Biochemical Pharmacology, Vol. 51, pp. 53–59, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02156-6

Antitumor 2,3-Dihydro-2-(aryl)-4(1*H*)-quinazolinone Derivatives

INTERACTIONS WITH TUBULIN

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ABSTRACT. A series of derivatives of 2,3-dihydro-2-(aryl)-4(1*H*)-quinazolinone (DHQZ) with known antitumor activity was re-evaluated in the National Cancer Institute cancer cell line screen. Analysis by the COMPARE algorithm suggested that their cytotoxicity derived from interactions with tubulin. Significant inhibition of tubulin assembly and of the binding of radiolabeled colchicine to tubulin was demonstrated with several of the compounds, particularly NSC 145669, 175635, and 175636. The DHQZ derivatives are structurally analogous to a number of antimitotic agents, flavonols and derivatives of 2-styrylquinazolin-4(3*H*)-one and of 2-phenyl-4-quinolone. Structure-activity analogies between these agents, the combretastatins, and the colchicinoids were analyzed and summarized. BIOCHEM PHARMACOL 51;1:53–59, 1996.

KEY WORDS. antimitotic agents; colchicine analogs; tubulin polymerization; styrylquinazolinone derivatives; phenylquinolone derivative; flavonols

The experimental *in vivo* and *in vitro* antitumor activities of a number of DHQZ¶ derivatives were first described over two decades ago, but efforts at that time to determine their mechanism of action were unsuccessful [1, 2]. Ample supplies of many of these agents (compounds **1–12,** structures summarized in Fig. 1) remain in the drug screening program of the National Cancer Institute, and the antileukemic activity of the most active agents in the series was sufficient to warrant their re-evaluation in the new NCI 60 human tumor cell line drug screen [3].

Data generated in the new screen are automatically entered into a data base for ready evaluation, including study by the COMPARE algorithm, which was devised to permit analysis of differential cytotoxicity patterns [4, 5]. COMPARE analysis has proven valuable for prediction of the mechanism of drug action [5]; and the patterns of cytotoxicity obtained with the DHQZ derivatives indicated that these compounds were inhibitors of tubulin function in cells [6]. For example, when compound **11** was used to probe the data base, Pearson correlation coefficients of 0.6 or greater were obtained for the remaining eleven DHQZ derivatives and for numerous agents known to inhibit tubulin polymerization (Table 1; cf. Ref. 6).

Moreover, the DHQZ derivatives have obvious structural analogies to other known antimitotic agents, especially SQZ [7, 8] (compounds **13–17;** and the related compounds **18** and **19**) and PQ derivatives [9–11] (structures in Fig. 2). In particular, the SQZ derivatives differ in the A/B ring system from the DHQZ derivatives only in the oxidation status of the bond between N(1) and C(2). The most striking structural difference between the SQZ and PQ derivatives on the one hand and the DHQZ derivatives on the other is the variety of apparently active C ring systems in the latter series, while in the former two series, for the most part, only compounds with phenyl C rings have been examined. For convenience, we will refer to DHQZ, SQZ, and PQ derivatives, together with antimitotic flavonols [12–14] (see below), as "heterocyclic ketones."

In this report, we describe studies examining interactions of the DHQZ derivatives with tubulin. These experiments were performed to confirm the mechanism of action predicted by the differential cytotoxicity data and for possible new insights into structural requirements for the binding of heterocyclic ketones to tubulin.

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¶ Abbreviations: DHQZ, 2,3-dihydro-2-(aryl)-4(1*H*)-quinazolinone; SQZ, 2-styrylquinazolin-4(3*H*)-one; PQ, 2-phenyl-4-quinolone; and CS-A4, combretastatin A-4.

Received 3 May 1995; accepted 7 August 1995.

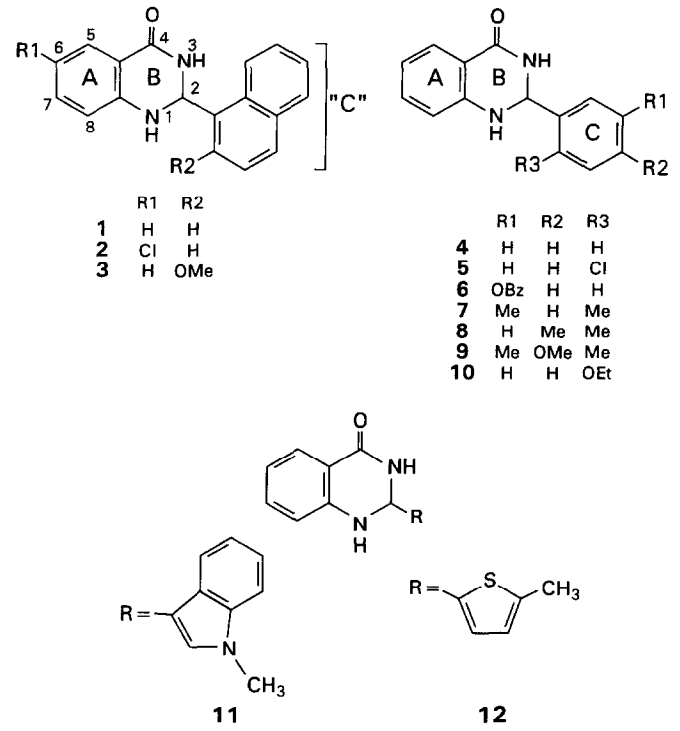


FIG. 1. Structural formulas of DHQZ derivatives (compounds 1–12).

MATERIALS AND METHODS

Materials

Electrophoretically homogeneous bovine brain tubulin was prepared as described previously [15]. Colchicine was from Sigma (St. Louis, MO), podophyllotoxin from Aldrich (Milwaukee, WI), monosodium glutamate from United States Biochemical (Cleveland, OH), and [^{3}H]colchicine and [^{3}H]vinblastine from DuPont (Boston, MA). Stock solutions (2.0 M) of monosodium glutamate were adjusted to pH 6.6 with HCl. CS-A4 was a gift of Dr. G. R. Pettit, Arizona State University, and SQZ derivatives and compound **18** were provided by Dr. D. Hesson, DuPont Corp. The DHQZ derivatives, the oxidized form of compound **4** (compound **19**; structure in Fig. 2), and maytansine were obtained from the Drug Synthesis & Chemistry Branch, NCI.

Methods

Inhibition of tubulin polymerization was evaluated following a 15-min drug-tubulin preincubation (in 0.24 mL) at 30° prior to the addition of the GTP (in 10 μL) required for tubulin assembly. All concentrations refer to the final reaction volume. Reaction mixtures contained 0.8 M monosodium glutamate, 1.0 mg/mL (10 μM) tubulin, 0.4 mM GTP, 4% (v/v) DMSO (the drug solvent), and various concentrations of drug. Reaction mixtures at 0° were transferred to 0° cuvettes in Gilford model 250 recording spectrophotometers equipped with electronic temperature controllers. Absorbance baselines were established at 350 nm, and the reaction temperature was set at 30° (temperature rose at about 0.5°/min). Turbidity development was followed for 20 min. The IC_{50} value for each drug is defined as the concentration (obtained by graphical interpolation) that inhibited the extent of assembly by 50%.

The binding of [^{3}H]colchicine to tubulin was followed by the DEAE-cellulose filter method, in reaction mixtures containing 0.1 mg/mL (1.0 μM) tubulin, 5.0 μM [^{3}H]colchicine, and potential inhibitor at either 5 or 50 μM. Additional reaction components (glutamate, GTP, albumin, $MgCl_2$, and glucose-1-phosphate) were as described previously [16] and used because they have been found to stabilize the colchicine binding activity of tubulin [17]. Incubation was at 37° for 10 min, a time point at which the uninhibited reaction is approximately 50% of maximum with about 0.3 mol colchicine bound/mol tubulin.

The binding of [^{3}H]vinblastine to tubulin was measured by centrifugal gel filtration as described previously [18]. Reaction mixtures contained 0.1 M 4-morpholineethanesulfonate (taken from 1 M stock solution adjusted to pH 6.6 with NaOH), 1 mM $MgCl_2$, 0.5 mg/mL (5 μM) tubulin, 5 μM [^{3}H]vinblastine, 4% (v/v) DMSO, and potential inhibitory agents at 50 μM. Incubation (10 min) and centrifugal gel filtration were done at room temperature.

Cytotoxicity evaluation of the DHQZ derivatives was performed by the NCI drug screening program [3]. The data were obtained from the NCI drug screen data base.

In vivo antileukemic evaluations were conducted in the i.p. P388 and L1210 murine leukemia models according to standard protocols used by the screening program of the Developmental Therapeutics Program of the NCI [19]. Mice, $C_{57}BL/6 \times DBA/2$ F_1 ($B_6D_2F_1$) or Balb/c × DBA/2 (CD_2F_1), and tumors

TABLE 1. Differential cytotoxicity analysis by COMPARE algorithm with compound 11 as the probe agent

Compound	Pearson correlation coefficient
11	1.00
6	0.84
8	0.84
1	0.82
3	0.79
Vinblastine	0.77
12	0.76
7	0.75
10	0.75
9	0.72
CS-A4	0.71
Nocodazole	0.70
4	0.68
Peltatin A	0.68
Dolastatin 10	0.68
2	0.66
Deoxypodophyllotoxin	0.65
Maytansine	0.64
10-Methylthiocolchicine derivative	0.64
Rhizoxin	0.63
Halichondrin B	0.61
Colchicine	0.61
5	0.60

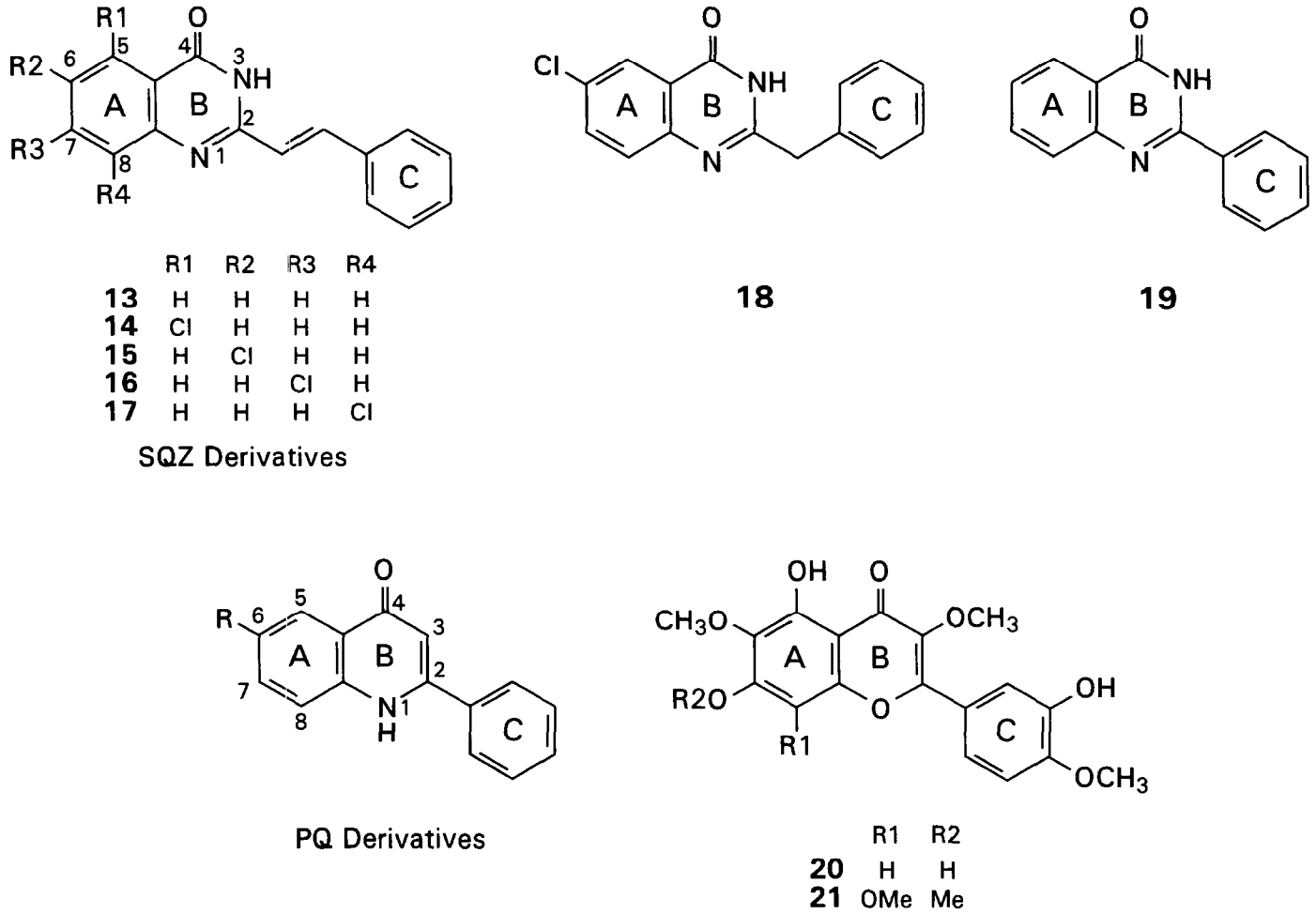


FIG. 2. Structural formulas of SQZ derivatives (compounds 13–17), related compounds (compounds 18 and 19), antimitotic flavonols (compounds 20 and 21), and PQ derivatives (general formula).

were obtained from the NCI animal program and tumor repository, respectively. All compounds were administered i.p. on a daily schedule as indicated. Colchicine was administered in water or 0.9% NaCl, and podophyllotoxin in 0.9% NaCl containing Tween 80 or in aqueous hydroxypropyl cellulose (Klucel). All other compounds were administered as suspensions, usually in Klucel, which was prepared free of silicon dioxide and was donated to the NCI by the Hercules Powder Co., Inc., Wilmington, DE.

RESULTS AND DISCUSSION

Table 2 summarizes NCI data for *in vivo* antileukemic activity and cytotoxicity for the DHQZ derivatives, colchicine, podophyllotoxin, CS-A4, and several related compounds. The cytotoxicity data are presented as the average GI_{50} (50% growth inhibition, equivalent to an IC_{50} value) obtained for the 60 cell lines tested (expressed as the logarithm of the molar GI_{50} value).

All DHQZ derivatives except compound 7 demonstrated antitumor activity against the i.p.-implanted P388 leukemia, although they were much less potent than colchicine and podophyllotoxin. The two most potent DHQZ derivatives (in terms of tolerated dose) in the *in vivo* P388 and L1210 studies were compounds 1 and 2, which were also the most cytotoxic agents in this group (midnanomolar GI_{50} values). Both 1 and 2 have a naphthyl substituent at position C(2). Moreover, aside from compound 11, compounds 1 and 2 were the only DHQZ derivatives active against L1210 leukemia *in vivo*. The only derivative with a substituted naphthalene ring (compound 3) was less active than 1 and 2. Compounds 3–6 and 8–12 showed similar activities against P388 leukemia *in vivo*. Four additional agents had average cytotoxicity GI_{50} values in the high nanomolar range (compounds 6, 9, 10, and 11), and three of these have a substituted phenyl ring at position C(2). Note that despite only modest differences in cytotoxicity between colchicine, podophyllotoxin, CS-A4, and compounds 1 and 2, their antileukemic effects *in vivo* were highly variable. Conversely, the similar *in vivo* activity among most of the remaining DHQZ derivatives occurred despite a wide range of cytotoxic activity.

Table 2 also presents data for inhibitory effects on tubulin assembly. The IC_{50} values for colchicine, podophyllotoxin, and CS-A4 were 1.2 to 1.6 μM, and three of the DHQZ derivatives had comparable activities (1, IC_{50} 1.7 μM; 2, IC_{50} 1.0 μM; and

TABLE 2. Antileukemic activity and inhibitory effects on cell growth, tubulin polymerization, and binding of colchicine and vinblastine to tubulin by DHQZ derivatives and related compounds

Compound	In vivo antileukemic activity [% T/C (Dose)]		Inhibition of cell growth (log GI_{50})	Inhibition of tubulin polymerization IC_{50} (μM)	Inhibition of colchicine binding (%)		Inhibition of vinblastine binding (%)
	P388	L1210			5 μM Drug	50 μM Drug	
Colchicine	178 (0.5)	Inactive (0.5)	−7.2	1.5 ± 0.3			
Podophyllotoxin	152 (8)	144 (5)	−7.6	1.6 ± 0.03	85 ± 4		
CS-A4	Inactive (100)		−8.1	1.2 ± 0.2	92 ± 2		
1 (NSC 145669)	194 (64)	164 (80)	−7.5	1.7 ± 0.4	17	62 ± 0.5	1
2 (NSC 175636)	160 (50)	131 (100)	−8.1	1.0 ± 0.4	34	79 ± 2	15
3 (NSC 175639)	166 (200)	Inactive (400)	−5.9	14 ± 1	0	19	
4 (NSC 113764)	170 (400)	Inactive (400)	−5.3	14 ± 0.9	4	5	
5 (NSC 175633)	135 (400)	Inactive (400)	−4.8	>40	1	1	
6 (NSC 175635)	166 (250)	Inactive (150)	−6.7	0.72 ± 0.3	10	58 ± 2	17
7 (NSC 158383)	Inactive (200)	Inactive (400)	−6.0	11 ± 2	7	17	
8 (NSC 158382)	120 (400)	Inactive (400)	−5.7	27 ± 4	3	10	
9 (NSC 154756)	185 (400)	Inactive (400)	−6.5	7.2 ± 2	9	33	
10 (NSC 158389)	188 (400)	Inactive (400)	−6.7	5.5 ± 1	5	35	
11 (NSC 339877)	146 (200)	151 (200)	−6.2	5.4 ± 1	6	24	
12 (NSC 175634)	190 (400)	Inactive (400)	−5.2	7.5 ± 2	0	0	
13				5.0 ± 0.6		17	
14				>40		0	
15 (NSC 377864)	185 (120)	Inactive (100)		1.1 ± 0.03		15	
16 (NSC 380849)	Inactive (200)			1.6 ± 0.3		16	
17				>40		0	
18 (NSC 382468)	Inactive (100)			>40		0	
19 (NSC 131274)				>40		0	

For the antileukemic studies (also see Materials and Methods), data are expressed as a percent T/C, based on the median (P388) or mean (L1210) day of death of mice in the control (C) and treated (T) groups. The best antitumor response obtained with each compound is shown in the table, with the dose in mg/kg shown in parentheses, except for colchicine. With this agent, the median optimal result from numerous studies is presented. Drugs were administered i.p. on days 1–9, except that drug administration was on days 1–5 only for podophyllotoxin and compounds **11**, **15**, **16**, and **18** with P388 leukemia and for compound **15** for L1210 leukemia. For inactive compounds the highest dose tested with no visible evidence of toxicity is shown. Inactivity in P388 leukemia is defined as a T/C value of less than 120%; and in L1210 leukemia, less than 125%. The cytotoxicity data were generated by the NCI drug screening program and obtained from the drug data base. The average log GI_{50} values (equivalent to IC_{50} value for growth inhibition) are presented, representing the average values obtained for the cell lines successfully tested, up to 60, with each agent. These average values are actually approximations, since the upper or lower limiting concentration was used to represent the GI_{50} value for specific cell lines when the actual value either exceeded the highest concentration examined or was less than the lowest concentration examined. For further details, see Ref. 5. All agents except compound **12** were evaluated at least two times, and the data from all tests were averaged. In the tubulin polymerization assay, each compound was evaluated at least three times, except for inactive compounds (IC_{50} > 40 μM), which were evaluated at least twice. Average values ± standard deviations are presented. In the colchicine binding assay each sample was performed in duplicate. Where indicated, a second independent experiment was also performed with selected compounds, and the range of values obtained in the two experiments is indicated in the table. A single vinblastine binding experiment was performed, and in a simultaneously performed control 50 μM maytansine inhibited vinblastine binding 90%.

6, 0.72 μM). Four derivatives had IC_{50} values in the 5–10 μM range, and only the least cytotoxic agent, compound **5**, had no effect on tubulin polymerization.

Since SQZ and PQ derivatives that inhibit tubulin polymerization inhibit the binding of colchicine to tubulin, the DHQZ derivatives were examined for this property (Table 2). In comparison to the potent inhibition obtained with podophyllotoxin and CS-A4, only weak effects were observed with the DHQZ derivatives. Nevertheless, the greatest inhibition occurred with the three best inhibitors of polymerization (**1**, **2**, and **6**). These DHQZ derivatives are better inhibitors of colchicine binding than structurally similar SQZ derivatives with similar potencies as inhibitors of assembly (Table 2). Compounds **1**, **2**, and **6** did not inhibit significantly the binding of vinblastine to tubulin (Table 2; maytansine, examined simultaneously, inhibited vinblastine binding 90%).

Strong inhibition of tubulin assembly combined with relatively weak inhibition of colchicine binding is not well understood. Comparable results have been observed with many other agents, including the SQZ [7, 8] and PQ [9–11] derivatives. Although the binding sites for these compounds may not completely overlap the binding site of colchicine, we believe the most reasonable explanation derives from differential binding and dissociation kinetics and differences in what the two assays actually measure. Assembly assays measure the formation of large aggregates of tubulin, and a transient interaction of drug with protein probably suffices to disrupt the reaction. This is particularly the case if the drug can also promote polymer disassembly. The colchicine binding assay, however, measures competition between two ligands for a drug binding site on a single tubulin molecule. Depending on how the assay is performed, the results obtained will reflect differential affinities of the ligands for tubulin and/or relative binding and dissociation rates. Particularly with a ligand like colchicine that barely dissociates once bound, the extent of inhibition by many competitors is exquisitely sensitive to

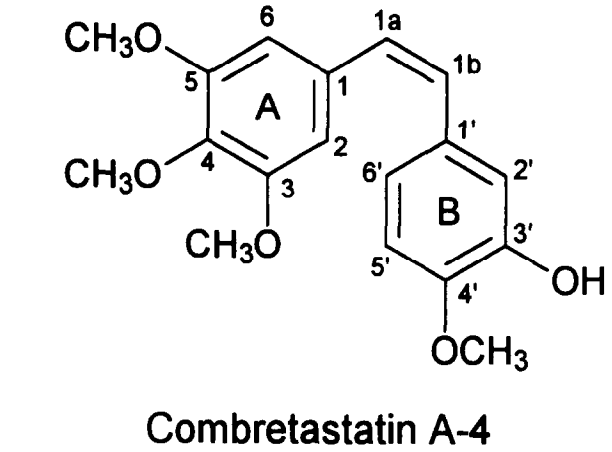
incubation time [8, 16, 20]. In support of this interpretation, we should note that a few PQ derivatives were quite potent as inhibitors of colchicine binding without a concomitant increase in potency in inhibiting assembly.

In conjunction with analysis of the DHQZ derivatives, we re-evaluated several previously studied SQZ derivatives [7], as well as the inactive analog **18** and compound **19,** obtained from the NCI collection after a computer search for structural analogs. These compounds were chosen for their structural similarity to the DHQZ derivatives **4–10** and to reiterate the apparent importance of the C(6)-substituent, which was only present in the DHQZ series with the 2-naphthyl derivatives (enhanced activity of **2** vs 1).

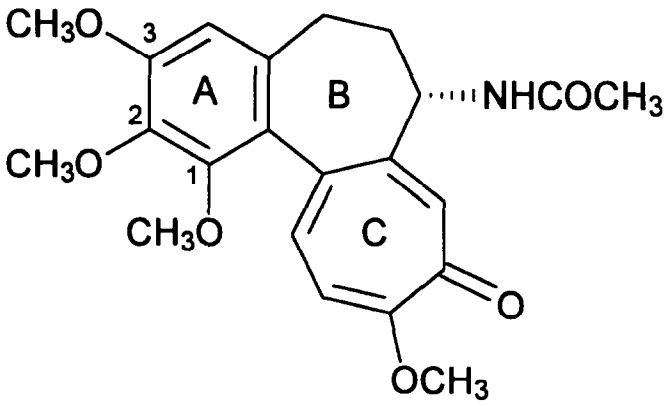
Oxidation of the N(2)-C(3) bond, converting the 2,3-dihydroquinazolinone ring to the quinazolinone ring (compound **19**), resulted in complete loss of activity in the tubulin-based assays. Substantial enhancement of activity occurred when the phenyl group of the inactive compound **19** was replaced with a styryl group (compound **13**). In the SQZ series of compounds, maximal activity required a C(6) substituent [7]; and a number of structure–activity studies were modeled around the 6-chloro derivative. In the current studies, this analog, compound **15,** was almost five times as active as **13** as an inhibitor of polymerization. In the earlier work [7], a 3-fold loss of inhibitory activity occurred when the double bond in the 2-carbon bridge was reduced; and complete loss of activity occurred when the styryl group was replaced with either a phenylethynyl or benzyl group (i.e., compound **18,** which was also inactive in the current studies). Finally, the styryl analog of compound **4** was found to have cytotoxic activity nearly equivalent to that of **4** [1]. These findings suggest that with a phenyl C ring maximal antitubulin activity would be observed in a compound with both a reduced N(2)-C(3) bond and a C(2)-styryl substituent.

In the series of DHQZ derivatives examined here, compounds **5** through **10** bore diverse substituents at the *ortho*, *meta*, and/or *para* positions of the phenyl C ring. Assuming that methyl, methoxy, and ethoxy substituents are nearly equivalent, compound **7** with *ortho-meta* disubstitution had activity equivalent to the unsubstituted **4,** while *ortho-para* disubstitution (compound **8**) resulted in loss of activity. Moderately enhanced activity occurred with trisubstitution (compound **9**) and with *ortho* monosubstitution (compound **10**). The only inactive compound in this group, **5,** bore an *ortho-chloro* substituent. Major enhancement of activity was observed only with compound **6,** which had a bulky benzyloxy group at the *meta* position of the phenyl C ring. An analogous *meta* substituent in the phenyl C ring of the PQ derivative series yielded an inhibitor with increased activity compared with the unsubstituted compound [11].

The activity of compounds **1** and **2,** combined with recent findings with combretastatin analogs [21] and PQ derivatives [10], suggest specific overlaps between the structures of the heterocyclic ketones and that of colchicine. We have suggested previously that the B ring of the combretastatins most likely corresponds to the C ring of colchicine, based on structural analogies ([22]; see Fig. 3). Recently, Medarde *et al.* [21] synthesized an active combretastatin analog in which the B ring was replaced with a naphthyl moiety. Li *et al.* [10] found that PQ derivatives with multiple methoxy groups in the C ring had little activity. In contrast, analogs with a single *meta*-methoxy group in the C ring retained full activity. These findings strongly suggest that the colchicine C ring, the combretastatin B ring, and the heterocyclic ketone C ring or moiety bind at a common site on tubulin.



Combretastatin A-4



Colchicine

FIG. 3. Structural formulas of CS-A4 and colchicine.

Among the available DHQZ derivatives, only compound **2** had a substituent at position C(6) in the A ring, and it was more active than the unsubstituted compound **1.** Substitutions in the A ring were studied extensively in both the PQ [9, 10] and the SQZ [7] series. Substituents at positions C(5) and C(8) substantially reduced or eliminated activity, as confirmed here for the SQZ derivatives **14** and **17.** Substituents at C(7) and C(6) enhanced activity relative to the unsubstituted par-

ent compounds. This is again shown here for the SQZ series with compounds **16** and **15.*** In addition, two antimitotic flavonol natural products, compounds **20** [12] and **21** [13] (structures in Fig. 2), inhibit tubulin polymerization, and both bear substituents at position C(6). In contrast to apparent limited ability to retain activity in heterocyclic ketones when A ring substitution is increased (with the apparent exception of the flavonols), maximum activity in both colchicinoids and combretastatins seems to require three methoxy groups in their A rings [22–24]. The heterocyclic moiety of the ketones may therefore not bind to tubulin in the same location as the A rings of colchicine and CS-A4, perhaps accounting for the relatively low activity of the heterocyclic ketones as inhibitors of colchicine binding.

Finally, their synthetic route indicates that compounds **1–12** should be racemic [1], and we found no optical rotation in compound **2.** If only one isomer is active, then these agents are still more potent than the data of Table 2 indicate. Efforts to resolve the chiral isomers of compound **2** by chiral chromatography have been unsuccessful thus far.

In summary, we found that a COMPARE analysis of differential cytotoxicity data predicted that antitumor DHQZ derivatives of unknown mechanism of action exerted their effect by interacting with tubulin. This prediction was confirmed by demonstrating that these agents, particularly compounds **1, 2,** and **6,** inhibited both tubulin polymerization and the binding of colchicine to tubulin. The DHQZ derivatives thus join three structurally similar groups of cytotoxic heterocyclic ketones (flavonols and SQZ and PQ derivatives) that interact with tubulin at the colchicine site. Structure-activity data from the available compounds lead to the following conclusions: (i) the importance of a C(6) substituent was again observed; (ii) reduction of the B ring double bond in active SQZ and PQ derivatives should be explored for possible enhancement of activity of these agents; and (iii) further exploration of structure–activity relationships in the C(2) substituent in any of these classes of heterocyclic ketone is merited.

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* In the current experiments, using a more sensitive reaction condition, there was only a small difference in the relative activities of compounds **15** and **16,** as opposed to over a 5-fold difference observed previously [7]. The origin of the discrepancy is not known at present.

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