Role of the Colchicine Ring A and Its Methoxy Groups in the Binding to Tubulin and Microtubule Inhibition†

Jose M. Andreu,‡ Bernardo Perez-Ramirez,§,|| Marina J. Gorbunoff,§ David Ayala,‡ and Serge N. Timasheff*,§

Department of Biochemistry, Brandeis University, 415 South Street, Waltham, Massachusetts 02254-9110, and Centro de Investigaciones Biológicas, CSIC, Velázquez 144, 28006 Madrid, Spain

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ABSTRACT: The roles of the methoxy substituents on ring A of two ring colchicine (COL) analogues were probed by the synthesis of a number of drugs and the examination of their effect on binding to tubulin, inhibition of microtubule assembly, and induction of GTPase activity. Selective elimination of ring A methoxy groups at positions 2, 3, and 4 weakened all three processes. The effects on binding and inhibition were independent of the nature of ring C (or C′). Specifically, excision of the 2- or 3-methoxy groups weakened binding by ca. 0.4 kcal mol^{-1}, while that of the 4-methoxy group of ring A was weakened by 1.36 ± 0.15 kcal mol^{-1}. The effect on the inhibition of microtubule assembly, expressed as the equilibrium constant for the binding of the tubulin−drug complex to the end of a microtubule, was more complex and strongly dependent on the nature of ring C (or C′). This was attributed to the abilities of various groups on ring C′ to overcome the wobbling in the tubulin−drug complex introduced by the weakening of the anchoring provided by ring A. It is concluded that ring A of COL is not germane to the mechanism of the inhibition of tubulin self-assembly. It serves only as a complex-stabilizing anchor. The control of this process resides in the interactions that key oxygen atoms of ring C of COL or C′ of structural analogues establish with the protein. It is proposed that the 4-methoxy group of ring A serves as a key attachment point for immobilization of the drugs on the protein.

The binding of the alkaloid colchicine (COL)[1] to tubulin inhibits microtubule formation substoichiometrically (*1*−*3*) and induces an assembly-independent GTPase activity in the protein directed at the E-site bound nucleotide (*4*−*7*). While the strength of the GTPase activity induced by COL and its structural analogues follows, in general, the strength of binding of the drugs to the protein (*7*, *8*), substoichiometric microtubule inhibition is linked only to the strength of binding of a tubulin−drug complex to a growing microtubule (*3*). Examination of a large number of structural analogues of COL, with variations in ring C (C′), has permitted us to determine that the presence of a methyl ketone carbonyl group in position 4′ of ring C′ of biphenyl analogues of COL is sufficient for the induction of strong substoichiometric inhibition (*3*, *8*). The observation that the microtubule inhibition constants, K_i, of TME (ring C of COL) and MTC (rings A−C) are essentially identical, while that of the ring A analogue, *N*-acetylmescaline, is indistinguishable from zero (3), has suggested that ring A is not essential for strong substoichiometric inhibition. To test this hypothesis, and to establish the contribution of the individual ring A methoxy groups to the binding strength of the COL family of drugs to tubulin, several COL analogues with a modified ring A were synthesized and the strengths of their binding to tubulin, microtubule inhibition, and induction of GTPase activity were compared (the structures are shown in Chart 1). This was amplified by the synthesis and examination of several molecules in which the number and positon of methoxy groups in both rings A and C were varied. The results of these studies are reported in this paper.

MATERIALS AND METHODS

Ligand and Other Materials

MTC was kindly provided by T. J. Fitzgerald (Florida State University, Tallahassee, FL). TCB, TKB, TMB, 2,2′,3,3′,4,4′-HMB, and 2,4,4′,6-TMB (formerly named HMB and TMB′, respectively) were synthesized as described previously (*9*−

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* To whom correspondence should be addressed.

‡ CSIC.

§ Brandeis University.

|| Present address: Genetics Institute Inc., One Burt Road, Andover, MA 01810.

[1] Abbreviations: COL, colchicine; MTC, 2-methoxy-5-(2,3,4-trimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; 2,4-MDC, 2-methoxy-5-(2,4-dimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; 3,4-MDC, 2-methoxy-5-(3,4-dimethoxyphenyl)-2,4,6-cycloheptatrien-1-one; TCB, 2,3,4-trimethoxy-4′-carbomethoxy-1,1′-biphenyl; 2,4-DCB, 2,4-dimethoxy-4′-carbomethoxy-1,1′-biphenyl; 3,4-DCB, 3,4-dimethoxy-4′-carbomethoxy-1,1′-biphenyl; TKB, 2,3,4-trimethoxy-4′-acetyl-1,1-biphenyl; 2,3-DKB, 2,3-dimethoxy-4′-acetyl-1,1′-biphenyl; TMB, 2,3,4,4′-tetramethoxy-1,1′-biphenyl; 2,4,4′-TMB, 2,4,4′-trimethoxy-1,1-biphenyl; 2,3,4′-TMB, 2,3,4′-trimethoxy-1,1′-biphenyl; 2′-CH_3-TMB, 2,3,4,4′-tetramethoxy-2′-methyl-1,1′-biphenyl; 2,3,3′,4′-TMB, 2,3,3′,4-tetramethoxy-1,1′-biphenyl; 2,2′,4,4′-TMB, 2,2′,4,4′-tetramethoxy-1,1′-biphenyl; 2,2′,3,3′,4,4′-HMB, 2,2′,3,3′,4,4′-hexamethoxy-1,1′-biphenyl; 3,3′,4,4′-TMB, 3,3′,4,4′-tetramethoxy-1,1′-biphenyl; 2,4,4′,6-TMB, 2,4,4′,6-tetramethoxy-1,1′-biphenyl; TME, tropolone methyl ether; MeO, methoxy group; PG buffer, 10 mM sodium phosphate and 0.1 mM GTP at pH 7.0.

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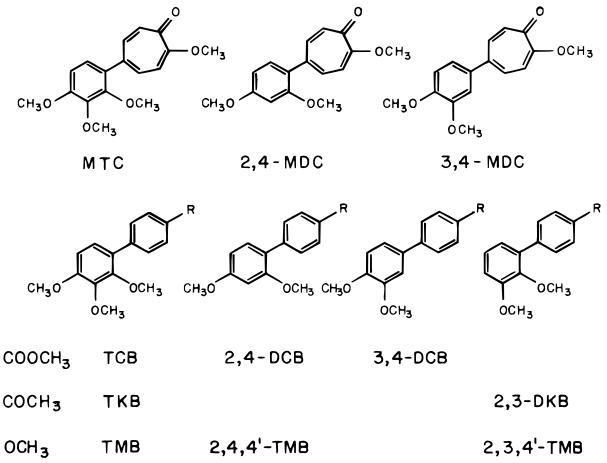
Chart 1



11). 2,4-MDC, 3,4-MDC, 2,3-DKB, 2,4-DCB, 3,4-DCB, 2,3,4′-TMB, 2,4,4′-TMB, 2-CH_3-TMB, 2,3,3′,4-TMB, and 3,3′,4,4′-TMB were synthesized as described below. The ligands were dissolved in dimethyl sulfoxide (spectroscopic grade, Merck) and stored at −20 °C. Residual dimethyl sulfoxide in experiments was less than 2.5%. GTP, dilithium salt, if not indicated otherwise, was from Boehringer-Mannheim, and other chemicals were as described (*3*, *10*). The concentration of each ligand was determined spectrophotometrically employing the following values for the extinction coefficients (in 10 mM sodium phosphate buffer at pH 7.0, in mM^{-1} cm^{-1}): MTC, 17.6 at 343 nm; 2,4-MDC, 17.3 at 350 nm; 3,4-MDC, 18.9 at 353 nm; TKB, 14.4 at 295 nm; 2,3-DKB, 17.4 at 278 nm; TCB, 12.1 at 284 nm; 2,4-DCB, 9.6 at 302 nm; 3,4-DCB, 8.0 at 305 nm; TMB, 16.1 at 256 nm; 2,3,4′-TMB and 2,4,4′-TMB, 16.0 at 256 and 254 nm, respectively; 2,2′,3,3′,4,4′-HMB and 2,4,4′,6-TMB, 12.7 at 248 nm and 10.8 at 251 nm, respectively; 2′-CH_3-TMB, 11.2 at 245 nm; 2,3,3′,4-TMB, 10.1 at 254 nm; 2,2′4,4′-TMB, 10.2 at 245 nm and 7.1 at 281 nm; and 3,3′,4,4′-TMB, 15.5 at 268 nm and 13.5 at 288 nm.

Synthesis and Characterization of the Ligands

2,3-Dimethoxy-4′-acetyl-1,1′-biphenyl (2,3-DKB) was synthesized according to a general procedure (*12*). Freshly distilled veratrole (0.1 M) in ether was treated under a N_2 atmosphere with stirring with 0.11 M butyllithium in an ice bath. After the mixture was stirred for 2 h at room temperature, the solvent was removed at low pressure and replaced by tetrahydrofuran (THF). $ZnCl_2$ (0.1 M) in ether (Aldrich) was warmed under vacuum to remove the ether, and THF was introduced instead. This solution was added to the above preparation of lithium veratrole. After the mixture was stirred for 2 h at room temperature, this was added to a three-necked flask that contained 0.1 M 4-bromoacetophenone and 1.0 g of tetrakis(triphenylphosphine)-palladium(0) in THF and the mixture left to stir at room temperature for 72 h. After workup, the material which had been extracted from the reaction mixture was distilled at ∼250 m (heating to 100 °C). The nondistillable residue was extracted by boiling with petroleum ether and chromatographed on a silica column. The material that had a λ_{max} of ≥270 nm was further purified by recrystallization from medium petroleum ether and chromatographed on silica plates. This resulted in crystalline material. Mp: 73.0−73.5 °C. MS: MW 256 (100%) (theory 256), 241 (86%). ^{1}H NMR ($CDCl_3$): δ 8.00 [d, J = 8.9 Hz, H-C(3′), H-C(5′)], 7.62 [d, J = 8.4 Hz, H-C(2′), H-C(6′)], 7.13 [t, J = 64,7.4 Hz, H-C(6)], 6.94 [d, J = 2.3 Hz, H-C(5)], 6.92 [m, H-C(4)], 3.90 (s, ring A *m*-OCH_3), 3.59 (s, ring A *o*-OCH_3), 2.63 (s, ketone CH_3). ^{13}C NMR ($CDCl_3$): δ 197.7 (C═O), 153.0 (C-1′), 146.4 (C-3), 143.0 (C-2), 135.5 (C-4′), 134.5 (C-1), 129.3 (C-3′, C-5′), 128.0 (C-6′, C-2′), 124.1 (C-5), 122.1 (C-6), 112.1 (C-4), 60.5 (ring A OCH_3), 55.8 (ring A OCH_3); 26.5(ketone CH_3). IR (CCl_4): 1682 (C═O), 1605 (aromatic), 1474 (aromatic), 1263 [C(C═O)C], 1120 (C−O), 1018 (C−O) cm^{-1}.

The following compounds were prepared by the mixed Ulman reaction, following a procedure described previously (*9*).

(1) 2,3,4,4′-Tetramethoxy-2′-methyl-1,1′-biphenyl (2′-CH_3-TMB). Starting materials were 1,2,3-trimethoxy-4-iodobenzene and 3-methyliodoanisole. Mp: 70.0 °C [lit. 69 °C (*13*)]. MS: MW 288 (100%) (theory 288), 289 (116%). ^{1}H NMR ($CDCl_3$): δ 7.13 [d, J = 8.25 Hz, H-C(6′)], 6.85 [d, J = 8.46 Hz, H-C(6)], 6.79 [d, J = 8.55 Hz, H-C(5)], 6.78 [dd, J = 8.31 Hz, J = 2.7 Hz, H-C(5′)], 6.83 [d, J = 2.7 Hz, H-C(3′)], 3.94 (ring A *m*-OCH_3), 3.91 (ring A *o*-CH_3), 3.84 (ring A *p*-OCH_3), 3.59 (ring C *o*-CH_3), 2.18 (ring C methyl). ^{13}C NMR ($CDCl_3$): δ 158.6 (C-4′), 152.7 (C-4), 151.5 (C-2), 142.1 (C-2′), 138.1 (C-1′), 131 (C-6′), 128.3 (C-1), 125.3 (C-6), 115.1 (C-3′), 110.6 (C-5′), 106.9 (C-5), 61.0 (ring A OCH_3), 60.7 (ring A OCH_3), 56.0 (ring A OCH_3), 55.1 (ring C′ OCH_3), 20.4 (ring C′ methyl). IR (CCl_4): 1599 (aromatic), 1488 (aromatic), 1460 (aromatic), 1241 (C−O), 1207 (aromatic), 1080 (C−O), 1051 (C−O), 1019 (C−O) cm^{-1}.

(2) 2,2′,4,4′-Tetramethoxy-1,1′-biphenyl (2,2′,4,4′-TMB). Starting materials were 1,3-dimethoxy-4-iodobenzene. Mp: 93.0−93.5 °C [lit. 93 °C (*14*)]. MS: MW 274 (100%) (theory, 274). ^{1}H NMR ($CDCl_3$): δ 7.21 [dm, H-C(6), H-C(6′)], 6.61 [s, H-C(3), H-C(3′)], 6.58 [m, H-C(5), H-C(5′)], 3.89 (2 *o*-OCH_3), 3.82 (2 *p*-OCH_3). ^{13}C NMR ($CDCl_3$): δ 160.0 (C-4, C-4′), 158.0 (C-2, C-2′), 131.9 (C-6, C-6′), 120.1 (C-1, C-1′), 104.1 (C-5, C-5′), 98.8 (C-3, C-3′), 55.8 (2 OCH_3), 55.3 (2 OCH_3). IR (CCl_4): 1607 (aromatic), 1578 (aromatic), 1496 (aromatic), 1465 (aromatic), 1209 (aromatic), 1035 (C−O) cm^{-1}.

(3) 3,3′,4,4′-Tetramethoxy-1,1′-biphenyl (3,3′,4,4′-TMB). The starting material was 1,2-dimethoxy-4-iodobenzene. Mp: 133.0−133.5 °C [lit. 133−134 °C (*15*)]. MS: MW 274 (theory, 274). ^{1}H NMR ($CDCl_3$): δ 7.11 [d, J = 2.1 Hz, H-C(2), H-C(2′)], 7.07 [d, J = 8.4 Hz, H-C(6), H-C(6′)], 6.94 [dd, J = 5.7 Hz, J = 2.1 Hz, H-C(5), H-C(5′)], 3.96 (s, 2 OCH_3), 3.92 (s, 2 OCH_3). ^{13}C NMR ($CDCl_3$): δ 149.0 (C-3, C-3′), 148.3 (C-4, C-4′), 134.2 (C-1, C-1′), 119.0 (C-6, C-6′), 111.4 (C-2, C-2′), 110.3 (C-5, C-5′), 55.9 (4 OCH_3). IR (CCl_4): 1603 (aromatic), 1502 (aromatic), 1250 (C−O), 1139 (aromatic), 1024 (C−O) cm^{-1}.

(4) 2,4-Dimethoxy-4′-carbomethoxy-1,1′-biphenyl (2,4-DCB). Starting materials were 1,3-dimethoxy-4-iodobenzene and methyl 4-iodobenzoate. Mp: 119.5−120.0 °C. MS: MW 272 (100%) (theory, 272), 241 (63%). ^{1}H NMR ($CDCl_3$): δ 8.04 [d, J = 8.1 Hz, H-C(3′), H-C(5′)], 7.57 [d, J = 8.1 Hz, H-C(2′), H-C(6′)], 7.25 [d, J = 8.7 Hz, H-C(6)], 6.58 [dd, J = 8.3 Hz, J = 2.4 Hz, H-C(5)], 6.56 [5, H-C(3)], 3.92 (s, ring A *o*-OCH_3), 3.84 (s, ring A *p*-OCH_3), 3.79 (s,

ester CH_3). ^{13}C NMR ($CDCl_3$): δ 167.1 (C═O), 160.9 (C-4), 157.5 (C-2), 143.2 (C-1′), 131.2 (C-6), 129.3 (C-3′, C-5′), 129.2 (C-2′, C-6′), 127.9 (C-4′), 122.3 (C-1), 104.8 (C-5), 99.0 (C-3), 55.5 (ring A OCH_3), 55.4 (ring A OCH_3), 52.0 (ester CH_3). IR (CCl_4): 1719 (C═O, ester), 1601 (aromatic), 1584 (aromatic), 1277 (C−O), 1215 (C−O), 1030 (C−O) cm^{-1}.

(5) 3,4-Dimethoxy-4′-carbomethoxy-1,1′-biphenyl (3,4-DCB). Starting materials were 1,2-dimethoxy-4-iodobenzene and methyl 4-iodobenzoate. Mp: 131.0−131.5 °C. MS: MW 272 (100%) (theory, 272). ^1H NMR ($CDCl_3$): δ 8.09 [d, J = 8.34 Hz, H-C(3′), H-C(5′)], 7.62 [d, J = 8.4 Hz, H-C(2′), H-C(6′)], 7.20 [dd, J = 8.3 Hz, J = 2.0 Hz, H-C(6)], 7.14 [d, J = 2.0 Hz, H-C(2)], 6.98 [d, J = 8.3 Hz, H-C(5)], 3.97 (s, ester CH_3), 3.94 (ring A OCH_3), 3.93 (ring A OCH_3). ^{13}C NMR($CDCl_3$): δ 167.0 (C═O), 149.3 (C-1′), 149.2 (C-3), 145.4 (C-4), 132.8 (C-1), 130.0 (C-3′, C-5′), 128.4 (C-4′), 126.6 (C-2′, C-6′), 119.7 (C-6), 111.4 (C-5), 110.3 (C-2), 56.0 (2 OCH_3), 52.1 (ester CH_3). IR (CCl_4): 1718 (C═O, ester), 1600 (aromatic), 1590 (aromatic), 1272 (C−O), 1145 (aromatic), 1025 (C−O) cm^{-1}.

The following ligands were prepared by cross coupling of Grignard reagents with aryl halides (*16*).

(1) 2,4,4′-Trimethoxy-1,1′-biphenyl (2,4,4′-TMB). A Grignard reagent prepared from 0.134 M 4-bromoanisole in 100 mL of THF was added dropwise with stirring at room temperature to 0.143 M 1,3-dimethoxy-4-bromobenzene that contained 0.002 M dichloro-1,3-bis(diphenylphosphino)-propane nickel(II) (*16*). After the mixture was heated under reflux overnight, the reaction was worked up. The material which was obtained by extraction with an organic solvent, such as petroleum ether, C_6H_6, or CH_2Cl_2, was subjected to distillation. The nondistillable residue was chromatographed on silica plates in CH_2Cl_2. After repeated recrystallizations from hexane, a crystalline material was obtained. Mp: 64.0−64.5 °C. MS: MW 244 (100%) (theory, 244), 229 (74%). ^1H NMR ($CDCl_3$): δ 7.49 [d, J = 8.6 Hz, H-C(2′), H-C(6′)], 7.26 [d, J = 9.0 Hz, H-C(6)], 6.99 [d, J = 4.38 Hz, H-C(3′), H-C(5′)], 6.55 [m, H-C(3), H-C(5)], 3.89(s, OCH_3), 3.88 (OCH_3), 3.84 (OCH_3). ^{13}C NMR ($CDCl_3$): δ 159.9 (C-4), 158.3 (C-2), 157.3 (C-4′), 130.9 (C-6), 130.7 (C-1′), 130.4 (C-2′, C-6′), 123.2 (C-1), 113.5 (C-5), 99.0 (C-3), 55.4 (ring A OCH_3), 55.3 (ring A OCH_3), 55.2 (ring C′ OCH_3). IR (CCl_4): 1606 (aromatic), 1498 (aromatic), 1302 (C−O), 1246 (C−O), 1207 (aromatic), 1031 (C−O).

(2) 2,3,4′-Trimethoxy-1,1′-biphenyl (2,3,4′-TMB). The same procedure that was used for 2,4,4′-TMB was used for this compound, but the second component was 1,2-dimethoxy-3-iodoveratrole. Mp: 63.0−63.5 °C. MS: MW 244 (100%) (theory, 244), 229 (74%). ^1H NMR ($CDCl_3$): δ 6.92 [dd, J = 8.1 Hz, J = 1.6 Hz, H-C(4)], 7.13 [t, J = 7.9 Hz, H-C(5′)], 6.98 [dd, J = 9.3 Hz, J = 1.6 Hz, H-C(6)], 7.55 [dd, J = 6.8 Hz, J = 2.2 Hz, H-C(2′), H-C(6′)], 7.00 [dd, J = 6.9 Hz, J = 2.1 Hz, H-C(3′), H-C(5′)], 3.93 (ring A m-OCH_3), 3.88 (ring A o-OCH_3), 3.62 (ring C OCH_3). ^{13}C NMR ($CDCl_3$): δ 158.7 (C-4′), 153.1 (C-3), 146.4 (C-2), 135 (C-1′), 130.5 (C-1), 130.3 (C-2, C-6′), 123.9 (C-6), 122.4 (C-5), 113.5 (C-3′, C-5′), 111.0 (C-4), 66.3 (ring A OCH_3), 55.8 (ring A OCH_3), 55.1 (ring C′ OCH_3). IR (CCl_4): 1608 (aromatic), 1473 (aromatic), 1265 (C−O), 1246 (C−O), 1023 (C−O) cm^{-1}.

(3) 2,3,3′,4-Tetramethoxy-1,1′-biphenyl (2,3,3′,4-TMB). The same procedure that was used for 2,4,4′-TMB was used for this compound, but the starting materials were a Gringard from 3-bromoanisole and 1,2,3-trimethoxy-4-bromobenzene. Recrystallization from low petroleum ether gave needles. Mp: 42.3 °C [Itoh et al. (*13*) reported an oil]. MS: MW 274 (100%) (theory, 274). ^1H NMR ($CDCl_3$): δ 7.31 [t, J = 7.5 Hz, H-C(5′)], 7.20 [s, H-C(2′)], 7.08 [dm, H-C(6′)], 7.04 [d, J = 8.6 Hz, H-C(6)], 6.86 [dm, H-C(4′)], 6.73 [d, J = 8.6 Hz, H-C(5)], 3.93 (ring A m-OCH_3), 3.89 (ring A o-OCH_3), 3.83 (ring A p-OCH_3), 3.68 (ring C OCH_3). ^{13}C NMR ($CDCl_3$): 159.3 (C-3′), 153.1 (C-1), 151.3 (C-4), 142.4 (C-2), 139.6 (C-1′), 129.0 (C-5′), 128.5 (C-3), 124.7 (C-6), 121.6 (C9-6′), 114.7 (C-4′), 112.3 (C-2′), 107.4 (C-5), 61.0 (ring A OCH_3), 60.9 (ring A OCH_3), 56.0 (ring A OCH_3), 55.2 (ring C′ OCH_3). IR (CCl_4): 1599 (aromatic), 1483 (aromatic), 1231 (C−O), 1089 (C−O) cm^{-1}.

(2′,4′- and (3′,4′-dimethoxyphenyl)-2-methoxycyclohepta-2,4,6-trien-1-one (2,4-MDC and 3,4-MDC) were prepared (17) by coupling the respective aryl methylstannanes with 5-bromotropolone, using dichlorobis(triphenylphosphine) palladium(II) as a catalyst. 2,4-MDC mp: 137.0−137.5 °C. 2,4-MDC MS: MW 272 (theory, 272). 3,4-MDC mp: 166.0 °C. 3,4-MDC MS: MW 272 (theory, 272).

Tubulin Preparation

Tubulin was purified from fresh calf brains, and concentrated solutions (0.5−1 mM tubulin) were stored in liquid nitrogen as described previously (*7*, *18*−*20*). Its concentration was determined spectrophotometrically in dilute neutral solutions in phosphate buffers (2−20 μM tubulin, 0.1−1 cm cells) after correction for light scattering, employing an extinction coefficient of 1.16 L g^{-1} cm^{-1} at 278 nm (*21*).

Measurements of Ligand Binding to Tubulin

All binding measurements were carried out spectrofluorometrically in 10 mM sodium phosphate buffer and 10 μM GTP at pH 7.0 (PG buffer) and 25 °C, employing a Shimadzu RF 540 fluorometer in the high sensitivity ratio mode with 5 mm (excitation) × 10 mm (emission) cells. The instrument stability was routinely checked with rhodamine cells. The arbitrary fluorescence intensity units given are normalized to the ×128 scale of the instrument. The excitation (2 nm slit) and emission (5 nm slit) wavelength pairs employed were the following: MTC, 345 nm (except where indicated as 380 nm) and 425 nm; 2,4-MDC, 345 and 430 nm; 3,4 MDC, 345 and 440 nm; TKB, 315 and 425 nm; TCB, 315 and 380 nm; and 2,4-DCB and 3,4-DCB, 315 and 390 nm. The three different methods employed are as follows.

Method 1 (*20*) was applied to those ligands which become fluorescent upon binding to tubulin and are soluble in PG buffer. Any fluorescence coming from the free ligand or protein was subtracted from the data, which were corrected by small dilution factors. The relative fluorescence intensity of bound ligand was determined by titrating with tubulin until saturation ligand solutions that gave no appreciable inner filter effect. The values obtained were essentially coincident for MTC, 2,4-MDC, and 3,4-MDC [7.1 ± 0.1 (4.2 ± 0.1 excitation at 380 nm), 6.9 ± 0.2, and 7.3 ± 0.2 arbitrary fluorescence units per micromolar bound ligand, respec-

tively]; tubulin-bound TKB gave a value of 2.1 ± 0.2 units μM^{-1}, and 2,3-DKB−tubulin solutions gave a very weak fluorescence. Tubulin solutions (concentration $[P]_0$, M_r = 100 000) were then titrated with known total concentrations of ligand ($[A]_0$). The inner filter effect was corrected by employing the equation

$$\ln F_{obs} = \ln F_{corr} - \epsilon L[A]_0 \quad (1)$$

where F_{obs} and F_{corr} are the observed and corrected fluorescence emission intensities, respectively. The product of the ligand extinction coefficient, ϵ, and the effective optical path, L, was determined by linear least-squares regression of ln F_{obs} versus $[A]_0$ in the binding saturation region (*22*). The concentration of bound ligand ($[A]_b$) was measured by its corrected fluorescence and the free ligand concentration ([A]) as the difference compared to the total ligand concentration. The values of the binding equilibrium constant, K, and the number of sites, n, were obtained by direct nonlinear least-squares fitting of the data to the equilibrium binding equation for independent sites

$$[A]_b/[P]_0 = nK[A]/(1 + K[A]) \quad (2)$$

employing a program based on the modified simplex algorithm (*23*), kindly provided by A. P. Minton (NIH, Bethesda, MD). The values of n determined for MTC, 2,4-MDC, 3,4-MDC, and TKB were 0.79 ± 0.07, 0.93 ± 0.06, 0.81 ± 0.07, and 0.75 ± 0.07, respectively, i.e., close to one binding site. Small errors in stoichiometry were found not to modify significantly the values of the standard free energy of binding $\Delta G°_{app} = -RT \ln K$. To facilitate comparison, the data are presented as the fractional saturation of binding $\alpha(A) = [A]_b/(n[P]_o)$.

Method 2 was employed for ligands which become fluorescent upon binding to tubulin, but whose poor solubility precludes the attainment of saturation of the protein binding sites. Unitary stoichiometry was assumed for the binding of TCB [n = 0.92 ± 0.09 (9)] and its analogues 2,4-DCB and 3,4-DCB. If the fraction of maximal fluorescence observed, F/F_{max}, is regarded to be identical to the fractional saturation of binding, the following equations hold for the titration of ligand with protein

$$F/F_{max} = K[P]/(1 + K[P]) \quad (3)$$

$$[P] = [P]_0 - F[A]_0/F_{max} \quad (4)$$

and for the titration of protein with ligand

$$F/F'_{max} = K[A]/(1 + K[A]) \quad (5)$$

$$[A] = [A]_0 - F[P]_0/F'_{max} \quad (6)$$

Each system of equations was solved iteratively by direct nonlinear least-squares fitting of the fluorescence versus total concentration data, seeking the best fit values of F_{max} and K, employing a Marquadt algorithm (Sigmaplot, Jandel Scientific). Titration of ligand with protein allows us to estimate the fluorescence intensity of bound ligand, F_{max}, and a value of K. Titration of protein with ligand should yield F'_{max} and a similar value of K. Since the TCB, 2,4-DCB, and 3,4-DCB data were in the low saturation region (nearly linear F vs $[A]_o$ plot) and not very accurate, it was necessary to constrain the value of F'_{max} (according to $F'_{max}/[P]_0 = F_{max}/[A]_0$), which yielded the approximate value of the equilibrium association constant K. As a test of this method, it was applied to MTC binding data, giving K values identical to those obtained with method 1.

Method 3 was applied to the binding of the nonfluorescent methoxy biphenyls TMB, 2,4,4′-TMB, 2,3,4′-TMB, etc. The fractional saturation of binding sites by a reference fluorescent ligand A of known equilibrium binding constant K(A) was measured as a function of the total concentration of competing ligand $[B]_0$, and a system of equations holding for the binding of A and B to the same site was iteratively applied to search for the value of K(B) that renders a minimal sum of least-squares deviations of the experimental values from the theoretical values of α(A) (*10*). The reference ligand employed was MTC (ref *20* and this work), which was excited at 380 nm. The method was also applied to the bindings of TKB, 2,3-DKB, TCB, 2,4-DCB, and 3,4-DCB, since their fluorescence upon excitation at 380 nm was negligible.

Other Procedures

The ligand-induced GTPase activity of tubulin was assayed by measuring the hydrolysis of [γ-^{32}P]GTP in 10 mM sodium phoshate buffer (pH 7.0), 4 mM $MgCl_2$, and 0.1 mM GTP (*7*). Self-assembly of tubulin into microtubules was monitored turbidimetrically as described elsewhere (*3*, *24*, *25*). The equilibrium binding constant (K_i) for the binding of the tubulin−drug complex to the end of a growing microtubule was calculated from the assembly inhibition data by solving eq 7 as described previously (*3*):

$$\text{fraction} = \frac{1}{1 + K_b K_i K_g^{-1}[A]} - \frac{K_b K_g^{-1}[A]}{(T_{total} - Cr)(1 + K_b K_i K_g^{-1}[A])} \quad (7)$$

In this equation, fraction is the ratio of the turbidity in the presence of the drug to that in its absence at identical tubulin concentrations, K_g is the normal microtubule growth constant, equal to Cr^{-1} (Cr is the critical concentration for assembly in the absence of drugs), K_b is the binding constant for the binding of drug to tubulin, K_i is the microtubule inhibition constant, which is the binding constant for the binding of the tubulin−drug complex to the end of a growing microtubule, T_{total} is the total tubulin concentration, and A is free drug. In the limiting case where the tubulin−drug complex does not bind to microtubules, $K_i = 0$ (*3*), eq 7 reduces to

$$\text{fraction} = 1 - \frac{K_b K_g^{-1}[A]}{T_{total} - Cr} \quad (8)$$

RESULTS

Binding of Des-2-MeO and Des-3-MeO Ring A Analogues to Tubulin. The interactions of MTC analogues from which the methoxy groups at positions 2 or 3 had been excised were examined fluorometrically in a rigorous comparison to their parent compound. The latter had been shown to bind specifically to the COL binding site (*20*, *26*). Panels B and

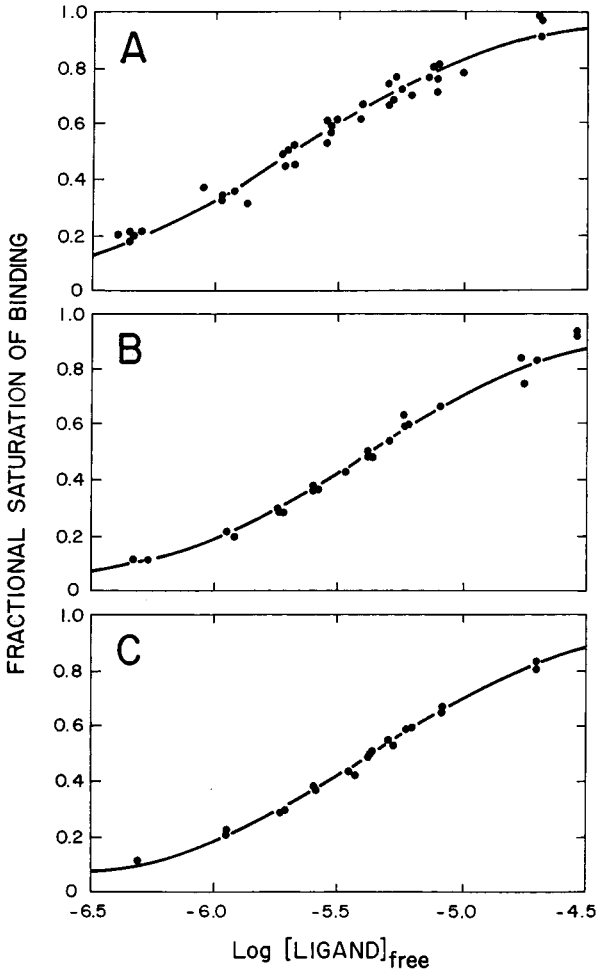
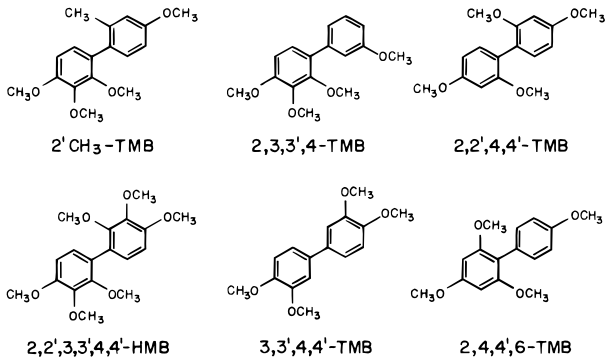


FIGURE 1: (A) Binding isotherm for binding of MTC to tubulin in PG buffer at pH 7.0 and 25 °C, determined fluorometrically (method 1, Materials and Methods). The results of five different experiments (protein concentrations of 5.9−6.7 μM) are shown. The solid line is the least-squares fit for an equilibrium binding constant K_b of $(4.7 \pm 0.6) \times 10^5$ M^{-1}. (B) Binding of 2,4-MDC under identical conditions (three experiments at tubulin concentrations of 6.9−7.1 μM). The line corresponds to a binding constant K_b of $(2.1 \pm 0.3) \times 10^5$ M^{-1}. (C) Binding of 3,4-MDC under the same conditions (two experiments at 7.1 and 7.4 μM tubulin). The line corresponds to a K_b of $(2.4 \pm 0.3) \times 10^5$ M^{-1}.

Chart 2



C of Figure 1 show the binding isotherms of 2,4-MDC and 3,4-MDC, respectively, in comparison to that of MTC (panel A) (for structures, see Charts 1 and 2). The deduced binding constants, K_b, are listed in Table 1. The results show that removal of methoxy groups at positions 2 or 3 of ring A does not abolish binding but lowers the equilibrium association constant by a factor of approximately 2; i.e., the apparent standard free energy change of association is weakened by nearly 0.5 kcal mol^{-1} in both cases.

Should the above results reflect weak interactions of the pertinent methoxy groups with the trimethoxybenzene binding subsite, they ought to be independent of the nature of ring C. This was tested by binding measurements of the corresponding analogues of TCB, which differed from those of MTC in the replacement of the tropolone methyl ether (ring C) by *p*-carbomethoxybenzene (ring C′). The results depicted in Figure 2 are an indication of the validity of this prediction, even though the poor solubility of 2,4-DCB and 3,4-DCB permitted only an approximate determination of their binding constants (see Table 1). For these analogues, binding was measured by two techniques (ligand fluorescence and competition with the binding of MTC). Figure 2A shows partial fluorometric titrations with protein at low ligand concentrations. The ligand fluorescence detected under these conditions was shown to be due specifically to binding to the COL site, since it was practically abolished both by the addition of an excess of podophyllotoxin to the tubulin−DCB solutions and by the substitution of the stable tubulin−COL complex for tubulin (data not shown). Figure 2B shows experiments in which the competition with MTC for the binding to tubulin was examined to the practical solubility limit of each compound, and the results were fitted to the pertinent equations (*10*) with the association binding constant of the ligand set as the adjustable parameter. The results in Table 1 show that the binding free energy increments for the two types of measurements are close to identical. Furthermore, both values of $\delta\Delta G°$ are similar to those obtained with the MTC analogues. This was further confirmed by binding measurements of the des-3-MeO analogue of TMB, with the use of the competition with MTC method. In Figure 3A, comparison of the isotherm of 2,4,4′-TMB (curve c) with that of TMB (curve a) shows a weakening of the binding by ca. 0.4 kcal mol^{-1} (Table 1). It is possible to conclude, therefore, that removal of either the 2- or the 3-methoxy group weakens binding by about 0.5 kcal mol^{-1}.

Binding of Des-4-MeO Ring A Analogues to Tubulin. To probe the role of the methoxy group at position 4 of ring A, the bindings of analogues 2,3-DKB and 2,3,4′-TMB were compared to those of TKB and TMB, respectively, with the assumption that the specificity of these compounds in their competition with MTC for binding to the COL binding site (*10*) is maintained in the des-4-methoxy compounds. The low affinity and/or low fluorescence of 2,3-DKB, when compared to TKB, precluded direct binding titration by ligand fluorescence. Figure 3B shows the equilibrium competition isotherms of TKB (line a) and 2,3-DKB (line b) for the binding of MTC to tubulin. The results (Table 1) indicate that excision of the 4-methoxy group of ring A in the ring C′ ketone lowers markedly the equilibrium association constant, roughly by a factor of 10, which reflects a binding weaker by ca. 1.5 kcal mol^{-1}. This result was confirmed by comparison of 2,3,4′-TMB with TMB. The competition isotherms are shown in Figure 3A (b and a). Their analysis gave a $\delta\Delta G°$ value of ca. 1.2 kcal mol^{-1} (Table 1), consistent with the value obtained with the TKB compounds.

Table 1: Binding to Tubulin of COL Analogues with Modified Rings A

ligand	K_b x 10^5 (M^{-1}) (ligand fluo)	$\Delta G°_{app}$ (kcal mol^{-1})	K_b x 10^5 (M^{-1}) (compet)[b]	$\Delta G°_{app}$ (kcal mol^{-1})	$\delta\Delta G°_{app}$ (kcal mol^{-1})[c]
MTC[a]	4.7 ± 0.6	−7.74 ± 0.08			0
2,4-MDC	2.1 ± 0.3	−7.26 ± 0.08			0.48 ± 0.08
3,4-MDC	2.4 ± 0.3	−7.34 ± 0.08			0.40 ± 0.08
TCB[a]	0.84[e]	−6.72[e]	1.5 ± 0.2	−7.06 ± 0.08	0
2,4-DCB[d]	0.35[e]	−6.20[e]	0.51[e]	−6.42[e]	0.58[e]
3,4-DCB[d]	0.26[e]	−6.02[e]	0.39[e]	−6.26[e]	0.75[e]
TKB[a]	2.5 ± 0.6	−7.36 ± 0.15	3.9 ± 0.4	−7.63 ± 0.06	0
2,3-DKB			0.28 ± 0.03	−6.07 ± 0.06	1.56 ± 0.06
TMB[a]			1.5 ± 0.4	−7.06 ± 0.16	0
2,3,4′-TMB			0.21 ± 0.08	−5.90 ± 0.25	1.16 ± 0.21
2,4,4′-TMB			0.82 ± 0.20	−6.70 ± 0.15	0.36 ± 0.18

[a] Previously determined $\Delta G°_{app}$ (kilocalories per mole) values for each of these ligands were as follows: MTC, −7.72 (*20*); TKB, −7.03 (ligand fluorescence) and −7.33 (competition with MTC) (*10*); TCB, −6.90 (*9*); and TMB, −6.98 (protein fluorescence) and −6.55 (competition with MTC) (*10*). [b] The competition assay (method 3, Materials and Methods) shows a trend to give $\Delta G°$ values more negative than the measurement from the bound ligand fluorescence (method 1). [c] The $\delta\Delta G°$ value for each analogue is defined as the difference from each head of a series so that possible systematic errors tend to be canceled. [d] Measurements of the binding of these ligands (employing methods 2 and 3, Materials and Methods) are only approximate, due to their low solubility. [e] Approximate values.

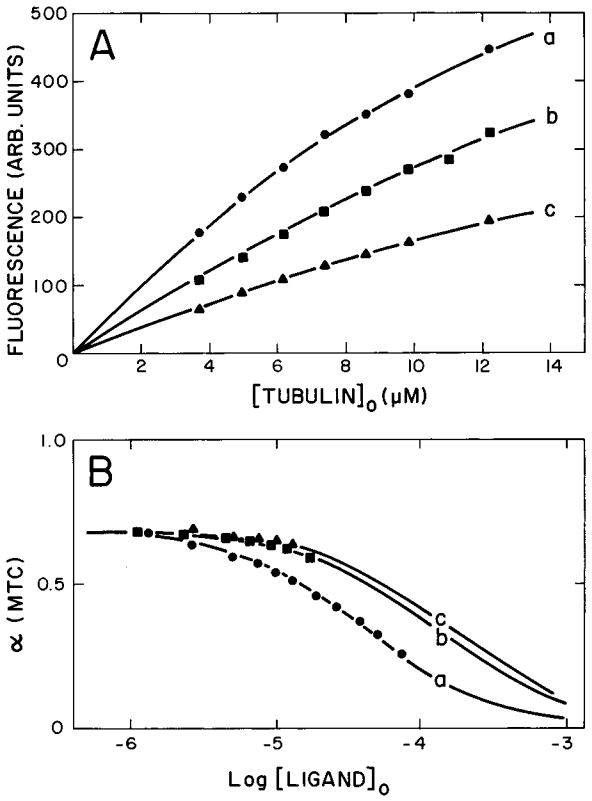


FIGURE 2: (A) Ligand fluorescence increase due to the interactions of tubulin with TCB (●, line a), 2,4-DCB (■, line b), and 3,4-DCB (▲, line c) in PG buffer at 25 °C. The concentration of each ligand was 4 μM. The lines shown correspond to best least-squares fits for 1/1 interactions (method 2, Materials and Methods), giving for this experiment the following approximate parameters: TCB, $K_b = (6.0 \pm 0.3) \times 10^4$ M^{-1} and $F_{max} = 1200 \pm 40$ (arbitrary fluorescence units); 2,4-DCB, $K_b = (1.7 \pm 0.5) \times 10^4$ M^{-1} and $F_{max} = 2000 \pm 500$; and 3,4-DCB, $K_b = (1.6 \pm 0.2) \times 10^4$ M^{-1} and $F_{max} = 1250 \pm 130$. (B) Competition of TCB (●, a), 2,4-DCB (■, b), and 3,4-DCB (▲, c) for the binding of the 10 μM reference ligand MTC to 8 μM tubulin. The lines correspond to least-squares fits assuming competition for the same site and unitary stoichiometry (method 3, Materials and Methods). The fitted equilibrium association constants were 1.5×10^5, 5.1×10^4, and 3.9×10^4 M^{-1}, respectively.

Binding of TMB Analogues with Modified Rings A and C′ to Tubulin. To explore further the roles in binding to tubulin of both rings A and C′, as well as that of the intramolecular rotation about the biaryl bond, analogues of TMB in which additional groups were introduced (Chart 2) or shifted in position were examined and compared to their parent compound. The method used in all cases was competition with MTC. The experimental results are shown in Figure 3C, and the corresponding binding parameters (K_b and $\Delta G°_{app}$) are listed in Table 2. In all cases, binding was weakened, which indicates that none of these additions or modifications improves the global interaction of TMB with the COL binding site. Introduction of a methyl group at position 2′ had little effect (possibly a slight weakening), while the larger methoxy group weakened binding by 1.1 kcal mol^{-1} (comparison of 2,2′,4,4′-TMB with 2,4,4′-TMB). This could be due either to steric interference with fitting into the COL binding site or to inhibition of biaryl rotation. Both of these results are in conflict with the conclusion of Itoh et al. (*13*) that both of these compounds were equipotent with COL in the inhibition of microtubule assembly. Shifting of the ring C′ methoxy to position 3′ weakened binding by 1.0 kcal mol^{-1}, while introducing it into TMB (3,3′,4,4′-TMB) weakened binding by ∼2.5 kcal mol^{-1}. Finally, the presence of methoxyls in both ortho positions of ring A 2,4,4′,6-TMB weakened binding by more than 2.7 kcal mol^{-1}. This last result suggests the need of free rotation about the biaryl bond or attainment of coplanarity for strong binding to tubulin.

Inhibition of Tubulin Self-Assembly by Colchicine Analogues with Modified Rings A and/or C′. The effects of the several analogues of MTC, TKB, and TMB on the in vitro microtubule assembly were also analyzed. Both analogues of MTC inhibited microtubule assembly with similar efficacies. The turbidity generated by the self-assembly of 2.1×10^{-5} M tubulin was reduced by 50% by 3.9×10^{-6} M 2,4-MDC (Figure 4A). The concentration of 3,4-MDC necessary to reduce by 50% the turbidity generated by the self-assembly of 2.5×10^{-5} M tubulin was 4.26×10^{-6} M (Figure 4B). A comparison of the total ligand/tubulin ratio that induced 50% inhibition, shown in Figure 5, gave ratios of 0.19 for 2,4 MDC and 0.17 for 3,4-MDC. Therefore, both analogues are weaker inhibitors than MTC, which induced a 50% inhibition at a total ligand/tubulin ratio of 0.07 (*3*). Inhibition of tubulin self-assembly by the TKB analogue in which the 4-methoxy group of ring A had been excised (2,3-DKB) is

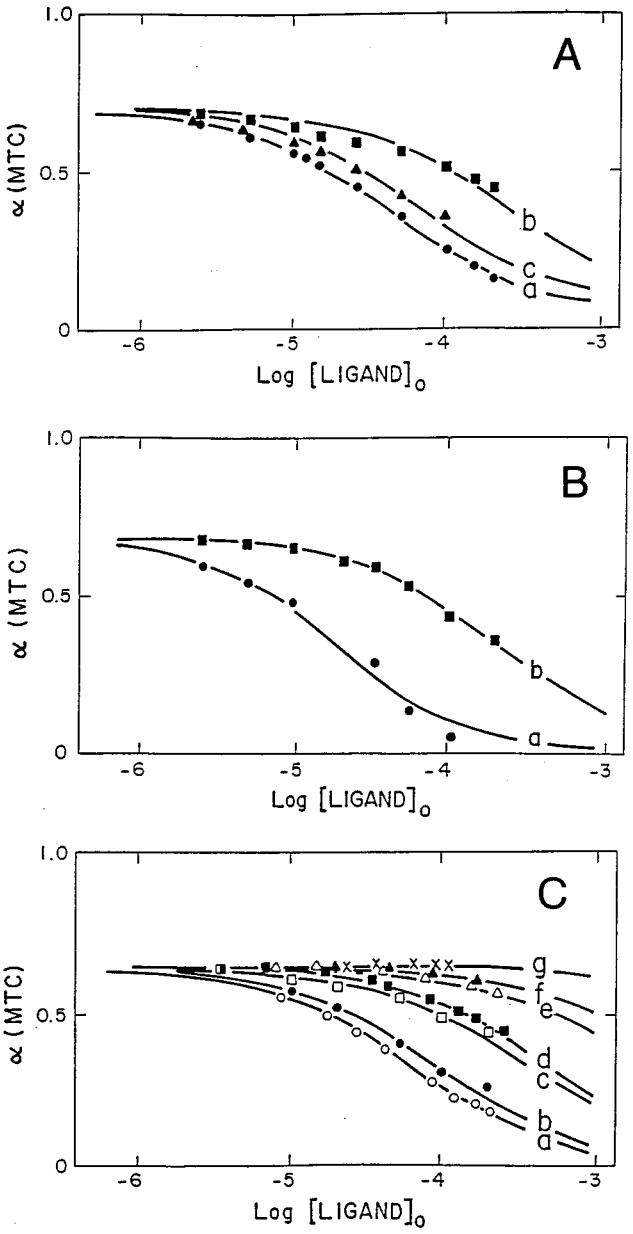


FIGURE 3: (A) Competition isotherms of TMB (●, a), 2,3,4′-TMB (■, b), and 2,4,4′-TMB (▲riangles, c) for the binding of 10 μM reference ligand MTC to 8 μM tubulin. The lines correspond to least-squares fits assuming competition for the same site and unitary stoichiometry (method 3, Materials and Methods). The fitted equilibrium binding constants were 1.5×10^5, 8.2×10^4, and 2.1×10^4 M^{-1}, respectively. (B) Competition isotherms of TKB (●) and 2,3-DKB (■) for the binding of 10 μM reference ligand MTC to 8 μM tubulin. Lines a and b correspond to least-squares fits, assuming competition for the same site and unitary stoichiometry (method 3, Materials and Methods). The fitted equilibrium association constants were 3.9×10^5 and 2.8×10^4 M^{-1}, respectively. (C) Competition isotherms of TMB (○, a), 2-CH_3-TMB (●, b), 2,3,3′,4-TMB (□, c), 2,2′,4,4′-TMB (■, d), 2,2′,3,3′,4,4′-HMB (△, e), 3,3′,4,4′-TMB (▲, f), and 2,4,4′,6-TMB (×, g) for the binding of 10 μM reference ligand MTC to 10 μM tubulin. The lines correspond to least-squares fits to the data (method 3, Materials and Methods). The fitted values of the equilibrium binding constants in this experiment were 1.0×10^5, 0.7×10^5, 0.17×10^5, 0.12×10^5, 0.03×10^5, $\sim 0.015 \times 10^5$, and $< 0.01 \times 10^5$ M^{-1}, respectively.

shown in Figure 4C. The concentration of drug needed to reduce the turbidity of 2.2×10^{-5} M tubulin by 50% was 1.98×10^{-5} M. As shown in Figure 5, the corresponding value of the total ligand/tubulin ratio (ca. 0.9) is considerably higher than that of 0.031 found for the parent compound TKB (*3*).

Table 2: Effects of TMB Structural Modifications on the Binding to Tubulin[a]

ligand	$K_b \times 10^5$ (M^{-1})	$\Delta G°_{app}$ (kcal mol^{-1})	$\delta\Delta G°_{app}$ (kcal mol^{-1})
TMB	1.01	−6.8	0
2′-CH_3-TMB	0.70	−6.6	0.2
2,3,3′,4-TMB	0.17	−5.8	1.0
2,2′,4,4′-TMB	0.12	−5.6	1.2
2,2′,3,3′,4,4′-HMB	0.03[b]	−4.7[b]	2.1[b]
3,3′,4,4′-TMB	0.015[b]	−4.3[b]	2.5[b]
2,4,4′,6-TMB	≤0.01	≥−4.1	≥2.7

[a] This is a series of experiments different from those in Table 1, hence the slightly different value of the binding constant of TMB. [b] Approximate values.

The analogues of TMB with one ring A methoxy excised were also tested as microtubule inhibitors. Figure 6A shows the results obtained with 2,3,4′-TMB where the position 4 methoxy of ring A was replaced by a hydrogen (see Chart 1). This compound is a very weak microtubule inhibitor, since 50% reduction of turbidity of 2.2×10^{-5} M tubulin required 3.7×10^{-5} M drug, which corresponds to a total ligand/tubulin ratio of 1.68 (Figure 5). However, the TMB analogue where the position 3 methoxy group of ring A was replaced by a hydrogen, namely, 2,4,4′-TMB, was close to the parent compound, TMB, in inhibiting efficacy. As seen in Figure 6B, the concentration of drug necessary to reduce by 50% the turbidity of 2.2×10^{-5} M tubulin was 1.23×10^{-5} M, which gives a total drug/tubulin ratio of 0.56, as shown in Figure 5, while that of the parent compound, TMB, was ca. 0.38 (*3*).

The microtubule inhibiting capacities of the analogues of TMB with modifications on ring C′ were also scrutinized. Panels C and D of Figure 6 show the results obtained with 2′-CH_3-TMB and 2,3,3′,4-TMB, respectively (for structures, see Chart 2). The turbidity generated by the assembly of 2.1×10^{-5} and 2.2×10^{-5} M tubulin was inhibited 50% by 8.1×10^{-6} and 2.3×10^{-5} M 2′-CH_3-TMB and 2,3,3′,4-TMB, respectively, which correspond to total drug/tubulin ratios of 0.39 and 1.1, respectively. It is clear that introduction of a methyl group in position 2′ did not affect inhibiting efficacy significantly. The analogues of TCB that lack the methoxy group in ring A at position 3 (2,4-DCB) or 2 (3,4-DCB) did not inhibit tubulin self-assembly significantly within their solubility limits (approximately 1.6×10^{-5} M).

Since the various ligands tested have different equilibrium binding constants for binding to the $\alpha\beta$-tubulin heterodimer, a rigorous analysis of the inhibition potency of each drug was carried out using the linked equilibrium analysis described previously (*3*). This procedure allows us to extract the binding constant, K_i, of the tubulin−drug complex for binding to the end of a growing microtubule. This binding stops additional polymer growth (*2*). The isotherms presented in Figure 7 show that the inhibitions by the various ring A-modified biphenyls could be expressed by the linked equilibrium model described by eq 7. The results of this analysis are given in Table 3. The application of this rigorous criterion clearly shows that excision of a methoxy group from ring A weakens inhibition of tubulin self-assembly relative to the those of parent compounds, MTC,

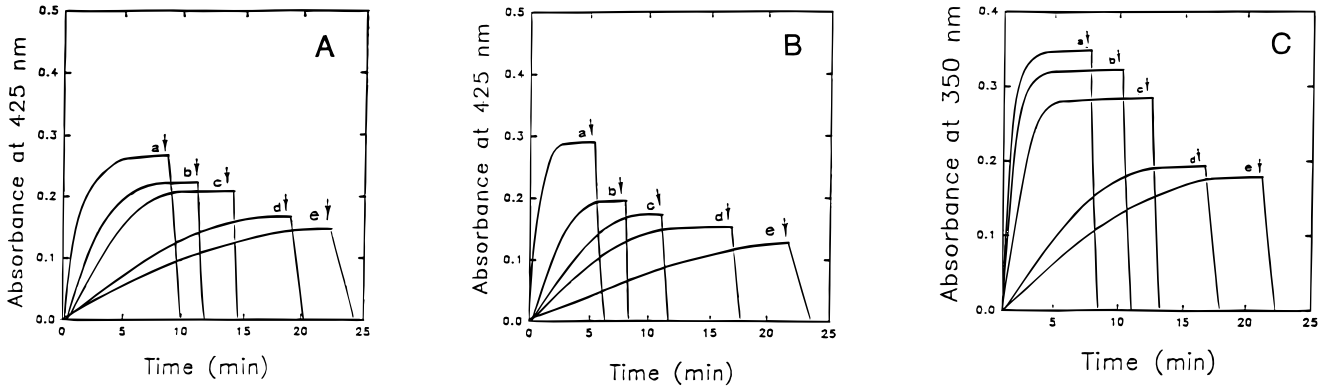


FIGURE 4: Effect of ring A-modified MTC analogues on the turbidity time course of the in vitro microtubule assembly: (A) (a) 2.1×10^{-5} M tubulin and (b−e) same as panel a with 6.72×10^{-7}, 1.58×10^{-6}, 2.87×10^{-6}, and 4.58×10^{-6} M 2,4-MDC, respectively; and (B) (a) 2.5×10^{-5} M tubulin and (b−e) same as panel a with 1.98×10^{-6}, 2.55×10^{-6}, 4.32×10^{-6}, and 5.1×10^{-6} M 3,4-MDC, respectively. (C) Inhibition of tubulin self-assembly by 2,3-DKB: (a) 2.2×10^{-5} M tubulin and (b−e) same as panel a with 1.73×10^{-6}, 6.87×10^{-6}, 1.37×10^{-5}, and 2.5×10^{-5} M 2,3-DKB, respectively. Tubulin was preincubated with the respective ligand at 20 °C for 30 min before initiation of the self-assembly reaction. Assembly was carried out by heating the samples from 10 to 37 °C in the assembly buffer [0.01 M sodium phosphate buffer (pH 7.0), 16 mM $MgCl_2$, 3.4 M glycerol, and 1 mM GTP]. The arrows indicate cooling of the samples to 10 °C. All assemblies reversed fully.

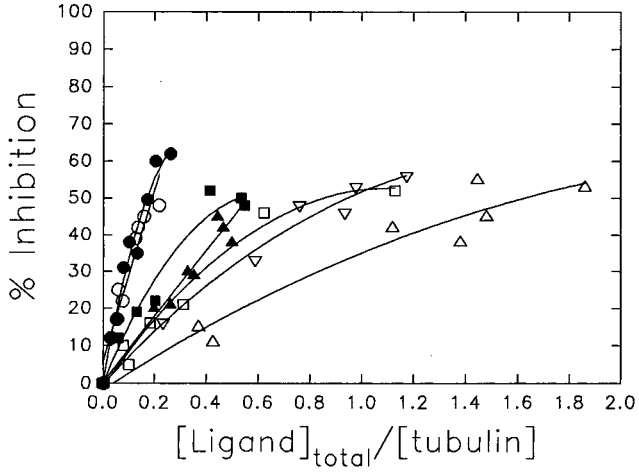


FIGURE 5: Fraction of the reduction of the plateau absorbance values (inhibition) as a function of the ratio of total ligand concentation to total protein concentration: 2,4-MDC (○), 3,4-MDC (●), 2,3-DKB (□), 2,3,4′-TMB (△), 2,4,4′-TMB (▲), 2,3,3′,4-TMB (▽), and 2′-CH_3-TMB (■).

TKB, and TMB. Removal of the ring A methoxy groups in position 3 (2,4-MDC) and position 2 (3,4-MDC) weakened the standard free energy of inhibition by almost identical amounts (0.63 and 0.58 kcal mol^{-1}, respectively). Removal of the ring A methoxyl in position 4 (2,3-DKB) had the greatest effect. The standard free energy of inhibition was weakened by 2.1 kcal mol^{-1} relative to that of the parent compound TKB. This was confirmed by the result obtained with the parallel analogue of TMB, 2,3,4′-TMB, for which K_i was not measurable, which indicates that there is essentially no binding of the tubulin−2,3,4′-TMB complex to the end of a microtubule. Yet, the binding affinities of the two analogues, 2,3-DKB and 2,3,4′-TMB, for binding to tubulin differ by only ∼0.17 kcal mol^{-1} (Table 3). Elimination of the TMB methoxy in position 3 of ring A, namely, 2,4,4′-TMB, weakened the standard free energy of inhibition by 1.03 kcal mol^{-1} M, a value somewhat higher than that obtained with 2,4-MDC, which lacks the same methoxy group (Table 3).

The role in microtubule inhibition of both rings (A and C′) and that of the intramolecular rotation around the biaryl bond were further explored using two analogues of TMB, one that contained a methyl group in position 2′ on ring C′ (2′-CH_3-TMB) and the other in which the methoxy group on ring C′ was in position 3′ (2,3,3′,4-TMB) (see Chart 2). With these analogues, the strength of inhibition was similar to that of TMB, as seen in Table 3.

An alternate way of comparing the microtubule assembly inhibiting capacity of a series of ligands is through the calculation of the extent of liganding of tubulin at 50% inhibition of self-assembly. This is expressed by the parameter *r* in column 7 of Table 3, where *r* is the percentage of tubulin liganded at 50% inhibition. These stoichiometry values were calculated from the binding constant, K_b, with the restriction that the equilibrium concentration of unliganded unassembled tubulin be equal to the critical concentration for assembly (*3*). An examination of Table 3 reveals that there is no correlation of the parameter *r* with the binding constant, K_b, and a good correlation with the inhibition constant, K_i, as had been found previously with other COL analogues (*3*). By this criterion, it is also clear that the desmethoxy COL analogues are weaker microtubule inhibitors. Thus, when the methoxy group at position 2 (3,4-MDC) is removed, the ratio is such that for every molecule of drug bound, 7.3 remain free. When the methoxy at position 3 is removed, as in compounds 2,4-MDC and 2,4,4′-TMB, the ratio of unliganded to liganded tubulins at 50% inhibition is ca. 6.7 and 3.0, respectively. In the case of the des-4-methoxy compounds, the ratio of unliganded to liganded tubulin is ca. 4 with 2,3-DKB and 0.4 with the TMB analogue 2,3,4′-TMB (Table 3).[2]

Ligand-Induced GTPase. One of the consequences of the liganding of tubulin to COL or its structural analogues is the induction of a weak assembly-independent GTPase activity directed to the E-site of the protein (*6*−*8*, *27*). This activity requires the binding of COL or of a structural analogue to the ring C (or C′) subsite in the protein, since podophyllotoxin, which binds to the ring A locus, does not induce this activity (*11*), while TME (ring C alone) has the

[2] These results indicate that 2,3,4′-TMB is a stoichiometric inhibitor. Its values of *r* should be regarded as a strictly qualitative indication of this fact.

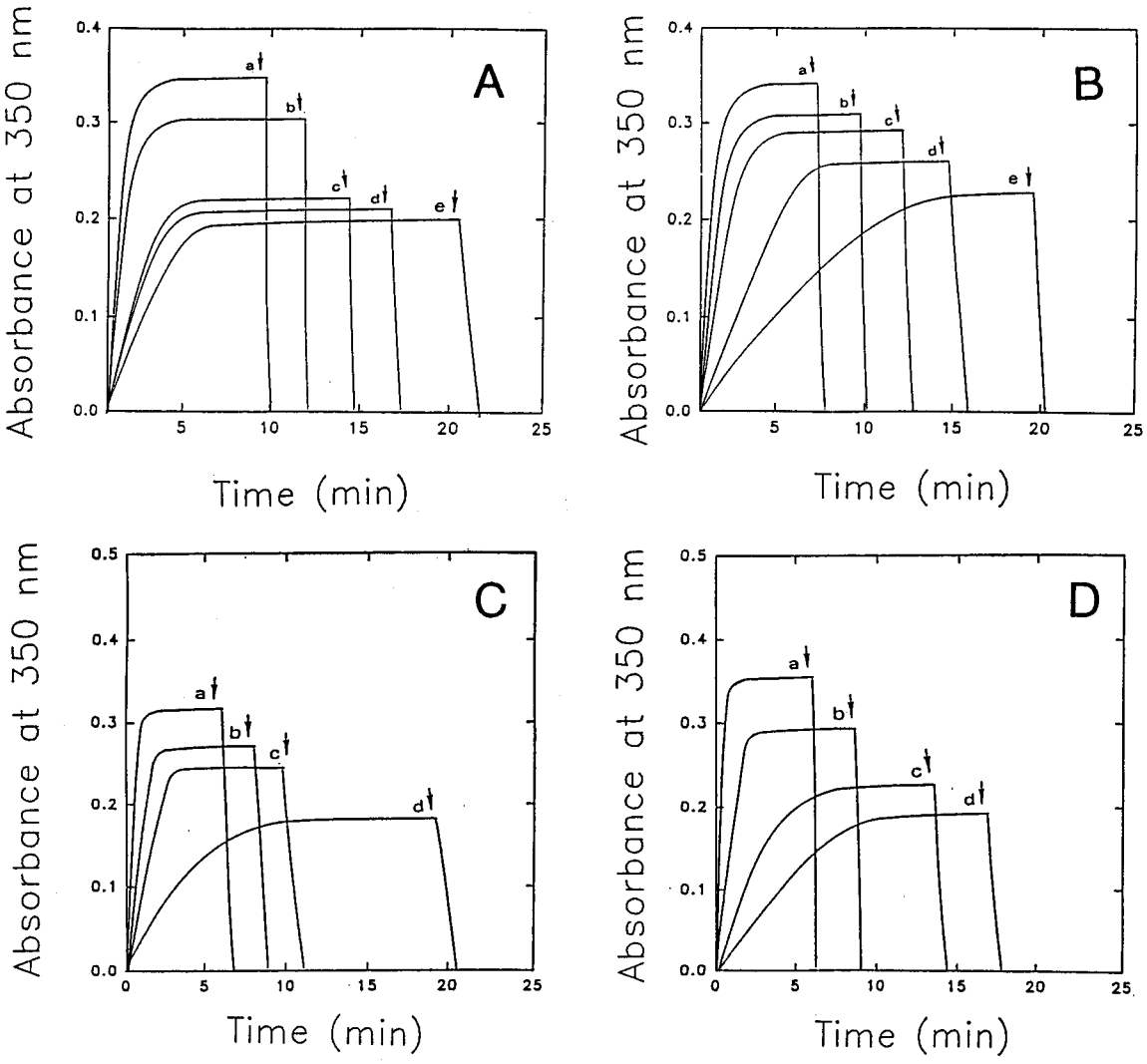


FIGURE 6: Effect of ring A- and C′-modified TMB analogues on the turbidity time course of the in vitro microtubule assembly: (A) (a) 2.2×10^{-5} M tubulin and (b−e) same as panel a with 8.18×10^{-6}, 3.04×10^{-5}, 2.46×10^{-5}, and 3.26×10^{-5} M 2,3,4′-TMB, respectively; (B) (a) 2.2×10^{-5} M tubulin and (b−e) same as panel a with 2.1×10^{-6}, 4.4×10^{-6}, 7.8×10^{-6}, and 1.0×10^{-5} M 2,4,4′-TMB, respectively; (C) (a) 2.1×10^{-5} M tubulin and (b−d) same as panel a with 1.23×10^{-6}, 4.27×10^{-6}, and 1.15×10^{-5} M 2′-CH_3-TMB, respectively; and (D) (a) 2.2×10^{-5} M tubulin and (b−d) same as panel a with 5.15×10^{-6}, 1.3×10^{-5}, and 2.05×10^{-5} M 2,3,3′,4-TMB, respectively. The conditions were the same as those in Figure 4.

ability to induce weakly the enzymatic activity (*8*). The GTPase is also induced by ions such as Ca^{2+} and Ga^{3+} (*28*), and modulated by preferentially excluded cosolvents (*7*, *27*). Table 4 shows that all the analogues tested induced a weak enzymatic activity which, for all the derivatives, was weakened relative to that of the head of the series. However, the great uncertainty in the absolute values of V_i/[liganded tubulin] due to the low solubility of most of the analogues and their weak binding constants for binding to tubulin precluded a quantitative comparison of the magnitudes of GTPase induction.

DISCUSSION

Contribution to Binding and Microtubule Inhibition of the Ring A Methoxy Groups. This analysis of the contribution of the ring A methoxys to the binding to tubulin, microtubule inhibition, and induction of GTPase activity has shown that all three processes are weakened, but not abolished, upon their excision. This raises the question of whether the presence of ring A is essential for the induction of these biochemical effects. The contributions of the individual methoxy groups of ring A to the free energies of binding for binding of the drugs to tubulin and of the tubulin−drug complexes to the end of a growing microtubule that induces microtubule inhibition are summarized in Table 5. Let us examine these in turn.

The increment of the apparent standard free energy change of binding to tubulin contributed by the 2-methoxy group in the ligands MTC and TCB is less than -0.5 kcal mol^{-1}. This free energy change increment reflects not only differences in the contacts made between protein and ligand whether the methoxyl is present or not but also any variation in the solvation and mobility changes in the system when the protein and ligand complex together. The des-2-methoxy compound is free to rotate about the biaryl bond. Introduction of this group is likely to hinder free intramolecular rotation and the attainment of nearly coplanar biaryl conformations, since it can collide with the H atom at position 2′ of ring C (C′). Therefore, the intrinsic contribution to binding of the 2-methoxy group may be substantially different from the apparent value, if the binding modes of COL and its analogues were in a conformation not far from

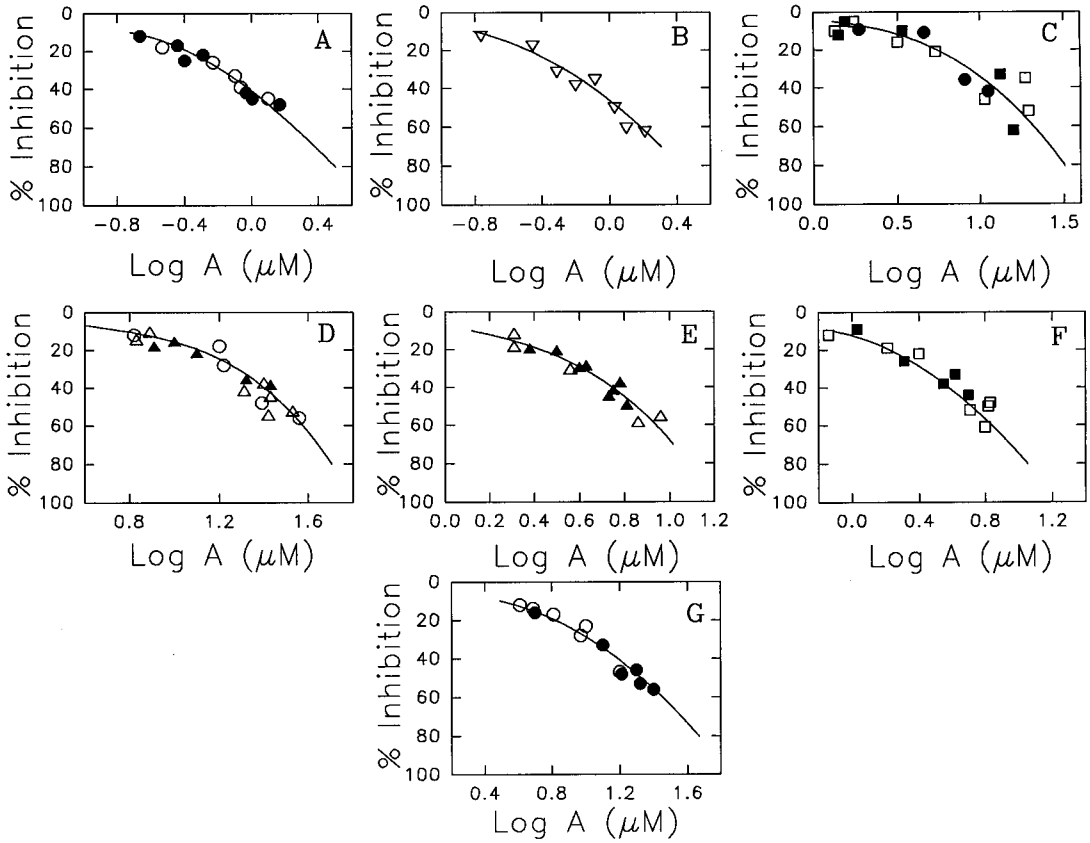


FIGURE 7: Microtubule inhibition isotherms of various biphenyl COL analogues. Percent inhibition is the reduction of the plateau absorbance values; [A] is the concentration of free drug. (A) Effect of 2,4-MDC: (●) 2.1×10^{-5} and (○) 2.5×10^{-5} M tubulin. (B) Effect of 3,4-MDC: (▽) 2.1×10^{-5} M tubulin. (C) Effect of 2,3-DKB: (□) 2.2×10^{-5}, (■) 2.3×10^{-5}, and (●) 2.5×10^{-5} M tubulin. (D) Effect of 2,3,4′-TMB: (△) 2.2×10^{-5}, (▲) 2.3×10^{-5}, and (○) 2.5×10^{-5} M tubulin. (E) Effect of 2,4,4′-TMB: (▲) 2.2×10^{-5} and (△) 2.4×10^{-5} M tubulin. (F) Effect of 2′-CH_3-TMB: (□) 2.1×10^{-5} and (■) 2.5×10^{-5} M tubulin. (G) Effect of 2,3,3′,4-TMB: (○) 2.2×10^{-5} and (●) 2.5×10^{-5} M tubulin. Free drug concentrations were calculated from the total amount of drug added with the use of the known equilibrium binding constants for the binding of drugs to unassembled tubulin, as described previously (*3*). The solid lines are the theoretical curves obtained by simultaneous fitting to eq 7 of the experimental values of K_b, K_i, and protein concentrations at different fractions of inhibition. Average values of K_i and protein concentrations were employed in the fitting, with a constant K_g^{-1} value of 1.0×10^{-5}. In the case of 2,3,4′-TMB, the fitting was carried out with eq 8. The parameters for each drug are as follows: 2,4-MDC, $K_b = 2.1 \times 10^5$ M^{-1}, $K_i = 1.9 \times 10^5$ M^{-1}, and $T = 2.23 \times 10^{-5}$ M; 3,4-MDC, $K_b = 2.4 \times 10^5$ M^{-1}, $K_i = 2.1 \times 10^5$ M^{-1}, and $T = 2.23 \times 10^{-5}$ M; 2,3-DKB, $K_b = 0.28 \times 10^5$ M^{-1}, $K_i = 6.9 \times 10^4$ M^{-1}, and $T = 2.33 \times 10^{-5}$ M; 2,3,4′-TMB, $K_b = 0.21 \times 10^5$ M^{-1}, $K_i = 0$, and $T = 2.33 \times 10^{-5}$ M; 2,4,4′-TMB, $K_b = 0.82 \times 10^5$, $K_i = 1.8 \times 10^4$ M^{-1}, and $T = 2.30 \times 10^{-5}$ M; 2′-CH_3-TMB, $K_b = 0.7 \times 10^5$ M^{-1}, $K_i = 1.2 \times 10^5$ M^{-1}, and $T = 2.30 \times 10^{-5}$ M; 2,3,3′,4-TMB, $K_b = 0.17 \times 10^5$ M^{-1}, $K_i = 1.3 \times 10^5$ M^{-1}, and $T = 2.35 \times 10^{-5}$ M (T is total tubulin concentration).

Table 3: Strength of Microtubule Assembly Inhibition by COL Analogues As Determined at 37 °C from the Assembly Data

ligand	K_b (M^{-1})	K_i (M^{-1})	$\Delta G_i^{\circ a}$ (cal mol^{-1})	protein concentration range (M)	$\delta\Delta G_i^{\circ b}$	r^c (%)
COL	$1.6 \times 10^{7\,d}$	$(2.2 \pm 0.2) \times 10^{6\,d}$	-9.00^d	$1.7-2.2 \times 10^{5\,d}$		1.9 ± 0.2^d
MTC	4.7×10^5	$(5.4 \pm 0.59) \times 10^{5\,e}$	-8.13^e	$1.8-3.0 \times 10^{5\,d}$	0	6.4 ± 0.72^e
2,4-MDC	2.1×10^5	$(1.9 \pm 0.21) \times 10^5$	−7.49	$2.1-2.5 \times 10^5$	0.63	13 ± 1.4
3,4-MDC	2.4×10^5	$(2.1 \pm 0.26) \times 10^5$	−7.55	$2.1-2.5 \times 10^5$	0.58	12 ± 1.7
TKB	$1.9 \times 10^{5\,d}$	$(2.1 \pm 0.34) \times 10^{6\,d}$	-8.97^d	$1.6-3.0 \times 10^{5\,d}$	0	1.9 ± 0.46^d
2,3-DKB	0.28×10^5	$(6.9 \pm 1.1) \times 10^4$	−6.86	$2.2-2.5 \times 10^5$	2.11	20 ± 3.4
TMB	$0.82 \times 10^{5\,d}$	$(9.6 \pm 1.34) \times 10^{4\,d}$	-7.07^d	$1.8-2.6 \times 10^{5\,d}$	0	17 ± 2^d
2,3,4′-TMB	0.21×10^5	$(<1 \pm 0.17) \times 10^3$	>-4.0	$2.2-2.5 \times 10^5$	>3.0	$\sim 70 \pm 11$
2,4,4′-TMB	0.82×10^5	$(1.8 \pm 0.21) \times 10^4$	−6.04	$2.2-2.4 \times 10^5$	1.03	25 ± 3.3
2′-CH_3-TMB	0.7×10^5	$(1.2 \pm 0.13) \times 10^5$	−7.20	$2.1-2.5 \times 10^5$	−0.13	16 ± 1.8
2,3,3′,4-TMB	0.17×10^5	$(1.3 \pm 0.18) \times 10^5$	−7.30	$2.2-2.5 \times 10^5$	−0.23	13 ± 1.7

[a] The standard free energy of inhibition was calculated from the K_i values. [b] Difference in ΔG_i° from the head of a series. [c] Liganded tubulin at 50% turbidity, calculated as described previously (*3*). [d] Data from ref *3*. [e] Parameters calculated from the data of ref *3* using the K_b value for MTC given in this table which differs by 0.31 kcal mol^{-1} from the one in Table 1 in ref *3*. However, the effect on K_i of MTC is minimal (0.018 kcal mol^{-1}).

coplanar [although this seems not to be the case for COL itself (*29*)]. In that case, the simple presence of the 2-methoxy group would hamper binding and weaken the free energy of interaction. This unfavorable contribution would have to be balanced then by an intrinsic contact contribution to binding that is considerably stronger than its apparent small value of −0.4 kcal mol^{-1}. The contribution of the 2-methoxy group to microtubule inhibition is −0.58 kcal mol^{-1}, a value very similar to its contribution to the binding process. Since substoichiometric microtubule inhibition

Table 4: Induction of GTPase Activity by Ring A- and C-Modified Biphenyl Analogues of COL

ligand[a]	ligand concentration (M)	liganded tubulin concentration[c] (M)	V_i[d]/liganded tubulin concentration (min^{-1})	V_i(ligand)/ V_i(head of a series) (%)	V_i(ligand)/V_i(COL)
COL	2×10^{-4}	3.00×10^{-6}	0.0095 ± 0.0004	−	100
MTC	2×10^{-4}	3.00×10^{-6}	0.0060 ± 0.0006	100	63 ± 6
2,4-MDC	1×10^{-3}	3.00×10^{-6}	0.0016 ± 0.0003	27 ± 5	17 ± 3
3,4-MDC	1×10^{-3}	3.00×10^{-6}	0.0024 ± 0.0004	40 ± 5	25 ± 3
TKB	2×10^{-4}	2.92×10^{-6}	0.0051 ± 0.0007	100	54 ± 7
2,3-DKB	7×10^{-4} [b]	2.85×10^{-6}	0.0015 ± 0.0009	29 ± 20	16 ± 11
TMB	2×10^{-4}	2.83×10^{-6}	0.0046 ± 0.0006	100	48 ± 8
2,3,4′-TMB	4×10^{-4} [b]	2.68×10^{-6}	0.0025 ± 0.0010	54 ± 20	27 ± 10
2,4,4′-TMB	1×10^{-4} [b]	2.67×10^{-6}	0.0017 ± 0.0007	37 ± 15	18 ± 7
2-CH_3-TMB	4×10^{-4} [b]	2.90×10^{-6}	0.0031 ± 0.0005	67 ± 10	33 ± 5
2,3,3′,4-TMB	4×10^{-4} [b]	2.61×10^{-6}	0.0024 ± 0.0007	52 ± 15	25 ± 7

[a] The ligand was preincubated with the protein for 30 min at 20 °C before the reaction was initiated. The GTPase activity measurements were carried out as described previously (*7*). [b] Apparent maximal solubility of the ligand. [c] Calculated from the K_b values for each ligand employing the total amount of tubulin in the reaction mixture (3×10^{-6} M). [d] Initial velocity.

Table 5: Observed Contributions of Ring A and Its Substituents to the Binding of COL Analogues to Tubulin and Microtubule Inhibition

group	ligand	$\delta\Delta G°_{b,app}$ for binding to tubulin (kcal mol^{-1})	$\delta\Delta G°_{b,app}$ for microtubule inhibition (kcal mol^{-1})
2-OCH_3	MTC or TCB	-0.40 ± 0.08[a]	-0.58[b]
3-OCH_3	MTC, TCB, or TMB	-0.42 ± 0.14[a]	-0.83[b] ± 0.20
4-OCH3	TKB or TMB	-1.36 ± 0.15[a]	-2.1; <-3[b]
ring A	MTC	-3.84 ± 0.30[c]	0.27[d]
ring A	COL	-5.70 ± 0.30[e]	-0.58[f]

[a] Values obtained from Table 1. [b] Values obtained from Table 3. [c] Difference between the free energies of binding of ring C (tropolone methyl ether, -3.9 ± 0.4 kcal mol^{-1}; *21*) and MTC. The latter contains an unfavorable entropic contribution that stems from the partial immobilization of the intramolecular rotation of the biaryl ring A−ring C bond during binding. This has been estimated as ∼2 kcal mol^{-1} (*20*), which makes the intrinsic contribution of ring A to the binding of MTC identical to that in COL. [d] Difference between the free energies of inhibition of ring C and MTC (*3*). [e] Difference between the $\Delta G°$ of binding of ring C and COL (-9.6 ± 0.1 kcal mol^{-1}; *34*). It is assumed that any productive contribution of ring B to binding is negligible (*21*), its role being the mutual immobilization of rings A and C. [f] Difference between the $\Delta G°$ of inhibition of ring C and COL (*3*).

requires the binding of the tubulin−drug complex to the end of the growing polymer (*2*, *3*), the difference in the standard free energy of inhibition between MTC and 3,4-MDC may be a reflection of the weakening of the induction of a surface conformational change in the protein that affects the heterodimer−heterodimer interactions. In the case of the ligand-induced GTPase, the decreased activity for the transformation MTC → 3,4-MDC is linked to the strength of the standard free energy of interaction between the drug and the protein. This may be related to the fact that the stronger the interaction between the drug and the protein, the better positioned are the catalytic groups and, therefore, the higher the enzymatic activity (*8*).

What is the role of the 3-methoxy group of ring A? This is determined by a comparison of 2,4-MDC, 2,4,4′-TMB, and 2,4-DCB with their parent compounds. All of these analogues share identical rings A, with differences in ring C (C′) (see Chart 1). The contribution of the three drugs to the binding to $\alpha\beta$-tubulin is remarkably similar, ca. -0.42 ± 0.14 kcal mol^{-1} (see Tables 1 and 5). This indicates strongly that the interactions that ring C or C′ establishes with particular residues in the protein are independent of ring A. Comparison of the effects of 2,4-MDC and 2,4,4′-TMB on the inhibition of tubulin self-assembly indicates that the contribution of the 3-methoxy group of ring A to inhibition is not large, and is on a similar order as its contribution to binding, as it amounts to -0.83 ± 0.25 kcal mol^{-1} (Table 5).

By contrast with methoxy groups 2 and 3, elimination of the 4-methoxy group from ring A of TKB or TMB had a much stronger effect on the standard free energy of the drug−protein binding interaction. As calculated from Table 1, the contribution to binding of the 4-methoxy group in the analogues 2,3-DKB and 2,3,4′-TMB is -1.36 ± 0.15 kcal mol^{-1}. This points to a relatively more specific interaction (such as, e.g., hydrogen bond formation) with the binding site than that of the 2- or 3-methoxy groups. In the biphenyl COL analogues, the 4-methoxy group is located diametrically opposite of the 4′ substituent (Chart 1), whose properly positioned oxygen atom is critical for their binding (*3*, *8*, *10*, *11*). This points to an endwise fitting of these ligands into the COL binding site. In the biphenyl COL analogues, free rotation around the A−C (or A−C′) bond should not affect significantly the interactions that the 4-methoxy group undergoes with the protein; hence, this group can act as an "anchor" of ring A to particular residues in the protein along the axis of the ligand. Compounds 2,3-DKB and 2,3,4′-TMB, which lack this methoxy, cannot position ring A in the binding pocket in a locked configuration.[3] The floppy state of the tubulin−ring A complex should be reflected in a weakening of free energy of binding that is entropic in nature. While free rotation about the A−C′ biaryl bond should not affect significantly the binding of the 4-methoxy group, a rotation of just a few degrees between the rings should hinder the groups in positions 2 and 3 from establishing optimally the appropriate interactions with the putative binding site in the protein. Nevertheless, the results obtained with the des-2- and des-3-methoxy compounds indicate that the interactions of these two methoxys with the protein may not to be critical for binding to tubulin and microtubule inhibition.

[3] That the locus of binding of the 4-methoxy group of ring A is not on the flat surface of the protein is supported by reports that introduction of bulky groups into that position suppresses the binding of colchicine (*30*) and podophyllotoxin (*31*) to tubulin, although their effect may not be specific.

Why is the contribution of the 4-methoxy of ring A to inhibition of microtubule assembly different in 2,3-DKB and 2,3,4′-TMB? Analysis of the data showed that with 2,3-DKB the standard free energy of inhibition is 2.1 kcal mol^{-1} weaker than that of the parent compound TKB. On the other hand, the excision of the same methoxy from TMB which gives the compound 2,3,4′-TMB had a considerably greater weakening effect on inhibition ($\delta\Delta G°_i$ > 3.0 kcal mol^{-1}) (Table 3). The answer to this difference in behavior may be found in the substituents in position 4′ of ring C′. While in 2,3-DKB the substituent on ring C′ is a methyl carbonyl group, in 2,3,4′-TMB it is a methoxy group (Chart 1). We have shown previously that a methyl carbonyl group in position 4′ induces maximal substoichiometric inhibition, while the methoxy compound is weakly substoichiometric (*3*, *8*). Comparison of the potency as microtubule inhibitors of the two and three ring keto analogues, TKB and KAC[4] (*8*), has shown that $\delta\Delta G°_{inhibition}$ ($=\Delta G°_{KAC} - \Delta G°_{TKB}$) is very small. Hence, the interactions that the carbonyl group establishes with particular residues in the protein can overcome effectively the unfavorable contribution of rotation about the A−C′ bond, and provide sufficient anchoring. The same must be true of the keto analogue 2,3-DKB. The additional general floppiness induced by the lack of the 4-methoxy group on ring A, however, should increase the entropy that must be overcome in maintaining the proper ring C′−protein contacts needed for inhibition. Therefore, 2,3-DKB is a weaker microtubule inhibitor than TKB. Let us analyze now in a similar manner the TMB derivative, 2,3,4′-TMB. If we compare the $\Delta G_i°$ values of the two and three ring COL analogues that bear a methoxy group in position 4′ of ring C′, namely, TMB and MAC[5] (*8*), the value of $\delta\Delta G_i°$ ($=\Delta G°_{MAC} - \Delta G°_{TMB}$) is −1.78 kcal mol^{-1}. The methoxy of TMB does not have the ability to anchor ring C′ with the rigidity needed to produce a strong interaction with the functional groups in the protein (*3*, *32*). Hence, the biphenyl TMB is a weak microtubule inhibitor. In the des-4-methoxy analogue (2,3,4′-TMB), it appears that the interactions of the methoxy group in position 4′ of ring C′ with functional groups in the protein do not have the strength needed for overcoming the additional floppiness due to the absence of anchoring by the methoxy group in position 4 of ring A. As a consequence, binding of this compound does not induce in the protein the conformation needed for binding of the tubulin−drug complex to the end of a microtubule ($K_i \sim 0$), a requirement for substoichiometric inhibition (*2*).

Possible Contribution to Binding and Inhibition of Phenyl Ring A. The intrinsic contribution of the complete trimethoxybenzene ring A to the binding of COL and MTC can be estimated as ca. −5.7 kcal mol^{-1} (see Table 5) (*20*, *33*). It is not known whether the contributions of the three methoxy groups and the phenyl ring are strictly additive. The probably weak binding of des-2,3,4-methoxy bicyclics has not been measured. Nevertheless, the theoretical increment of free energy change provided by the plain phenyl ring can be calculated as the difference between the contribution of ring A (−5.7 kcal mol^{-1}) and the sum of the contributions of the three methoxy groups (−2.2 kcal mol^{-1}). This gives a contribution of the plain phenyl ring of −3.5 kcal mol^{-1}. This is larger than the sum of the values of the three methoxy groups. As a whole, the binding of the trimethoxyphenyl ring A appears to be compatible with a relatively loose hydrophobic interaction (*21*, *33*) with some specificity provided by the 4-methoxy group and perhaps by the 2-methoxy group. Let us calculate in a similar manner the contributions of the entire ring A to inhibition of tubulin self-assembly by COL and MTC. As shown in Table 5, in the case of COL, the intrinsic contribution to inhibition of ring A (plus ring B), taken as the difference between the $\Delta G°_i$ values of COL and pure ring C, can be estimated as −0.58 kcal mol^{-1}. In the case of MTC, the same calculation results in a contribution ($\Delta G°_{MTC} - \Delta G°_{TME}$) of 0.27 kcal mol^{-1} (*3*). Thus, both in COL and in MTC, ring A makes no significant contribution to the strength of inhibition. This is supported by the observation that the ring A analogue, *N*-acetylmescaline (NAM), has an immeasurable value of K_i; i.e., it cannot induce substoichiometric inhibition (*3*). It may be concluded, therefore, that the presence of ring A is not required for the induction of microtubule inhibition.

Is There a Linkage between Drug Binding to Tubulin and Microtubule Inhibition? In previous studies (*3*, *8*), it had been concluded that the strength of microtubule inhibition is not thermodynamically linked to the strength of drug binding to tubulin. An examination of Tables 1 and 3 of this study supports this general conclusion. For example, in the TMB series, 2,4,4′-TMB and 2′-CH_3-TMB bind with similar affinities, yet their inhibitory capacities differ by 1.15 kcal mol^{-1}. On the other hand, 2,3,3′,4-TMB and 2′-CH_3-TMB have identical inhibitory capacities, but their binding affinities for tubulin differ by 1 kcal mol^{-1}. It may seem surprising that a shift of the ring C′ methoxy group from position 4′ to position 3′ had no effect on inhibitory capacity, but did weaken binding by 1 kcal mol^{-1}. This is consistent with the proposal (*3*) that the strength of inhibition is modulated by the ability of an oxygen on ring C (or C′) to make the proper specific contact with a group in the protein. In the case of TMB, the 4′-methoxy occupies a position in space that is removed from that of the COL ring C methoxy group by 1.0 Å (*32*). A model calculation was carried out on 2,3,3′,4-TMB using the same method used previously (*11*). It was found that a methoxy group in position 3′ of ring C′ is removed from the space locus of the COL ring C methoxy by a distance comparable to that when it is in position 4′. Therefore, its oxygen appears to be able to enter into the same interaction as that of the 4′-methoxy group of TMB. As a consequence, both compounds are weak substoichiometric inhibitors of microtubule assembly.

The general conclusion about the absence of a thermodynamic linkage seems to be contradicted, however, by the finding that removal of particular methoxy groups from ring A has very similar thermodynamic consequences on both binding ($\delta\Delta G°_{b,app}$) and inhibition ($\delta\Delta G°_{i,app}$). The answer to the apparent dilemma may be found in the fact that inhibition is a postbinding event. The binding of COL and its analogues to tubulin follows a bidentate mechanism in which the bindings of ring A and C (or C′) to their specific subsites on tubulin are thermodynamically independent (*21*, *33*). This is supported by the current observation that removal of a given methoxy group from ring A has very

[4] KAC is the analogue of allocolchicine in which the ring C′ carbomethoxy has been replaced by acetyl (transformation from acetyl ester to methyl ketone).

[5] MAC is the allocolchicine analogue in which the ring C′ carbomethoxy has been replaced by a methoxy.

similar consequences for the binding of compounds that differ in rings C or C′ (e.g., the des-3-methoxy analogues of MTC, TCB, and TMB). In all the previous studies, all the compounds had identical rings A and all the changes occurred in ring C. The strength of inhibition has been attributed to the formation of specific contacts between oxygen-containing groups on ring C′ or C that, in the presence of identical anchoring by ring A (trimethoxy), manifest large differences. These differences between different ring C (or ring C′) compounds remain when individual methoxy groups are excised from ring A, and the independence between the strengths of binding and inhibition remains. Substoichiometric inhibition, however, requires proper anchoring of the molecule within the particular site on tubulin. As shown in this study, this anchoring is weakened by the excision of any of the three methoxy groups of ring A. This introduces floppiness into the protein−drug complex. This floppiness will, per force, reduce the strength of the ring C (or C′) group−protein group interaction needed for the induction of microtubule inhibition, since free energy will have to be expended to overcome the entropic contribution of the floppiness of the complex and stabilize the inhibition-inducing interaction. As a result, the free energy of inhibition becomes affected to an extent similar to that of the standard free energy of binding, because of the kinetic linkage between the two processes.

Conclusion. From these studies, it may be concluded that the structural moieties of ring A, namely, the phenyl ring and the three methoxyl groups, make additive contributions to the strength of binding of COL and its analogues to tubulin.

The strength of binding of the drugs to tubulin is provided by the sum of the free energies of interaction of ring A and C (or C′) with tubulin. The strength of the inhibition is determined by specific interactions of oxygen atoms in the groups on ring C (C′) with the appropriate groups within the COL binding pocket on tubulin, as ring A serves only as an anchor that maintains the two-ring molecules in the proper orientation within the binding locus. Weakening of the anchoring may affect the strength of inhibition by allowing some wobbling of the A−C molecule within the binding pocket, which causes ring C (C′) to use up free energy for stabilizing the complex in the proper orientation. Ring A, however, per se does not play a role in the mechanism of inhibition. The processes of binding of the drug to tubulin and of microtubule inhibition by the tubulin−drug complex are linked only kinetically, and not thermodynamically.

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