from the effects of colchicine, podophyllotoxin or nocodazole. Figure 2 shows the concentration-dependence of the effect of TN-16. Half-maximal suppression was observed at a TN-16 concentration of 7 μM, comparable to colchicine and podophyllotoxin which give half-maximal inhibition at 3-4 μM and 5 μM, respectively (6).

Figure 2 also shows that TN-16 inhibits the formation of the β* cross-link by EBI, using tubulin prepared in the presence of GTP, where only the β* cross-link can be produced. The suppression of β* formation is about 90% complete at 30 μM TN-16 and is half-maximal at a TN-16 concentration of 6 μM. This is com-

TABLE I

EFFECTS OF TN-16 AND OTHER ANTI-MITOTIC DRUGS ON THE ALKYLATION OF TUBULIN BY IODO[^{14}]ACETAMIDE[a]

Addition	Incorporation of ^{14}C: Moles ^{14}C/mole Tubulin	% Control	P[c]
None	1.21 ± .04[b]	100 ± 3[b]	<.001
TN-16	0.97 ± .06	81 ± 6	
Colchicine	0.97 ± .02	80 ± 3	NS[d]
Podophyllotoxin	0.81 ± .18	67 ± 15	NS
Nocodazole	0.89 ± .06	74 ± 5	NS
Vinblastine	0.74 ± .03	61 ± 3	<.001

[a] Aliquots (250 μl) of tubulin (0.66 mg/ml) were incubated for 1 h at 37°C in the presence of 100 μM concentrations of the indicated drugs and of 1.36 mM iodo[^{14}C]acetamide (0.51 Ci/mole). The incorporation of ^{14}C into tubulin was calculated by precipitating the tubulin with trichloroacetic acid, filtering the aliquots and determining the radioactivity of the filter (8). Incorporation is expressed on a mole/mole basis assuming a molecular weight of 100,000 for tubulin. All incubations were done in quadruplicate.

[b] Standard deviation.

[c] The results were analyzed by Student's *t*-test and compared for significance to the results obtained with TN-16. The resulting P-values are shown in the table. All the drugs gave significant effects when compared to the control (podophyllotoxin, $P < .005$; the other drugs, $P < .001$).

[d] Not significant.

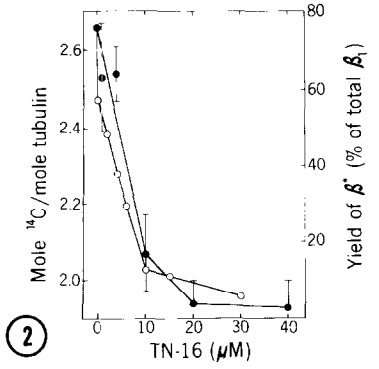

Figure 2: Concentration-dependence of the Effect of TN-16 on the Reaction of tubulin with EBI and iodo[^{14}C]acetamide.

Open circles (O): aliquots of tubulin (0.66 mg/ml), purified in the presence of GTP, were incubated for 1 h at 30°C in the presence of 0.91 mM EBI and the indicated concentrations of TN-16. The yield of β* was calculated as described previously (5). Closed circles (●): triplicate aliquots of tubulin (0.66 mg/ml) purified in the presence of GTP, were incubated for 1 h at 37°C in the presence of 1.36 mM iodo[^{14}C]acetamide (0.51 Ci/mole) and of the indicated concentrations of TN-16. The incorporation of ^{14}C into tubulin was calculated as in Table I.

Figure 3: Effect of TN-16 and Other Anti-tubulin Drugs on the Reaction of Tubulin with EBI.

Aliquots (250 µl) of tubulin (0.66 mg/ml), purified in the absence of GTP, were incubated for 1 h at 30°C in the absence (samples 1,8) and presence (samples 2-7) of 0.91 mM EBI and of the indicated drugs. Samples were then reduced and carboxymethylated (4) and run on a 5.5% polyacrylamide gel slab in the system of Laemmli (10). The gel was stained with Coomassie blue. Samples were prepared as follows: 1, no EBI; 2, EBI only; 3, EBI and 100 µM TN-16; 4, EBI and 100 µM colchicine; 5, EBI and 100 µM podophyllotoxin; 6, EBI and 100 µM nocodazole; 7, EBI and 5 µM maytansine; 8, no EBI. Note that EBI causes the appearance of the β_1^S, β_1*, and β_1^{S}* bands. TN-16, colchicine, podophyllotoxin and nocodazole inhibit formation of β_1* and β_1^{S}* but enhance that of β_1^S. In contrast, maytansine inhibits formation of β_1^S and β_1^{S}* but enhances that of β_1*. Electrophoresis was from top to bottom.

parable to the effects of colchicine, which inhibits β* formation half-maximally at 3 µM and by 13% at 100 µM, and podophyllotoxin, whose half-maximal effect is observed at a drug concentration of 2-5 µM and which blocks β* formation by 92% at 100 µM (6)

Figure 3 shows the effect of TN-16 on the cross-linking by EBI of tubulin prepared in the absence of GTP, which allows both the β^S and the β* cross-links to form. As can be seen, TN-16 inhibits β* formation and enhances that of β^S, as do colchicine, podophyllotoxin and nocodazole. These results are given quantitative expression in Table II, which shows that under these conditions, TN-16 is slightly more effective than the other drugs at blocking β* formation but is somewhat less effective at enhancing β^S formation. TN-16 also slightly inhibits

TABLE II

EFFECTS OF TN-16 AND OTHER ANTI-MITOTIC DRUGS ON THE CROSS-LINKING OF TUBULIN BY EBI[a]

	Yield of β_1 Derivatives					
	β^*		β^s		$\beta_1$agg	
	b	c	b	c	b	c
None[d]	17.3 ± 1.3[e]	100 ± 7[e]	12.2 ± 0.5[e]	100 ± 4[e]	58.7 ± 3.3[e]	100 ± 6[e]
TN-16[d]	2.9 ± 0.1[e]	17 ± 1[e]	14.8 ± 0.7[e]	122 ± 8[e]	47.2 ± 1.5[e]	80 ± 5[e]
Colchicine	4.7	27	22.7	186	34.9	59
Podophyllotoxin	4.7	27	27.6	227	0	0
Nocodazole	4.9	28	16.9	139	43.9	75

[a] Aliquots (250 µl) of tubulin (0.66 mg/ml), purified in the absence of GTP, were incubated for 1 h at 30°C in the presence of 0.91 mM EBI and 100 µM concentrations of the indicated drugs. Samples were processed as described previously (5). The yields of cross-linked derivatives of β_1-tubulin are shown.

[b] % of total β_1.

[c] % of control.

[d] These samples were done in duplicate.

[e] Standard deviation.

formation of $\beta_1$agg, about as much as does nocodazole but not as effectively as do colchicine and podophyllotoxin.

It is very clear from Figure 3 that TN-16 is acting in a manner similar to that of colchicine, podophyllotoxin and nocodazole, but very differently from GTP, vinblastine, or maytansine each of which enhances β^* formation while inhibiting that of β^s (5). It appears likely, therefore, that TN-16 binds to the colchicine binding site, as Arai has suggested (1). We have previously found that colchicine's inhibition of β^* formation is due to its trimethoxyphenyl (A) ring (7). This would also explain why podophyllotoxin and nocodazole inhibit β^* formation, since they contain, respectively, a trimethoxyphenyl and a phenyl group, which presumably bind to the site on tubulin where colchicine's A-ring binds. It would appear likely, therefore, that the binding site of one of the phenyl groups of TN-16 overlaps that of colchicine's A-ring.

We have also reported that the inhibition by tubulin ligands of the alkylation of tubulin by iodo[^{14}C]acetamide is likely to be a conformational effect. TN-16 suppresses this about as much as do podophyllotoxin and colchicine, but not as much as does vinblastine. TN-16 thus appears to have similar conformational

effects on tubulin as do many of the other anti-mitotic drugs, at least insofar as the accessibility of tubulin's sulfhydryl groups is concerned.

ACKNOWLEDGMENTS

Supported by grants AQ-726 from the Robert Welch Foundation and CA 26376 from the National Institutes of Health to R.F.L. We gratefully acknowledge the skilled technical assistance of Phyllis Smith and helpful conversations with Dr. Takao Arai.

REFERENCES

1. Arai, T. (1983) FEBS Lett. 155, 273-276.
2. Cortese, F., Bhattacharyya, B., and Wolff, J. (1977) J. Biol. Chem. 252, 1134-1140.
3. Hoebeke, J., Van Nijen, G., and DeBrabander, M. (1976) Biochem. Biophys. Res. Commun. 69, 319-324.
4. Luduena, R.F. and Roach, M.C. (1981) Biochemistry 20, 4437-4444.
5. Roach, M.C. and Luduena, R.F. (1984) J. Biol. Chem. 259, 12063-12071.
6. Luduena, R.F. and Roach, M.C. (1981) Biochemistry 20, 4444-4450.
7. Roach, M.C., Bane, S., and Luduena, R.F. (1985) J. Biol. Chem. 260, 3015-3023.
8. Luduena, R.F. and Roach, M.C. (1981) Arch. Biochem. Biophys. 210, 498-504.
9. Fellous, A., Francon, J., Lennon, A.M., and Nunez, J. (1977) Eur. J. Biochem. 78, 167-174.
10. Laemmli, U.K. (1970) Nature 227, 680-685.