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Investigation of the Mechanism of the Interaction of Tubulin with Derivatives of 2-Styrylquinazolin-4(3*H*)-one

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SUMMARY

A new class of antimitotic agents, derivatives of 2-styrylquinazolin-4(3*H*)-one (SQZ), was recently described [*J. Med. Chem.* 33:1721–1728 (1990)]. Because they appeared to interact at a new ligand binding site on tubulin, we attempted to determine their mechanism of action as inhibitors of tubulin polymerization. Although in initial studies inhibition of colchicine binding was negligible, substantial and competitive inhibition of this reaction could be demonstrated with very short incubation times (<5 min), provided that a relatively low colchicine to tubulin ratio was used. The initial apparent failure to inhibit colchicine binding resulted from extremely rapid binding to tubulin and dissociation from tubulin by the SQZ derivatives, in comparison with the slow, temperature-dependent, poorly reversible binding of colchicine. The most inhibitory of the SQZ derivatives in the colchicine binding assay was 6-methyl-2-styrylquinazolin-4(3*H*)-one (NSC 379310), and its interaction with tubulin, particularly as an inhibitor of colchicine binding, was compared with that of 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone (MTPT), because the binding parameters of MTPT with tubulin have been well described. The data indicate that NSC 379310 binds to tubulin and dissociates from the protein about 3 times as rapidly as MTPT. The other SQZ derivatives with equal or greater potency as inhibitors of tubulin polymerization but apparently less potency as inhibitors of colchicine binding presumably bind to and/or dissociate from tubulin even more rapidly than does NSC 379310.

The microtubule system of eukaryotic cells is an important target for cytotoxic agents. The most dramatic effect of these drugs is the accumulation of tetraploid cells with condensed chromosomes. Virtually all antimitotic agents interact with tubulin, the major constituent of microtubules, and these compounds are important as potential antineoplastic agents (for a recent review, see Ref. 1). Clinically, the most important drugs that bind to tubulin are the *Vinca* alkaloids, which inhibit microtubule assembly, cause microtubules to depolymerize, and cause formation of tubulin aggregation products. In contrast, taxol, a promising agent currently in clinical trials, enhances tubulin polymerization and stabilizes microtubules.

Recently, we described a new class of antimitotic compounds (2), derivatives of SQZ (Fig. 1). At least one of these agents, NSC 381272 [Fig. 1, $R = OCH_3$; 6-methoxy-2-styrylquinazolin-4(3*H*)-one], has promising activity in several model tumor systems. Our first studies indicated that these drugs might act at a new binding site on tubulin. Even though a number of SQZ derivatives inhibited assembly, they had little effect on the binding of other ligands, including colchicine, to tubulin. Attempts to synthesize a radiolabeled SQZ derivative failed, so indirect studies were initiated to explore the mode of interaction of SQZ derivatives with tubulin. These experiments strongly suggested an interaction at the colchicine site. We then found that inhibition of colchicine binding to tubulin could be demonstrated with short incubation times. The SQZ derivatives bind to and dissociate from tubulin extremely rapidly, with the rate constants for both reactions being greater than those obtained for the two-ring colchicine analog MTPT (3–5). Identification of the SQZ site as the colchicine site should aid in defining molecular features that are important for binding of drugs at this site and in designing more potent analogs.

Experimental Procedures

Materials. SQZ derivatives were prepared as described previously (2). The bovine brain tubulin preparations used in these studies were electrophoretically homogeneous (6, 7). Nonradiolabeled colchicine, GTP, and monosodium glutamate were from Sigma, podophyllotoxin from Aldrich, [*ring C*-^{3}H-*methoxy*]colchicine from DuPont, [8-^{14}C]GTP from Moravek Biochemicals, and [^{3}H]vinblastine from Amersham. Stock 2 M glutamate solutions were adjusted to pH 6.6 with HCl. Stock 1 M MES solutions were adjusted to pH 6.9 with NaOH. CS-A2 and CS-A4 and MTPT were generous gifts, respectively, from Drs. G. R.

ABBREVIATIONS: SQZ, 2-styrylquinazlin-4(3*H*)-one; MES, 4-morpholineethanesulfonate; MTPT, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; CS-A2, combretastatin A-2; CS-A4, combretastatin A-4; EBI, *N,N′*-ethylenebis(iodoacetamide).

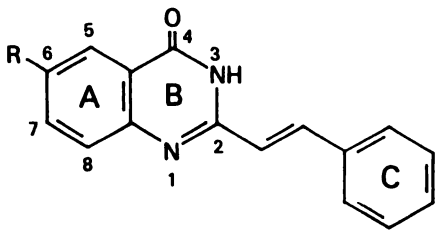

Fig. 1. Structural formula of SQZ derivatives.

TABLE 1

Effects of the SQZ derivative NSC 381272 on binding of vinblastine, GTP, and colchicine to tubulin

Drug added	Binding		
	Vinblastine[a]	GTP[b]	Colchicine[c]
μM	% of control		
Podophyllotoxin, 5			21
Podophyllotoxin, 50	105	117	3
Maytansine, 50	4	3	101
NSC 381272, 5			92
NSC 381272, 50	105	110	91
MTPT, 5			72
MTPT, 50			20
CS-A2, 5			40
CS-A2, 50			6
CS-A4, 5			11
CS-A4, 50			1

[a] Each 0.32-ml reaction mixture contained 0.5 mg/ml tubulin, 5 μM [^{3}H]vinblastine, inhibitor as indicated, 0.1 M MES (pH 6.9), 0.5 mM $MgCl_2$, and 2% (v/v) dimethyl sulfoxide. Incubation was for 10 min at 22°. Triplicate 0.1-ml aliquots were analyzed by centrifugal gel filtration chromatography, performed at 22° (8). Control was 0.41 pmol of vinblastine/pmol of tubulin.

[b] Each 0.32-ml reaction mixture contained 1.0 mg/ml tubulin, 50 μM [8-^{14}C]GTP, inhibitor as indicated, 0.1 M MES (pH 6.9), 0.5 mg/ml $MgCl_2$, and 2% (v/v) dimethyl sulfoxide. GTP was the last component added to the reaction mixtures (13). Incubation was for 10 min at 0°. Triplicate 0.1-ml aliquots were analyzed by centrifugal gel filtration chromatography, performed at 4° (8). Control was 0.55 pmol of GTP/pmol of tubulin.

[c] Each 0.1-ml reaction mixture contained the components described in the text, 0.2 mg/ml tubulin, 5 μM [^{3}H]colchicine, inhibitor as indicated, and 5% (v/v) dimethyl sulfoxide. Incubation was for 1 hr at 37°. Each value represents the average of triplicates. Control was 0.48 pmol of colchicine/pmol of tubulin.

Pettit and T. J. Fitzgerald. Maytansine was obtained from the Natural Products Branch, National Cancer Institute, and thiocolchicine from Roussel-Uclaf.

Methods. Tubulin polymerization was followed turbidimetrically, at 350 nm, in Gilford spectrophotometers equipped with electronic temperature controllers (0 to 37° temperature jump in 75 sec). GTP hydrolysis was measured by following the formation of [8-^{14}C]GDP from [8-^{14}C]GTP, by thin layer chromatography on polyethyleneimine-cellulose (8). Binding of [8-^{14}C]GTP to tubulin was measured by centrifugal gel filtration (8). Tubulin-drug complexes were separated from free drug with Sephadex G-50 (superfine) by column chromatography or by centrifugal gel filtration. The binding of radiolabeled colchicine to tubulin was measured on DEAE-cellulose filters (9). Reaction mixtures contained 1.0 M monosodium glutamate (pH 6.6), 0.1 M glucose-1-phosphate, 1 mM $MgCl_2$, 1 mM GTP, 0.5 mg/ml albumin, and tubulin, colchicine, and inhibitor as indicated. Reactions of tubulin with iodo[^{14}C]acetamide (10) and with the bifunctional alkylating agent EBI (11) were examined and quantitated as described previously.

Results and Discussion

Indirect studies indicated the interaction of SQZ derivatives occurs at the colchicine site. Initial work was with NSC 381272, because of its activity in experimental tumors and its potent inhibition of tubulin polymerization (2). Table 1 demonstrates that NSC 381272, in contrast to maytansine (12, 13), did not affect the binding of either vinblastine or GTP to tubulin.[1] Negligible inhibition of colchicine binding was observed, compared with that obtained with podophyllotoxin (14, 15), CS-A2 and CS-A4 (16, 17), and MTPT (18, 19), and inhibition of colchicine binding with NSC 381272 changed little as a function of concentration.[2] We concluded that the SQZ derivatives did not bind at a previously described site on tubulin.

Because drugs that interact with tubulin usually stimulate or inhibit tubulin-dependent GTP hydrolysis (20–22), the most potent SQZ derivatives were examined for effects on this reaction. They all stimulated GTP hydrolysis, despite their inhibition of polymerization, and their effects most closely resembled those of colchicine. This is shown in Fig. 2, which compares stimulation of GTP hydrolysis by NSC 379310 [Fig. 1, R = CH_3; 6-methyl-2-styrylquinazolin-4(3*H*)-one] with the stimulation obtained with colchicine, as a function of drug concentration.[3]

Several SQZ derivatives were examined for their effects on tubulin sulfhydryl reactivity with iodoacetamide and EBI. Characteristic patterns have been obtained with different classes of antitubulin agents, and all SQZ derivatives examined produced results most consistent with their binding at the colchicine site (Tables 2 and 3). Alkylation of tubulin sulfhy-

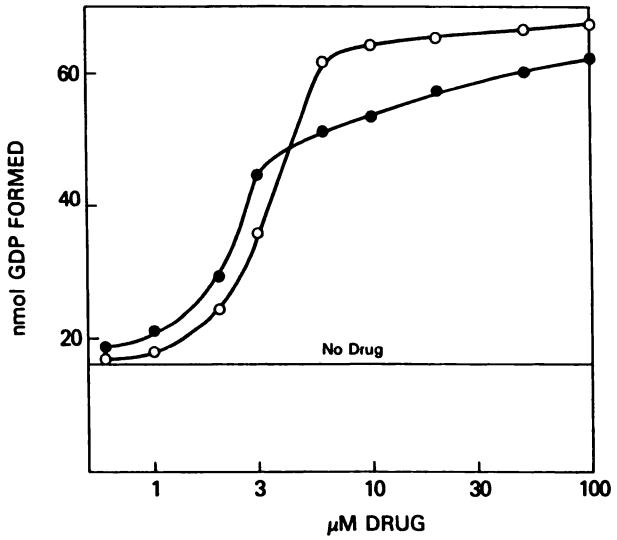

Fig. 2. Comparison of colchicine and NSC 379310 in stimulating tubulin-dependent GTP hydrolysis. Reaction volume was 40 μl during the preincubation and 50 μl during the incubation. All concentrations refer to the final volume. Reaction mixtures contained drug as indicated, 1 mg/ml tubulin, 1 mM $MgCl_2$, 1.0 M monosodium glutamate (pH 6.6), and 4% (v/v) dimethyl sulfoxide and were preincubated for 15 min at 37°. After they were chilled on ice, [8-^{14}C]GTP (final concentration, 100 μM) was added, and the incubation was resumed for 30 min at 37°. Data are expressed as nmol of [8-^{14}C]GDP formed/ml of reaction. ○, Colchicine; ●, NSC 379310.

[1] The conditions chosen for these initial screening experiments were those we had previously found to give optimal results with the different ligands. Vinblastine and GTP binding were not studied under additional conditions, because subsequent experiments indicated that the SQZ derivatives bound at the colchicine site.

[2] The inhibitory effect of NSC 381272 on tubulin polymerization is essentially equivalent to those obtained with podophyllotoxin, CS-A2, CS-A4, and MTPT and about twice that of maytansine.

[3] The effect of lower concentrations (below 20 μM) of colchicine on the stimulation of GTP hydrolysis is distinctly enhanced by the preincubation of drug and tubulin used in the experiment presented in Fig. 2 (30, 31). No significant enhancement was observed by a drug-tubulin preincubation with any of the SQZ derivatives examined.

TABLE 2

Effects of SQZ derivatives on the reaction of tubulin with iodo[^{14}C] acetamide

Aliquots (250 μl) of tubulin (0.66 mg/ml) purified in the presence of 1 mM GTP (6) were incubated with drugs as indicated, at 50 μM, and 1.36 mM iodo[^{14}C]acetamide, for 1 hr at 37°. Incorporation of ^{14}C radiolabel into tubulin was determined as described previously (10). Values are mean ± standard deviation.

Drug added	Incorporation of iodoacetamide into tubulin	
	Experiment I[a]	Experiment II[b]
	dpm/pmol	
None	3.28 ± 0.11 (100%)[c]	2.21 ± 0.08 (100%)
Podophyllotoxin	2.37 ± 0.37 (72%)	
Vinblastine		1.33 ± 0.05 (60%)
NSC 379310	2.87 ± 0.28 (87%)	
NSC 380836	2.50 ± 0.02 (76%)	

[a] Quadruplicate samples.
[b] Triplicate samples.
[c] Numbers in parentheses, percentage of control.

TABLE 3

Effects of SQZ derivatives on the formation of the β* and β* cross-links

Aliquots (250 μl) of tubulin (0.66 mg/ml) containing reduced and carboxymethylated conalbumin (0.2 mg/ml) were incubated at 30° for 1 hr with 0.91 mM EBI with drugs as indicated, at 50 μM, except that the GTP concentration was 1 mM. Samples were processed, and the yields of the β* and β* cross-links were calculated as described previously (11). Yields are expressed as a percentage of total β_1-tubulin [75% of total β-tubulin, the β_I, β_{II}, and β_{IV} isotypes; the remaining 25%, the β_{III} isotype, is not cross-linked by EBI (11, 34, 35)].

Drug added	Experiment I, yield of β*[a]	Experiment II, yield of β*[b]
	% of total β_1	
None	60, 69[c]	40, 40[c]
Podophyllotoxin	4	
Vinblastine		19
GTP		7
NSC 380836	31	40
NSC 379310	15	53
NSC 381272	30	49

[a] The tubulin used in this experiment contained 1 mM GTP, which inhibits formation of the β* cross-link. Polyacrylamide gel electrophoresis was performed as described by Laemmli (36).
[b] GTP was removed from the tubulin by gel filtration chromatography on Sephadex G-25 (11). Podophyllotoxin (50 μM) was used to prevent β* formation. Polyacrylamide gel electrophoresis was performed as described previously (37).
[c] Duplicate samples.

dryls by iodoacetamide (Table 2) was weakly inhibited by NSC 379310. The effect of NSC 380836 (Fig. 1, R = I) was identical to that of podophyllotoxin, rather than to the more extensive inhibition of alkylation obtained with vinblastine (10, 23, 24).

When tubulin reacts with EBI, two intrachain cross-links form in β-tubulin, i.e., β*, between Cys^{239} and Cys^{354}, and β*, between Cys^{12} and either Cys^{201} or Cys^{211} (25–27). Formation of β* is inhibited by colchicine, podophyllotoxin, and MTPT (23, 24), whereas formation of β* is inhibited by vinblastine, maytansine, and GTP (11). We found that three SQZ derivatives inhibited formation of β*, whereas none of them reduced formation of β* (Table 3). The most inhibitory SQZ derivative for β* formation was NSC 379310. Half-maximal inhibition was obtained at 5 μM (data not presented), about twice the IC_{50} value of 2 μM obtained for inhibition of tubulin polymerization (2). Extent of inhibition of β* formation by NSC 379310 was significantly less than that obtained with podophyllotoxin (Table 3), consistent with our conclusion (see below) that the interaction of SQZ derivatives with tubulin is rapidly reversible. In terms of their effects on the alkylation of tubulin sulfhydryl groups, the SQZ derivatives thus most closely resemble ligands that bind at the colchicine site.

Direct studies of the interaction of SQZ derivatives at the colchicine site. These findings led us to reexamine the effects of SQZ derivatives on the binding of [^{3}H]colchicine to tubulin. We found that shorter incubation times and lower colchicine concentrations resulted in extensive inhibition of the reaction. There was reasonable correlation between the ability of a SQZ derivative to inhibit tubulin polymerization and its ability to inhibit colchicine binding (data not presented), but NSC 379310 was disproportionately active as an inhibitor of colchicine binding. It was, therefore, studied in detail.

If incubation time was reduced to 2 min and the tubulin to colchicine ratio made 1:1, NSC 379310 could completely inhibit the binding of [^{3}H]colchicine to tubulin (Fig. 3), consistent with its potent inhibition of polymerization. Under these reaction conditions, we demonstrated competitive inhibition of colchicine binding by NSC 379310 (Lineweaver-Burk analysis), with an apparent K_i value of 2.5 μM (Dixon analysis) (data not shown).

Estimation of the dissociation rate constant of NSC 379310 from tubulin. We wanted to obtain insight into the association and dissociation rates of the SQZ-tubulin interaction. Without a radiolabeled agent, the dissociation rate was more readily approachable. Inhibition of colchicine binding decreased as incubation time increased, so the SQZ derivatives must bind to tubulin faster than colchicine and must dissociate readily. Thus, a measure of the dissociation constant of NSC 379310 from tubulin could be made by determining the rate of [^{3}H]colchicine binding to a preformed complex of tubulin with NSC 379310 and comparing it with rates of colchicine binding to unliganded tubulin and to other drug-tubulin complexes with known dissociation rate constants. Such a study is presented in Fig. 4. Tubulin was preincubated with a 10-fold molar excess of thiocolchicine, CS-A2, MTPT, or NSC 379310, to allow formation of the protein-drug complexes. Because gel filtration

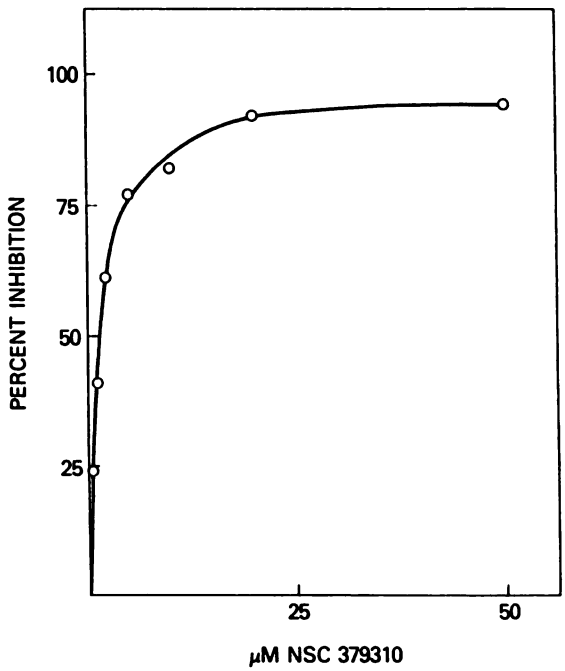

Fig. 3. Extensive inhibition of colchicine binding by NSC 379310. Each 0.1-ml reaction mixture contained the components described in the text, 0.1 mg/ml tubulin, 5% (v/v) dimethyl sulfoxide, 1.0 μM [^{3}H]colchicine, and NSC 379310 as indicated. Incubation was for 2 min at 37°. Control was 0.070 pmol of colchicine bound/pmol of tubulin.

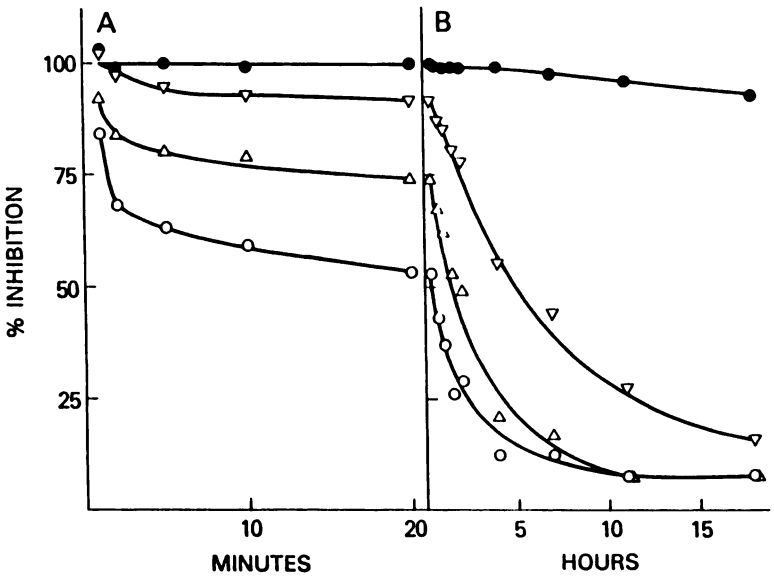

Fig. 4. Effect of incubation time on the extent of inhibition of colchicine binding by various colchicine site drugs. *A, Early time points (up to 20 min). B*, Later time points (20 min to 18 hr) Reaction mixtures (4.8 ml) were prepared containing the components described in the text, 0.1 mg/ml tubulin, 5% (v/v) dimethyl sulfoxide, and no drug, 10 μM thiocolchicine (●), 10 μM CS-A2 (▽), 10 μM MTPT (△), or 10 μM NSC 379310 (○). They were incubated at 37° for 30 min and chilled on ice. Aliquots (0.1 ml) of the reaction mixtures were distributed into tubes, and 10 μl containing 200 pmol of [^{3}H]colchicine were added to each tube. The samples were incubated at 37° for the indicated times. Triplicate data points were obtained for each incubation time. For each time point, reaction mixtures containing each inhibitory drug were compared with the control reaction mixtures without inhibitor, with the data expressed as percentage of inhibition of colchicine binding. In the control reaction mixtures, stoichiometry of binding ranged from 0.014 pmol of colchicine bound/pmol of tubulin at 1 min to 0.47 pmol/pmol at 2 hr.

results in substantial loss of MTPT and NSC 379310 from tubulin (see below), [^{3}H]colchicine was added directly to reaction mixtures. Its binding was measured and compared with binding to tubulin preincubated without drug. Data are expressed as percentage of inhibition of colchicine binding, compared with the control (early time points in Fig. 4A, late points in Fig. 4B). The reaction components used in this experiment minimize the decay of tubulin (28) and do not greatly affect the kinetic parameters for colchicine binding (9).

Thiocolchicine binds to tubulin more tightly than does colchicine (9, 29), and little colchicine was bound to tubulin preincubated with thiocolchicine even after 18 hr. A different pattern was observed with the other drugs, with the extent of inhibition of colchicine binding being highly time dependent. Inhibition was maximal at early time points and negligible at late time points; 50% inhibition occurred at 30 min with NSC 379310, 1.75 hr with MTPT, and 5 hr with CS-A2. Analysis of data with semilogarithmic plots (assuming 100% formation of tubulin-drug complex) demonstrated biphasic decay patterns for all three inhibitors. The half-lives obtained from both phases were in a similar ratio to the 50% inhibition times, implying that the half-life of the tubulin-CS-A2 complex is about 3 times that of the tubulin-MTPT complex, which is, in turn, about 3 times that of the tubulin-NSC 379310 complex.

The reported half-life of the tubulin-CS-A2 complex is 2.4 min at 37° (17) and that of the tubulin-MTPT complex is 12 sec at 24–25° (3, 5) and 17 sec at 37° (4). Although differences in association reaction rates may affect the inhibition data presented in Fig. 4, the relationship of the half-lives of the tubulin-CS-A2 and tubulin-MTPT complexes (the former being about 8–12-fold longer than the latter) does not differ greatly from the 3-fold longer time it takes tubulin to reach 50% inhibition of colchicine binding in the presence of CS-A2, compared with MTPT. Thus, by analogy (ignoring association rates), the 3-fold faster decay to 50% inhibition of colchicine binding that occurs in the presence of excess NSC 379310, compared with MTPT, indicates that the tubulin-NSC 379310 complex is less stable than the tubulin-MTPT complex. As a first approximation, the half-life of the tubulin-SQZ derivative complex is no greater than 5 sec (corresponding to a dissociation rate constant of 1.4×10^{-1} sec^{-1}).

Estimation of the association rate constant for the binding of NSC 379310 to tubulin. Indirect estimation of association rates can be difficult, but, because inhibition of colchicine binding occurs most extensively at early time points, the binding reaction of NSC 379310 to tubulin must be fast. Further, effects of NSC 379310 on GTP hydrolysis and polymerization were not enhanced by a tubulin-drug preincubation. Such observations with other drugs have correlated well with rapid binding reactions (9, 17, 30, 31).

Although originally performed to confirm the rapid dissociation of NSC 379310, as well as MTPT, from tubulin, experiments on the behavior of tubulin incubated with these drugs and then subjected to gel filtration chromatography actually indicated that NSC 379310, like MTPT, must bind rapidly and provided suggestive evidence about their relative binding rates. Evaluation of data obtained with the SQZ derivative, in comparison with data obtained with MTPT, strongly indicates that NSC 379310 must bind to tubulin more rapidly than does the phenyltropolone, which has a known binding rate constant (5).

Tubulin at 30 μM was incubated at 37° without drug or with 300 μM NSC 379310, MTPT, CS-A2, or colchicine. (Except for the control, the tubulin in all reaction mixtures was unable to polymerize when GTP was added.) Each mixture (including the control) was gel filtered at 4°, to separate protein and unbound drug. The ability of the reisolated protein to polymerize upon addition of GTP was examined (Fig. 5). Tubulin preincubated with colchicine or CS-A2 was totally inactive. Preincubation with either MTPT or NSC 379310, followed by gel filtration, however, resulted in only partial inhibition of polymerization, with the tubulin treated with MTPT polymerizing more like the control tubulin than protein treated with the SQZ derivative (confirmed in repetitions of the experiment). Maximum rates of turbidity development are summarized in Table 4.

These were unexpected results, because the dissociation of NSC 379310 from tubulin is so rapid. Although we have previ-

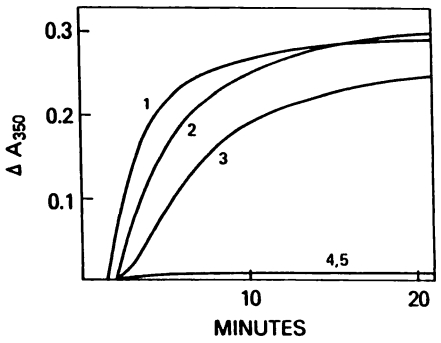

Fig. 5. Polymerization of tubulin after preincubation with drugs and removal of unbound drug by gel filtration chromatography. Reaction mixtures (1.0 ml) contained 3.0 mg/ml tubulin, 1.0 M monosodium glutamate (pH 6.6), 4% (v/v) dimethyl sulfoxide, and, if present, 300 μM drug. Incubation was for 15 min at 37°, and the reaction mixtures were placed on ice. Each reaction mixture was applied to a 1.5- × 45-cm Sephadex G-50 (superfine) column, which was equilibrated and developed with 1.0 M monosodium glutamate (pH 6.6) at 4°. Fractions (0.5 ml) were collected, the two fractions from each column containing the highest protein concentrations were pooled, and the protein concentrations of the pools were determined. The polymerization reaction mixtures contained 1.0 M monosodium glutamate (pH 6.6), 0.8 mM GTP, and 0.7 mg/ml tubulin. The drug present during the preincubation was as follows: *curve 1*, none; *curve 2*, MTPT; *curve 3*, NSC 379310; *curve 4*, CS-A2; *curve 5*, colchicine.

TABLE 4

Properties of tubulin preincubated with drugs and separated from excess drug by gel filtration chromatography

Drug added	Maximum rate of polymerization[a]	Colchicine bound[b]		GTP hydrolyzed[c]
		2-min incubation	45-min incubation	
		% of control		
Colchicine	0	5	6	337
CS-A2	0	50	78	145
MTPT	58	100	104	135
NSC 379310	33	110	110	172

[a] The data from the experiment of Fig. 5 were evaluated to determine the maximum rate of polymerization. Control was 0.099 A_{350} unit/min.

[b] Each 0.1-ml reaction mixture contained the components described in the text, 0.08 mg/ml tubulin/tubulin-drug complex isolated by gel filtration chromatography (Fig. 5), and 1.0 μM [^{3}H]colchicine. Incubation was at 37° for the times indicated. Control was 0.014 pmol of colchicine/pmol of tubulin at 2 min and 0.24 pmol/pmol at 45 min.

[c] Each 40-μl reaction mixture contained 1.0 M monosodium glutamate (pH 6.6), 1 mM $MgCl_2$, 100 μM [8-^{14}C]GTP, and 0.4 mg/ml tubulin/tubulin-drug complex isolated by gel filtration chromatography (Fig. 5). Incubation was at 37° for 20 min. Control was 6.9 nmol/ml [8-^{14}C]GDP formed. The values in the table represent the average of five data points in three independent experiments, with standard errors ranging from 7 to 15% of the mean values presented.

ously noted the persistence of residual MTPT and CS-A2 with tubulin after gel filtration (17), the more rapid dissociation of NSC 379310 (Fig. 4) led us to predict that the drug would be totally released from tubulin by this procedure. Instead, the recovered tubulin after NSC 379310 treatment was even less active than MTPT-treated tubulin. Factors other than relative dissociation rates, thus, must affect retention of residual drug by the tubulin.

Tubulin obtained in the experiment of Fig. 5 was also evaluated for residual drug by inhibition of colchicine binding and by stimulation of tubulin-dependent GTP hydrolysis (Table 4). Although tubulin treated with CS-A2 or nonradiolabeled colchicine was deficient in binding [^{3}H]colchicine, the former experiment failed to confirm that residual NSC 379310 or MTPT remained bound to the tubulin. The GTPase assay, like the polymerization assay, was more sensitive to small amounts of drug. Relative to the control tubulin, enhanced formation of GDP occurred with all drug-treated tubulin preparations.

The 3.4-fold stimulation of GTP hydrolysis obtained with the colchicine-treated tubulin indicates near-saturation of tubulin with drug (Fig. 2). CS-A2 has only a weak stimulatory effect on net GTP hydrolysis (17), and our current results agree with this earlier finding. Net GTP hydrolysis in the presence of saturating MTPT (19) or NSC 379310 (Fig. 2) is nearly identical to that observed with saturating colchicine. Thus, the feeble enhancement of GTP hydrolysis after gel filtration of the tubulin treated with these agents demonstrates a low concentration of residual drug, consistent with the weakly inhibited polymerization reactions. The preincubated and gel-filtered tubulin samples were compared with tubulin containing known amounts of MTPT and NSC 379310 in the GTPase assay (32). This study indicated that these preparations contained amounts of MTPT and NSC 379310 equivalent to molar saturations of 0.11 and 0.094, respectively. Adding similar molar concentrations of the two drugs to tubulin produced partial inhibition of polymerization, comparable to the data of Fig. 5.[4]

Comparing the findings with MTPT and NSC 379310, the latter dissociates from tubulin 3 times faster than the former (see above), whereas the gel filtration rates for the tubulin samples containing either drug were the same. Moreover, nearly identical amounts of the two drugs remained with the tubulin after gel filtration. Because dissociation of drug from tubulin cannot be the only factor determining whether drug will remain protein associated during gel filtration, we conclude that there was ample time for drug molecules to rebind repeatedly to tubulin as the sample passed through the column. Rebinding reactions must be significantly more rapid than diffusion of drug into the beads. Further, the binding of the SQZ derivative to tubulin is probably faster than the binding of MTPT. Assuming that the residual amount of drug bound to tubulin after gel filtration is proportional to the association constant (K_A value) of the drug, then the K_A values of MTPT and NSC 379310 must be nearly identical. Because K_A = association rate constant ÷ dissociation rate constant, the association rate constant for NSC 379310 (in parallel with the dissociation rate constant) is about 3 times that of the association rate constant of MTPT. The apparent second-order rate constant for the binding of MTPT to tubulin at 37° is 5.2×10^4 M^{-1} sec^{-1} (3), implying that the value for NSC 379310 is about 2×10^5 M^{-1} sec^{-1}. The dissociation constant (K_D value) for MTPT obtained by dividing the dissociation rate constant by the association rate constant is about 2–3 μM (3, 5, 33), within the range where 50% inhibition of tubulin polymerization is observed. Similarly, NSC 379310 would have a comparable K_D value, again within the range where 50% inhibition of polymerization occurs.

Conclusions. Despite initially puzzling results, the SQZ derivatives bind in the colchicine site of tubulin as competitive inhibitors. Their apparently weak inhibition of the reaction derives from the binding properties of the two types of drugs; colchicine binds slowly in a temperature-dependent reaction and dissociates even more slowly from tubulin, whereas the SQZ derivatives bind rapidly even at low temperatures, in a readily reversible reaction. Similarly, NSC 379310 was deficient

[4] IC_{50} values for inhibition of polymerization for MTPT and NSC 379310 are 2.6 and 2.0 μM, respectively, so that with identical concentrations of the two agents greater inhibition of polymerization will occur in the reaction mixture containing NSC 379310.

as an inhibitor of β^* formation because the rapid and reversible SQZ-tubulin interaction was in competition with the covalent reaction of EBI with tubulin. Inhibition of colchicine binding was greatest with NSC 379310, indicating that the other derivatives with comparable inhibitory effects on tubulin polymerization (2), including NSC 381272, bind to tubulin and dissociate from the protein even more rapidly than NSC 379310. By comparing the properties of NSC 379310 with those of MTPT and CS-A2, compounds known to bind to and dissociate from tubulin rapidly, we concluded that the SQZ derivative's rates of binding and dissociation exceeded those of MTPT by about 3-fold. Previous work with MTPT (3–5) allowed us to estimate the association and dissociation rate constants for the interaction of NSC 379310 with tubulin.

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