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A NEW ANTI-TUBULIN AGENT CONTAINING THE BENZO[*b*]THIOPHENE RING SYSTEM

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Abstract: A new type of inhibitor of tubulin polymerization was discovered based on the 3-aroyl-2-arylbenzo[*b*]thiophene molecular skeleton. The lead compound in this series, 2-(4'-methoxyphenyl)-3-(3',4',5'-trimethoxybenzoyl)-6-methoxybenzo[*b*]thiophene **1**, inhibited tubulin polymerization, caused an increase in the mitotic index of CA46 Burkitt lymphoma cells, and inhibited the growth of several human cancer cell lines.

Introduction

An aggressive chemotherapeutic strategy toward the treatment of human solid-tumor-type cancers requires the development of new agents. One such class of drugs is those that arrest cells in mitosis through a direct interaction with tubulin. A variety of clinically promising compounds target this protein.[1] Such compounds undergo an initial binding interaction with tubulin, either unpolymerized or in microtubules, which in turn prevents formation/function of the microtubules essential for maintenance of cellular integrity and cell division.[2] Currently, the most clinically useful members of this class of drugs are the vinca alkaloids[3] and the taxoids.[4] Additionally, the natural products rhizoxin,[5] combretastatins A-4 and A-2,[6] halichondrin B,[7] and cryptophycin 1[8] (to name just a few) as well as certain synthetic analogues including the 2-styrylquinazolin-4(3H)-ones[9] and highly oxygenated derivatives of *cis*- stilbene and dihydrostilbene[10] are all known to mediate their cytotoxic activity through a binding interaction with tubulin (selected structures shown in Figure 1).

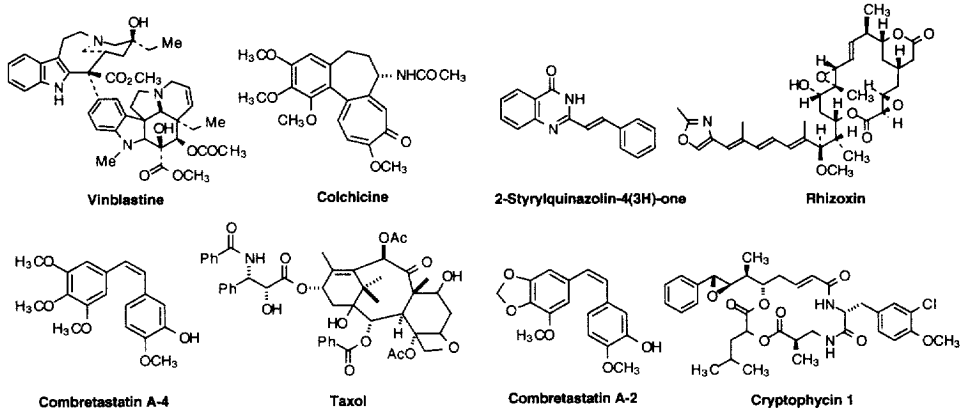

Figure 1. Representative Antimitotic Compounds which Interact with Tubulin.

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Design Rationale

An intriguing approach to the design of new antimitotic agents relies on incorporating structural features of compounds that are estrogenic in nature combined with the methoxy aryl functionality required for maximal interaction at the colchicine binding site. Such templates could be either estrogens or antiestrogens that bind to the estrogen receptor. A relevant example of this combination is 2-methoxyestradiol, which has been shown to inhibit tubulin polymerization with an IC_{50} value of 2–3 μM.[11a] In addition, several novel steroidal ligands have been prepared in an effort to identify new estradiol-based inhibitors of tubulin polymerization.[11b,c] Both 2-ethoxyestradiol and 2-(1-(*E*)-propenyl)estradiol are excellent inhibitors of tubulin polymerization, with IC_{50} values of about 1 μM. Estradiol is among the most important estrogens in humans, and it is interesting and instructive that the addition of these alkoxy aryl substituents renders this compound interactive with tubulin. This structural correlation could be based on homologies between the hormone binding site on the estrogen receptor and the colchicine site on tubulin. Alternatively, it is possible that the tubulin system plays some role in estrogen receptor binding, although 2-methoxyestradiol and its analogs have greatly reduced binding to the estrogen receptor.[11b]

We initially chose the benzo[*b*]thiophene molecular skeleton as a template since this class of compounds is known to have a very high affinity for the estrogen receptor and to function biochemically as very effective antiestrogens or selective estrogen receptor modulators.[12] In addition, two photoreactive probes for the estrogen receptor contain this ring system, and their X-ray structures have been determined.[13] The design of the new benzo[*b*]thiophene-based inhibitors (Figure 2) for tubulin incorporates structural motifs of the 3-aroyl-2-arylbenzo[*b*]thiophene ring system and the methoxy-aryl substitution pattern that seems important for the potent activity of combretastatin A-4 and colchicine (Figure 1).

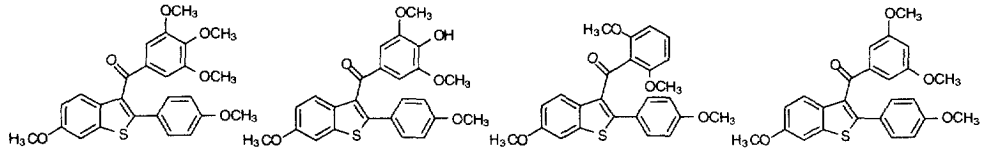


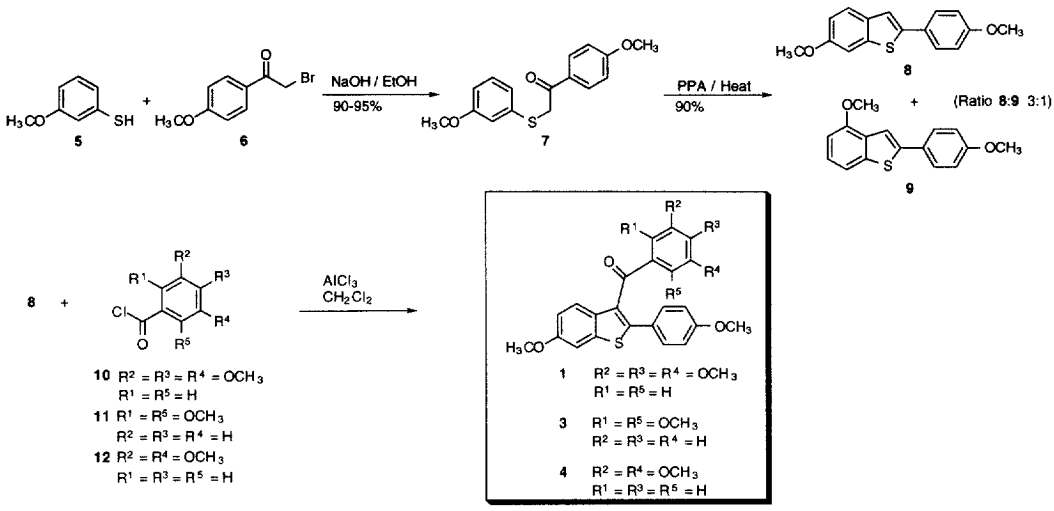
Figure 2. Benzo[*b*]thiophene Ligands Designed to Interact with Tubulin.

Results and Discussion

A series of ligands based on the benzo[*b*]thiophene molecular skeleton was prepared utilizing Friedel-Crafts aroylation as a key synthetic step (Scheme 1).[12,13] Reaction of 3-methoxybenzene thiol **5** with bromoacetophenone **6** afforded sulfide **7** in excellent yield.[12] Cyclization and concomitant aryl ring migration in the presence of polyphosphoric acid (PPA) gave a mixture of regioisomers **8** and **9**.[12] These were separated by trituration in acetone. Friedel–Crafts aroylation of 2-(4'-methoxyphenyl)-6-methoxybenzo[*b*]thiophene with the appropriate methoxy benzoyl chloride **10–12** gave the requisite benzo[*b*]thiophenes **1**, **3**, and **4** in good yield.[12,14] The phenolic derivative **2** was readily prepared by treatment of trimethoxy ligand **1** with $AlCl_3$. Alternatively, this transformation may be achieved directly during the Friedel–Crafts step (by using excess $AlCl_3$). The structure of phenol **2** was confirmed by X-ray crystallographic analysis.[15]

Ligands **1, 2, 3,** and **4** were initially screened for cytotoxic activity against the murine P388 lymphocytic leukemia cell line. The trimethoxyaroylbenzo[*b*]thiophene **1** was marginally inactive with an ED_{50} of 22 μg/mL,

Scheme 1 - Synthesis of Benzo[*b*]thiophene Antimitotic Agents



while compounds **2, 3,** and **4** were inactive (ED_{50} >100 μg/mL).

The trimethoxy aroyl ligand **1** was further evaluated against a selection of human cancer cell lines (Table 1).[16] The compound had significant (GI_{50} < 10 μg/mL) inhibitory effects on the growth of all cell lines, and it was especially effective against the colon cancer cell line KM20L2 (GI_{50} = 0.049 μg/mL).[16] In the NCI 60 cell line human tumor screen, benzo[*b*]thiophene ligand **1** demonstrated a mean panel GI_{50} = 3.31 x 10^{-7} M, with especially strong activity noted against breast cancer cell lines (for example, MCF7 (GI_{50} = 3.52 x 10^{-8} M)) as well as colon, leukemia, and ovarian cancer cell lines.[17] Based in part on this strong in vitro activity, this compound was selected (in 1998) by the National Cancer Institute for further evaluation in the form of an in vivo hollow fiber assay. Results from this study will be reported in due course.

Table 1. Activity of Trimethoxy Ligand **1** Against Selected Human Cancer Cell Lines.

		3, 4, 5 -Trimethoxybenzo[*b*]thiophene **1**
Cell Type	Cell Line	GI_{50} (μg/mL)[a]
Ovarian	OVCAR - 3	0.19
CNS	SF - 295	0.20
Renal	A498	0.46
Lung - NSC	NCI - H460	0.13
Colon	KM20L2	0.049
Melanoma	SK - MEL - 5	0.48

[a] GI_{50} = Drug concentration that inhibits cell growth by 50%

Compounds **1–4** were initially evaluated for effects on tubulin polymerization under a restrictive reaction condition that minimizes, but does not eliminate, formation of structurally aberrant polymers, such as those containing twisted spiral filaments.[18] Negligible effects on the reaction were observed with compounds

2–4, but compound **1** clearly altered the assembly reaction. The effect varied from experiment to experiment, but the reaction rate was always more substantially altered than the reaction plateau. Generally, inhibition of extent of assembly has been the parameter measured to compare drug IC_{50} values, but, as shown in Figure 3A, this proved impossible with compound **1**. Repeatedly, we observed significant reduction in the assembly rate with low concentrations of **1** (3 μM, curve 2), but even very high concentrations of the compound failed to show substantial progressive inhibition (40 μM, curve 3). Experiments with colchicine showed typical progressive inhibition and yielded an IC_{50} value of 1.4 ± 0.08 (S.D., n = 3) μM. A variety of other reaction conditions were examined, but none proved more useful for evaluation of compound **1**. An experiment performed to compare drug effect on the maximum assembly rate is shown in Fig. 3B. With **1**, 50% inhibition of the maximum rate was observed with 1.1 μM drug, compared to 0.76 μM with colchicine.

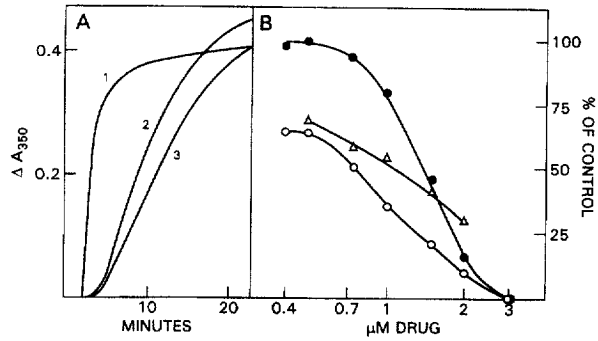


Figure 3. Inhibition of Tubulin Assembly by Compound **1**.

Reaction mixtures contained (final concentrations) 10 μM tubulin, 0.8 M monosodium glutamate (pH 6.6 with HCl), drug concentrations as indicated, and 10 μM GTP. All components except GTP were preincubated at 30 °C for 15 min in 0.24 mL. Reaction mixtures were chilled on ice, and 10 μL of 10 mM GTP was added to each mixture. The reactions were transferred to cuvettes held at 0 °C by electronic temperature controllers in Gilford model 250 spectrophotometers. Baselines were established, and the temperature was increased to 30 °C over about 60 sec. In panel A the concentrations of compound 1 were none (curve 1), 3 μM (curve 2), and 40 μM (curve 3). For panel B, the plateau readings for colchicine (•) were taken at 20 min, whereas the rate data for compound 1 (Δ) and colchicine (o) represent the maximum interval changes in turbidity, which occurred at different time points during the reactions.

These unusual assembly effects of compound **1** led us to consider that the compound might induce formation of polymer with aberrant morphology, even though there was no significant alteration in polymer temperature stability in the presence of the agent (cf. refs 11a and 18). Electron microscopy failed to demonstrate any morphological difference in polymer formed in the presence or absence of compound **1**. In both cases a mixture of microtubules and ribbons composed of parallel protofilaments was observed.

The structural analogies of benzo[*b*]thiophene **1** to colchicine and combretastatin A-4, together with the inactivity of the hypomethoxylated analogs **2–4**, indicated that benzo[*b*]thiophene **1** interacts at the colchicine binding site of tubulin. Modest inhibition (23%) of the binding of [^{3}H]colchicine to tubulin was observed with 5 μM compound **1** (Table 2), as compared with the total inhibition caused by combretastatin A-4.[6a] Increasing the concentration of **1** to 50 μM resulted in little additional inhibition.

Compounds that interact with tubulin at the colchicine site often induce a GTPase reaction uncoupled from tubulin polymerization,[19] although with some drugs the apparent stimulation of GTP hydrolysis

Table 2. Effects of Compound **1** on the Binding of [^{3}H]Colchicine to Tubulin and on Tubulin-Dependent GTP Hydrolysis.

	Experiment I Colchicine Binding [a] % Inhibition		Experiment II GTP Hydrolysis [b] % of Control	
Compound Added	10 μM	50 μM	10 μM	50 μM
Compound **1**	23	28	122	135
Combretastatin A-4	100	—	161	160
Podophyllotoxin	—	—	43	31

[a] Reaction mixtures contained 1 μM tubulin, 5 μM [^{3}H]colchicine, and inhibitor as indicated. Incubation was for 10 min at 37 °C. Without inhibitor the amount of colchicine bound to tubulin corresponded to 0.18 mol/mol. See ref 6a for further experimental details. [b]Reaction mixtures contained 10 μM tubulin, 1 mM $MgCl_2$, 1.0 M monosodium glutamate (pH 6.6 with HCl), 100 μM [8-^{14}C]GTP, and drug as indicated. Incubation was for 5 min at 37 °C. In the control reaction mixture 8.4 nmol/mL of [8- ^{14}C]GDP was formed. See ref 6a for further details.

(compared to a control reaction with a concurrent assembly reaction) is minimal (e.g., combretastatin A-2[6a]) and with others (e.g., podophyllotoxin[19]) inhibition of net hydrolysis occurs. Table 2 presents data in which the effects of compound **1** were compared with those of combretastatin A-4 and podophyllotoxin. Slight stimulation of net GTP hydrolysis occurred, which was somewhat greater with 50 μM than with 10 μM compound **1**.

In view of the relatively modest effects of compound **1** on tubulin-dependent reactions as compared with other antimitotic agents, we also considered the possibility that tubulin might not be the intracellular target of the agent. The human Burkitt lymphoma line CA46 was treated with varying concentrations of **1**, and 50% growth inhibition occurred at 2 μM. Cells treated with 10 μM had a marked increase in the mitotic index, from 3% to 30%. Such an antimitotic effect, when combined with the tubulin assembly data, is strong evidence that tubulin is the intracellular target of compound **1**. In addition, compound **1** demonstrates negligible (<0.0003%) relative binding affinity (RBA) for the estrogen receptor as compared to estradiol (RBA = 100%).[20]

Suitable crystals were grown, and the X-ray structures were solved for ligands **1**,[21] **2**,[15] and **3**.[15] In the solid state, each of these ligands has a propensity to orient its pendant aroyl ring in a pseudo-stacking interaction with the aryl ring at C-2 of the benzo[*b*]thiophene skeleton. For molecules of relatively small size, such as ligands **1**, **2**, and **3**, it is often an accurate assumption to predict that the solid state structure will approximate the structure adopted in solution.[22] Presumably, the molecular cleft formed by the pseudo-stacking orientation of these aryl rings plays a key role in the binding interaction with tubulin.[23]

Conclusions

We have discovered a new type of inhibitor of tubulin polymerization based on the 3-aroyl-2-arylbenzo[*b*]thiophene molecular skeleton. The first promising compound in this series, 2-(4'-methoxyphenyl)-3-(3',4',5'-trimethoxybenzoyl)-6-methoxybenzo[*b*]thiophene **1**, most likely interacts with tubulin at the colchicine site and has significant human cancer cell growth inhibitory activity (GI_{50} < 10 μg/mL).

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