B ring regulation of colchicine binding kinetics and fluorescence

(B ring analogues/binding rate/reversibility/atropisomerism)

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ABSTRACT   Several properties of the colchicine–tubulin interaction such as association rate, reversibility, and the promotion of drug fluorescence have been related to the B ring of colchicine. The B ring itself retards the binding rate, and substitution at C-7 leads to further binding rate decreases that appear to be related to both substituent bulk and the presence of a N-acyl group. Thus, the decreasing order of binding rates is 2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone > deacetamidocolchicine > deacetylcolchicine ≥ colcemid > colchicine > N-benzoyldeacetylcolchicine, etc. The apparent irreversibility of the binding seems more closely related to the presence of an N-acyl group rather than the bulk of the substituent at C-7. Substitution at C-7 also affects the tropolone fluorophore. Thus, amines (deacetylcholchicine, colcemid, or N-methylcolcemid) fluoresce poorly in the presence of tubulin, whereas substitution of the amino group with an acyl group enhances fluorescence. The presence of an N-acyl group at C-7 is essential for enhanced fluorescence. We conclude that, in addition to A- and the C-ring portion of the molecule, the B ring of colchicine is a third determinant recognized by the binding site on tubulin.

Structure–function relationships in colchicine (Fig. 1) and its congeners can be conveniently described in terms of the three rings of the molecule, the A ring or trimethoxybenzene moiety, the seven-membered B ring, and the methoxytropone moiety or C ring. Substantial evidence has accumulated that the colchicine-binding site of tubulin, through which most of the desired drug effect presumably must operate, contains a domain that recognizes the A ring and a second domain that recognizes the C ring (1–3). This was first concluded from the finding that podophyllotoxin competed for colchicine, presumably through the mutual trimethoxybenzene moiety (4). Tropolone and methoxytropone block colchicine, but not podophyllotoxin, binding (5). It was thus proposed that colchicine has at least two attachment points to its binding site on tubulin, one for the A ring and one for the C ring (4, 5). Subsequently, it was shown (6) that these domains can be independently occupied by single ring analogues such as mescaline (A ring) or methoxytropone (C ring). However, affinities for these single rings are low and marked enhancement of binding affinity is attained when these are linked as in colchicine, etc. The sum of the individual binding energies (plus a correction for the cratic entropy resulting from incorporation of these rings into a single molecule) was similar to that of colchicine.

Because of the finding that an analogue containing the A and C rings but lacking the B ring [2-methoxy-5-(2',3',4'-trimethoxyphenyl)tropone], referred to hereafter as the A-C compound, has high biological and tubulin binding activity (7), this compound has been widely used as a simple and flexible model for colchicine. (Colchicine congeners used in this study are shown in Table 1; for identification, hereafter,
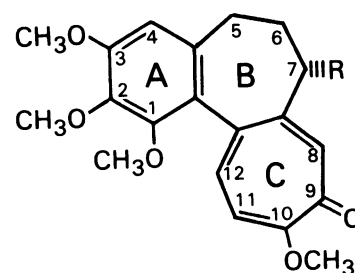
FIG. 1.   Structure of colchicine analogues used in this study. For colchicine, R = $-NHCOCH_3$.

these compounds will be followed by their number in Table 1.) It binds rapidly, possibly due to the facile rotation about the biaryl bond and with an activation energy lower than colchicine (8) (9, 10). Like colchicine (8), the A-C compound (16) exhibits marked enhancement of fluorescence upon binding, and free energies of binding are consistent with the A- and C-ring contributions (9–11). The contributions of the B ring to the binding of colchicine congeners to tubulin have been considered to be of a minor nature. Thus, using the A-C compound (16) as a model, a bifunctional ligand model has been proposed for colchicine (8) in which the bulk of the interactions with the tubulin site, including the conformational changes in tubulin, are accomplished by the A and C rings (10, 11). Colchicine (8) binding to tubulin is a two-step process (12, 13), and it has been proposed that the tropolone moiety binds before the remainder of the molecule and promotes the conformational change in the protein that now facilitates binding of the A ring.

When present, however, B-ring substituents at position C-7 have major effects on the association and dissociation rates of the drugs and on the temperature dependence of binding as exemplified by the difference between colchicine (8), colcemid (4), and deacetamidocolchicine (1) (8, 9, 14). Moreover, the unnatural enantiomer, (+)colchicine, shows no tubulin binding activity (unpublished results). The present study was undertaken to elucidate the contributions of the B ring and substitutions at C-7 to the kinetics, binding constants, and fluorescence properties of colchicine analogues.

MATERIALS AND METHODS

Tubulin was prepared by phosphocellulose purification of rat brain microtubule protein, prepared by two cycles of temperature-dependent polymerization (15) and stored at −70°C following drop freezing in liquid nitrogen. Fluorescence measurements were carried out in thermostated cuvettes at

37°C in a Perkin–Elmer MPF 3L instrument with an excitation wavelength of 353 nm and emission at 430 nm except when stated. GTP was deleted to prevent polymerization. Corrected emission spectra were measured with a modified Bowman Aminco spectrofluorometer made available through the kindness of Raymond F. Chen. Spectra in 99.5% (vol/vol) glycerol/0.5% dimethyl sulfoxide were obtained in 5-mm cells in a Perkin–Elmer MPF 66 instrument at 27°C and were corrected for instrument variation and for solvent contributions by difference. The quantum yields of colchicine analogue–tubulin complexes were calculated by comparison with quinine sulfate in 0.1 M H_2SO_4, whose quantum yield was taken as 0.546 at 25°C (16).

The binding of colchicine analogues to tubulin was determined by competition for 1 μM [^{3}H]colchicine (8) binding. Aliquots (250 μl) of rat brain tubulin (0.25–0.5 mg/ml) in 100 mM Mes buffer (pH 7.0), 1 mM $MgCl_2$, 1 mM EGTA, [^{3}H]colchicine (8), and analogues (added at the beginning of the incubation to concentration ranges from 1 to 100 μM) were incubated for 60 min at 37°C. The extent of binding was measured by the DE 81 filter disc method (17). Apparent K_i values, the amount of colchicine analogue required to inhibit the [^{3}H]colchicine (8) binding to tubulin by 50%, were then determined from the semi-log plot of colchicine (8) binding activity against the concentration of analogue. Colchicine congeners with different substituents at C-7 were prepared as described (18–20); the quaternary amine was generously provided by M. Rösner; it was prepared from colcemid by reaction with methyl iodide (mp 213–215°C, $M^++1 = 385$). Deacetamidocolchicine (1) and the A-C compound (16) were gifts of T. J. Fitzgerald (Florida A & M University).

Association Rate Constant. The bimolecular association rate constant can be represented as follows:

$$k_1 = \frac{d(CT)/dt}{[C][T]}$$

where $d(CT)/dt$ is the rate of formation of the complex CT (colchicine analogue–tubulin) and [C] and [T] are the concentrations of free colchicine analogue and unbound tubulin, respectively. Conditions were adjusted such that <10% of the reactants were consumed during the reaction and progress curves were linear. We have thus assumed that $[C] = [C]_0$ and $[T] = [T]_0$ where $[C]_0$ and $[T]_0$ are the initial concentrations of colchicine analogue and tubulin, respectively. The amount of analogue-bound protein was determined by fluorescence as follows: $[CT] = (F_c/F_o) \times C_o$ where [CT] = the amount of complex, F_c is the fluorescence of a given solution of analogue–tubulin complex, and F_o is the fluorescence of an equal concentration of analogue in excess tubulin, such that all the analogue is bound. Fluorescence of the analogues in water was negligible at the concentrations used. Each value of the rate constant is an average of three determinations. In general, analogue concentration was held constant (3–5 μM) and linear curves were produced over a concentration range of 3–8 μM of tubulin.

RESULTS

Equilibrium Constants. Binding constants for colchicine congeners with different substitutions at C-7 of the B-ring analogues obtained by displacement of 1 μM [^{3}H]colchicine (8) are listed in Table 1. It is apparent that substitution on the nitrogen attached to C-7 has an effect on the binding constants obtained but that, despite large variations in the bulk of the substituents, all constants are within less than an order of magnitude of colchicine (8). It is of interest that an even bulkier group (fluorescein isothiocyanate) has been reported to bind with about 0.10 the affinity of colchicine (8) (21), and an azidoaromatic photoaffinity label at C-7 with a long spacer (22) and a spin label at C-7 of allocolchicine (23) have good

specificity for the site. It appears, therefore, that this portion of the B ring has relatively modest effects on this binding parameter although the introduction of a positive charge through formation of a quaternary nitrogen causes loss of binding activity. Another conclusion permitted by the present results is that hydrogen bonding between the amino group and the tropolone carbonyl group is not essential (24) since the disubstituted analogues N-methylcolchicine (9), N-methylcolcemid (5), and N-trifluoroacetylcolcemid (11) show good binding activity. Comparison of these results with the one point assays performed (2, 20) shows some discrepancies in the order of affinities, particularly with deacetylcolchicine (3), N-trifluoroacetylcolcemid (11), N-methylcolchicine (9), and colcemid (4). Part of these differences can be ascribed to the fact that highly active analogues were compared on the nonlinear portion of the dose response curve. It should be noted that the total absence of the B ring, as in the A-C compound (16) listed in Table 1, yields an equilibrium binding constant of the same order as a number of the analogues containing the intact B ring (Table 1), as also reported by others (10, 11).

Binding Rates. Association rate constants were measured by enhancement of analogue fluorescence that occurs upon binding to tubulin (25). At the concentrations used, there was only negligible fluorescence of these compounds in buffer alone. Quantum yields differ markedly (see below), and all rates are expressed as a function of the maximal attainable fluorescence for that particular analogue in the presence of an 8- to 10-fold excess of tubulin, where all of the compound may be assumed to be bound (see below).

Although colchicine (8) binding has long been known to exhibit very slow kinetics, there is a very wide range of binding rates for the colchicine analogues. This is demonstrated for B-ring analogues at C-7 in Table 1. Association rate constants vary by nearly four orders of magnitude as we move from no B ring at all, as exemplified by the A-C compound (16), to the N-retinoyl (15) derivative at the seven position. The bare, unsubstituted B ring of deacetamidocolchicine (1) causes a 20-fold reduction in the association rate constant. Substitution at C-7 in the B ring further lowers the association rate constant, and this occurs as a rough function of the size of the substituent. It seems possible to propose, therefore, that the seven-membered B ring itself retards binding and that substitution at C-7 leads to further rate losses that appear to be functions both of the bulk and the presence of a N-acyl group.

Fluorescence. The very slow dissociation of the tubulin–colchicine complex has proved a boon in binding studies and has been explained (10) by an activation energy barrier for dissociation that is substantially larger than for the reversible analogue, A-C compound (16). The binding of colchicine to tubulin is accompanied by a marked enhancement of fluorescence from the methoxytropone moiety with characteristics of a $\pi^* \rightarrow \pi$ transition (25, 26) and a lifetime, τ, of 1.14 nsec (13). A substantial fraction of this fluorescence is caused by the immobilization of this drug in the binding site on tubulin, rather than being due solely to the hydrophobic environment that the site may provide (27) since emission can be elicited from colchicine (8) contained in a sheet of solid polyvinyl alcohol (26). It has also been suggested that stabilization of the drug in a conformation that makes the A and C rings more nearly coplanar contributes to fluorescence (10).

B-ring substitution at C-7 has a profound effect on the fluorescence properties of the methoxytropone fluorophore of colchicine derivatives. This was first suggested (14) by the poor fluorescence of colcemid. The quantum yields of analogues of colchicine are listed in Table 1 for assays carried out with an 8- to 10-fold molar excess of tubulin after incubation

Table 1. Properties of B-ring analogues of colchicine

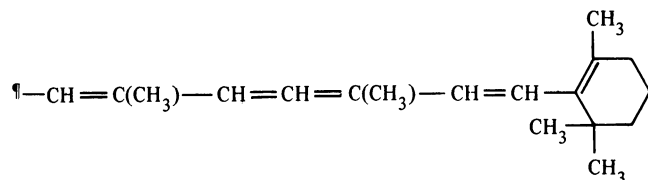| No.	R group on C-7 of the B ring	Name	Binding parameters K_i, μM	k_1 M^{-1}·hr (×10^6)	Reversibility,* %	Quantum yield of tubulin complex	Fluorescence in glycerol Rel. intensity†	λ_{max}, nm EX	EM
1	H	7-Deacetamidocolchicine	5	6.0	≈30	0.023	89	364	465
2	H, (5,6—CH=CH—)	5,6-Dehydro-7-deacetamidocolchicine	—	5.8	≈40	0.013	—	—	—
3	NH$_2$	N-deacetylcolchicine	12	1.8	≈40	0.0057	66	365	465
4	NHCH$_3$	Colcemid (demecolcine)	60	1.8‡	67‡	≈0	64	365	465
5	N(CH$_3$)$_2$	N-methylcolcemid	30	—	—	≈0	57	367	465
6	N$^+$(CH$_3$)$_3$I$^-$	N-dimethylcolcemid iodide	Inactive	—	—	≈0	158	364	465
7	N-(CH$_3$)CH$_2$C$_6$H$_4$OH	Speciosin	—	—	—	≈0	32	364	465
8	NHCOCH$_3$	Colchicine	9.4	0.34	≈0	0.030	100	365	465
9	N(CH$_3$)COCH$_3$	N-methylcolchicine	30	0.25	≈0	0.032	70	366	465
10	NHCOCF$_3$	N-deacetyl(trifluoracetyl)colchicine	10	0.30	≈0	0.026	67	366	465
11	N(CH$_3$)COCF$_3$	N-(trifluoracetyl)colcemid	12	0.18	≈0	0.028	73	365	465
12	NHCOC$_6$H$_5$	N-deacetyl-N-benzoylcolchicine	24	0.09	≈0	0.036	59	364	465
13	NHCOC$_6$H$_2$(OCH$_3$)$_3$	N-deacetyl-N-trimethoxybenzoyl colchicine	54	0.07	≈0	0.029	59	363	465
14	NHCOC(CH$_3$)$_3$	N-deacetyl-N-pivaloylcolchicine	70	0.05	≈0	0.0175	105	364	460
15	NH (retinoyl)¶	N-deacetyl-N-retinoylcolchicine	50	0.01	≈0	0.018	—	—	—
16	A-C compound	A-C compound	(2, 3)§	120	>90	0.021	293	365	464

Rel., relative; EX, excitation; EM, emission.
*One hundred times excess of podophyllotoxin for 60 min at 37°C.
†Intensity relative to that of colchicine.
‡Taken from ref. 8.
§Values obtained by fluorescence measurements.

¶—CH = C(CH$_3$)—CH = CH = C(CH$_3$)— CH = CH— [structure with CH$_3$ groups and cyclohexene ring]

at 30°C for 60–90 min (to attain plateau values), followed by separation of the complex and checking for unbound drug.

The unsubstituted B ring or deacetamidocolchicine (1) gives a fluorescence yield about two-thirds that of colchicine (8). When a double bond is introduced as in 5,6-dehydro,7-deacetamidocolchicine (2), the red shift expected from addition of a double bond conjugated to an aromatic center (28) occurs in the absorption spectrum (λ_{max}, 358 nm; λ_{max} emission, 431 nm), but there is a reduction in the quantum yield to ϕ = 0.013. Dreyding models suggest that this may be due to the fact that neither atropisomer can attain the near coplanar biaryl angle that is possible with the saturated B ring. This is consistent with the postulate of Bane *et al.* (10). Addition of the free amino or alkyl amine groups at position seven yields analogues that fluoresce very poorly or not at all when bound to tubulin. By contrast, the N-acyl-containing derivative exhibits quantum yields on the order of colchicine (8), and fluorescence is not particularly sensitive to the bulk of the substituent once the complex is formed. Dreyding models show that, in one conformation of colchicine (8), the N-acyl group could approach the π electrons over the A ring. To test whether this could enhance fluorescence by supplying extra electrons to the A ring and thus to the tropolone ring by extended conjugation, we tested N-deacetylsuccinylcolchicine (prepared from (−)-deacetylcolchicine and succinic anhydride—mp 255°C, M$^+$+1 = 468). However, this compound had slightly lower fluorescence than colchicine under identical conditions (data not shown).

Attempts were made to determine whether the differences in quantum yield between N-alkyl and N-acyl derivatives of

colchicine were intrinsic properties of the fluorophore or were the result of the binding interaction with tubulin. To this end we employed glycerol, which is known to promote colchicine fluorescence (provided a threshold viscosity of ≈3 cP is exceeded), albeit less efficiently than tubulin (27). There is little correlation between the ability of the C-7 substituted analogues to fluoresce in viscous solvents and the quantum yields of the tubulin complex (Table 1). It is apparent that substantially less variation occurs in the relative fluorescence of the analogues in glycerol than in the tubulin complex. Note, however, the low fluorescence intensity of speciosin (7) and the high intensity of the A-C compound (16). Excitation and emission maxima also show only small deviations from colchicine under these conditions (Table 1). This suggests that the intrinsic fluorescence of the colchicine analogues (as measured in glycerol) is not the primary factor determining the quantum yields resulting from binding to the tubulin site. The enhancement by acyl groups is also less apparent in glycerol. For example, the relative fluorescence intensity of deacetylcolchicine (3) in glycerol contrasts sharply with the low quantum yield of its tubulin complex, despite the fact that this compound binds well (Table 1). Similarly, the absence of fluorescence of the quaternary N-derivative (6) in the presence of tubulin can be ascribed to the absence of binding to the site, because it fluoresces well in glycerol. Such differences between glycerol and tubulin strongly suggest that B-ring interactions within the binding site contribute to fluorescence and this contribution does not derive strictly from the affinity of the congener for tubulin.

DISCUSSION

One of the peculiar properties of colchicine binding to tubulin is the slow equilibration, which often takes 60–90 min. Garland (12) first proposed a two-step model in which rapid, reversible binding to tubulin is followed by slow conformational changes that convert the initial complex to a more stable, less easily reversible state. This model, with some modifications, is the currently most used one, and it has been shown (29) that the promotion of fluorescence best coincides with the formation of the second complex. The conformational changes leading to the more stable state have been postulated to be due to changes in the tubulin molecule, the colchicine molecule (30, 31), or both. The assumption made is that initial binding occurs with the skewed conformation whereas binding to the second state occurs with the more planar conformer (10).

Is it possible that the B ring or substitutions at C-7 contribute to these conformational changes? Certainly the B ring provides constraints on the spatial relationship of the A and C rings to each other. It contributes three properties to the drug: (*i*) bulk (both the three carbon chain of the ring and substituents at C-7), (*ii*) the N-acyl group, and (*iii*) atropisomerism. The present studies clearly show that bulk at C-7 impedes the binding process, and this effect is greater on the association rate than on the "equilibrium" constant (Table 1). On the other hand, the apparent irreversibility of the binding seems more closely related to the presence of a N-acyl group, although the nature of its interaction with the binding site is not clear at present. Since the presence of a N-acyl group is also associated with higher quantum yields of the colchicine congener-tubulin complex (Table 1), the question of the relation of the irreversibility to the quantum yield may well be raised. The lower quantum yield (0.021) for the A-C compound (16), despite its high fluorescence in glycerol (Table 1), is consistent with a connection between the two phenomena.

The role of the B ring itself has been more difficult to rationalize. The drug loses its negative circular dichroic band at 340 nm, and it was postulated that binding stabilizes rotation about the biaryl bond from an angle of 53° to 19°, thereby facilitating extended conjugation and, hence, fluorescence (10, 30). Such a mechanism would explain the 20-fold greater association constant of the A-C compound (16) compared to deacetamidocolchicine (1) (Table 1), provided it is assumed that the more planar conformation forms the stable complex. Additional impediments to approaching coplanarity appear to occur in 5,6-dehydro,7-deacetamido-colchicine (2), and the quantum yield is reduced (Table 1). To what extent these considerations pertain to the excited state remains to be determined. Whether this effect, or the reduction of vibrational energy dissipation from the excited state, is the chief factor promoting fluorescence is difficult to determine, since both occur as a consequence of binding to tubulin. In any case, it seems likely that the B ring of colchicine with the acetamido group at C-7 is a third deter-minant recognized by the binding site. The domain plays a major role in the kinetics of binding, reversibility, and the induction of fluorescence.

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