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MORPHOLINO DERIVATIVES OF BENZYL-BENZODIOXOLE, A STUDY OF STRUCTURAL REQUIREMENTS FOR DRUG INTERACTIONS AT THE COLCHICINE/PODOPHYLLOTOXIN BINDING SITE OF TUBULIN

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Abstract—We performed a structure–activity evaluation of the effects of methoxy substituents in the benzyl moiety of a series of morpholinyl Mannich base derivatives of 6-benzyl-1,3-benzodioxol-5-ol ("morpholino compounds") on the ability of these compounds to inhibit tubulin polymerization *in vitro*. Structurally these agents are most similar to the natural product podophyllotoxin and, like podophyllotoxin, they inhibited *in vitro* tubulin polymerization, tubulin-dependent GTP hydrolysis, and the binding of colchicine to tubulin. The benzyl ring (C ring) of these compounds appeared to be analogous to the trimethoxybenzene ring (E ring) of podophyllotoxin (with its methoxy substituents at the 3′, 4′ and 5′ positions), but the morpholino compound superficially most similar to podophyllotoxin (with 3′, 4′ and 5′ methoxy substituents) was the least active in the series. The most potent methoxy-substituted morpholino compounds bear these substituents either at the 2′ and 4′ positions (NSC 370277) or at the 2′, 4′ and 6′ positions (NSC 381577). NSC 370277 and NSC 381577 were essentially identical in their inhibitory effects on tubulin polymerization, but the latter compound was considerably more effective as an inhibitor of the binding of colchicine to tubulin. The most active of the monomethoxy substituted compounds bore this group at position 4′. A number of compounds with alternate substituents at this position (in particular, alkyl-substituted amines) also had significant *in vitro* inhibitory effects on tubulin polymerization. Although the morpholino compounds appear to possess only limited cytotoxicity, these findings suggest possible modifications of the antimitotic benzyl-benzodioxole compounds described previously [Batra *et al.*, *Molec. Pharmac.* **27**, 94 (1985)] to enhance their antineoplastic activity.

Among the many functions of microtubules in eucaryotic cells is their participation in cell division, for they form the framework of the mitotic spindle. As a consequence, virtually all antimitotic drugs interfere with normal formation of microtubules. This generally occurs through a specific interaction with their major component, the protein tubulin. Although of all antimitotic agents only the vinca alkaloids have a well-defined role in the therapy of neoplastic diseases, a great deal of attention has also been directed at colchicine and its interactions with tubulin. Indeed, the essentially irreversible binding of colchicine to tubulin was an important property exploited in the initial purification of tubulin from mammalian brain [1]. Subsequently a large number of antimitotic compounds have been discovered to have specific interactions with tubulin which include interference with the binding of colchicine to the protein. The most potent agents include the natural products podophyllotoxin [2, 3], steganacin [4], and combretastatin [5], and the following synthetic compounds: a large number of benzimidazole carbamates including nocodazole [6, 7]; the 1-deaza-7,8-dihydropteridine carbamate NSC 181928 [8]; the bicyclic compound MTPT§ [9]; 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione (TN-16) [10]; and a number of derivatives of benzyl-benzodioxole [11].

The benzyl-benzodioxole compounds have been of particular interest to us because of their apparent structural analogy to podophyllotoxin, steganacin and colchicine (see Fig. 1) and because their synthesis is relatively facile. This permits a detailed examination of structure–function relationships which should provide insights into key features of the colchicine/podophyllotoxin binding site on tubulin and may assist in the design of optimally active antineoplastic drugs. In this report we describe *in vitro* interactions of a new series of benzyl-benzodioxole derivatives [12] with tubulin. This series of compounds all bear a morpholino substituent on the benzylic bridge carbon between the benzodioxole and phenyl rings ("morpholino compounds", see Fig. 1).

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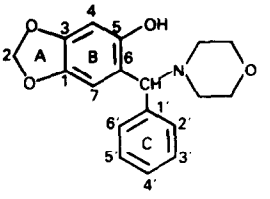
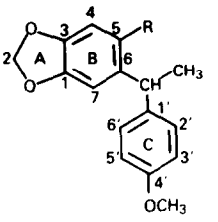
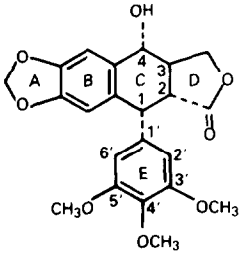
§ Abbreviations: MTPT, 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone; MAPs, microtubule-associated proteins; and Mes, 2-(*N*-morpholino)ethanesulfonate.



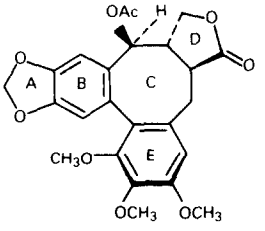
MORPHOLINYL MANNICH BASE DERIVATIVE
OF 6-BENZYL-1,3-BENZODIOXOL 5 OL



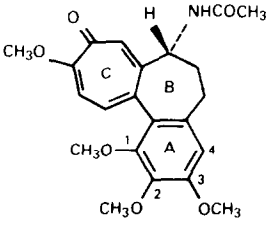
NSC 321567 : R = $-OCH_2CH_3$
NSC 350102 : R = $-OCH_3$



PODOPHYLLOTOXIN



STEGANACIN



COLCHICINE

Fig. 1. Structural formulas of benzyl-benzodioxole derivatives and related natural products.

MATERIALS AND METHODS

Heat-treated MAPs and electrophoretically homogeneous tubulin from calf brain were prepared as described previously [13]. GTP and [8-^{14}C]GTP were obtained from the Sigma Chemical Co. and Moravek Biochemicals, respectively, and both preparations were repurified by gradient chromatography on DEAE-Sephadex A-25. Colchicine was from Sigma, podophyllotoxin from the Aldrich Chemical Co.,

Table 1. Effect of phenyl ring substituents on the inhibition of tubulin polymerization with morpholinyl Mannich base derivatives of 6-benzyl-1,3-benzodioxol-5-ol*

Drug added (NSC number)		Substituents†	IC_{50}‡
	IC_{50}‡ 5–10 μM		
368269		$N(CH_3)_2$ at 4′	6.7 ± 0.2§
370277		OCH_3 at 2′ and 4′	9.1 ± 0.2
381577		OCH_3 at 2′, 4′ and 6′	7.4 ± 0.3
600216		$N(CH_2CH_3)_2$ at 4′	6.1 ± 0.3
	IC_{50}‡ 10–15 μM		
364724		OCH_3 at 4′	13.5 ± 0.2
381575		OCH_3 at 2′, 3′ and 4′	10.5 ± 0.3
	IC_{50}‡ 15–25 μM		
368252		OCH_3 at 3′ and 4′	16.6 ± 0.4
368275		Cl at 4′	19.0 ± 0.2
368277		F at 4′	17.0 ± 0.2
381579		OCH_3 at 2′	17.0 ± 0.2
	IC_{50}‡ 25–50 μM		
381576		OCH_3 at 2′ and 3′	27.0 ± 0.3
604289		OCH_3 at 3′	38.5 ± 0.4
	IC_{50}‡ 50–100 μM		
368260		OCH_3 at 3′, 4′ and 5′	90.0 ± 0.6

* The structural formulas of these compounds are presented in Fig. 1.

† All substituents are on the phenyl ring (C ring) as indicated in Fig. 1.

‡ Defined as the drug concentration required for 50% inhibition of the turbidity plateau after a 20-min incubation at 37°. Reaction conditions were as described in the text. IC_{50} values (mean ± S.D.) were based on a minimum of two independent determinations at the drug concentrations heading each subgroup of compounds.

§ Statistical evaluation of the data was by probit analysis as described by Goldstein *et al.* [19].

Table 2. Effects of morpholino compounds on tubulin-dependent GTP hydrolysis*

Drug added†	GDP (nmoles formed/ml) Experiment I‡ (1.0 M glutamate)	Experiment II§ (0.1 M Mes − MAPs)	Experiment III‖ (0.1 M Mes + MAPs)
None	9.1	2.4	29.1
Podophyllotoxin	3.2	1.7	3.1
NSC 321567	6.1	1.8	4.1
Colchicine	30.6	26.6	29.3
Steganacin	20.7	15.7	18.8
NSC 368269	1.4	1.3	1.8
NSC 370277	5.7	1.3	2.2
NSC 381577	3.5	1.4	2.5
NSC 600216	0.8	1.4	2.1

* All data are expressed as nmoles of [8-^{14}C]GDP formed per ml of reaction mixture. Incubations were for 20 min at 37°. In all experiments, 50 μl reaction mixtures contained 1.0 mg/ml of tubulin, 0.1 mM [8-^{14}C]GTP, and 0.1 mM drug, if present. Each experiment was performed at least three times (although incubation times varied), and qualitatively identical results were obtained in all experiments.

† Drug structures are as in Fig. 1 and Table 1.

‡ Reaction mixtures contained 1.0 M monosodium glutamate (adjusted to pH 6.6 with HCl).

§ Reaction mixtures contained 0.1 M Mes (adjusted to pH 6.4 with NaOH) and 0.5 mM $MgCl_2$.

‖ Reaction mixtures contained 0.1 M Mes (pH as above), 0.5 mM $MgCl_2$ and 0.5 mg/ml of heat-treated MAPs.

and [^{3}H]colchicine from Amersham. Steganacin was a gift of Dr. J-P. Robin. NSC 321567 and NSC 350102 [14] and the morpholino compounds [12] were synthesized as described previously. All drugs were dissolved in dimethyl sulfoxide, and control reaction mixtures contained equivalent amounts of the solvent.

Tubulin polymerization was followed turbidimetrically [15] as described previously [11]. The 0.25-ml reaction mixtures contained 1.0 mg/ml tubulin, 1.0 M monosodium glutamate, 0.1 mM GTP, 1.0 mM $MgCl_2$, and drugs as indicated. Colchicine binding to tubulin was measured by the DEAE-cellulose filter assay [16] essentially as described previously [8]. GTP hydrolysis was measured by following the formation of [8-^{14}C]GDP from [8-^{14}C]GTP, using thin-layer chromatography on polyethyleneimine-cellulose and autoradiography [17].

RESULTS

Figure 1 presents the structural analogies between podophyllotoxin, steganacin, colchicine, the series of benzyl-benzodioxole derivatives previously described [11], and the morpholino compounds we will discuss in detail here. Most of the active compounds described earlier have a 1-3 carbon substituent at position 5 (an ethoxy group in NSC 321567, a methoxy group in NSC 350102), a methyl group at the benzylic bridge carbon (all compounds studied were racemic mixtures at this position), and a single methoxy group at position 4′. Further, we found that additional methoxy groups at positions 3′ and 5′, which superficially would seem to enhance the analogy of these agents to podophyllotoxin, steganacin and colchicine, practically eliminated interactions of this class of compounds with bovine brain tubulin.

* The IC_{50} values in this system for podophyllotoxin and steganacin are 0.5 to 1.0 and 5 μM respectively.

The morpholino compounds all have a hydroxyl substituent at position 5, and a morpholino group replaces the methyl group at the bridge carbon. Again, all compounds are racemic mixtures, and there is strong hydrogen bonding of the 5-hydroxyl group to the morpholino group [12].

Derivatives with a wide variety of substituents on the phenyl ring were examined for inhibitory effects on glutamate-induced [18] tubulin polymerization (Table 1). As in the previous studies [11], we have defined the IC_{50} as the drug concentration that reduces the turbidity reading after 20 min at 37° by 50%.* (It should be noted that much lower values would be obtained if the IC_{50} were defined in terms of inhibiting the rate of turbidity development, but the relative activities of the derivatives would be similar.) Morpholino compounds with substituents in the phenyl ring analogous to those studied earlier—i.e. with a single methoxy group at the 4′ position (NSC 364724; IC_{50} 10–15 μM), with two methoxy groups at 3′ and 4′ (NSC 368252; IC_{50} 15–25 μM), and with three methoxy groups at 3′, 4′ and 5′ (NSC 368260; IC_{50} 50–100 μM)—were similar in their relative inhibitory effects: increasing the superficial analogy to the natural products resulted in a decreasing interaction with tubulin.

To gain insight into this apparent paradox, the additional compounds described in Table 1 were synthesized and evaluated. Of the compounds with a single methoxy group on the phenyl ring (NSC 364724, NSC 381579, and NSC 604289), the greatest inhibitory effect on tubulin polymerization occurred with the substituent at the 4′ position and the least with the substituent at the 3′ position. An additional methoxy group at the 3′ position invariably reduced the inhibitory effect of compounds in this series: cf. NSC 381576 to NSC 381579; NSC 368252 to NSC 364724; and NSC 381575 to NSC 370277. The most active compounds with phenyl ring methoxy substituents were NSC 370277

Table 3. Effects of morpholino compounds on the binding of colchicine to tubulin*

Drug added	Colchicine bound (% of control)
Podophyllotoxin	2.7
NSC 321567	5.9
Steganacin	17
NSC 368269	47
NSC 370277	96
NSC 381577	34
NSC 600216	30

* Reaction mixtures (0.1 ml) contained 0.1 mg/ml tubulin, 5 μM [^{3}H]colchicine, and the indicated drug at 25 μM. Other reaction components and the DEAE-cellulose paper filtration procedure were as described previously [8]. Incubation was for 10 min at 37°. All values represent the average of triplicate determinations which were within 10% of each other.

(methoxy groups at the 2′ and 4′ positions) and NSC 381577 (methoxy groups at the 2′, 4′ and 6′ positions). The inhibitory effects of these latter two agents on glutamate-induced tubulin polymerization were similar to those of the active drugs described earlier, including NSC 321567 and NSC 350102 [11].

In the course of these studies, two additional analogs with equally potent inhibitory effects were identified. These compounds both had amine substituents at the 4′ position of the phenyl ring (NSC 368269, dimethylamine; NSC 600216, diethylamine). Significant, but weaker, inhibitory effects on tubulin polymerization were also observed in agents with a halide substituent at the 4′ position (NSC 368275 and NSC 368277).

To further characterize this group of drugs, the four most active morpholino compounds were examined for their effects on tubulin-dependent GTP hydrolysis under three reaction conditions—in 1.0 M glutamate and in 0.1 M Mes–0.5 mM $MgCl_2$ both with and without heat-treated MAPs*—and compared to colchicine, steganacin, podophyllotoxin and NSC 321567 (Table 2). Under all three conditions, the morpholino compounds inhibited GTP hydrolysis, as did podophyllotoxin and NSC 321567, while colchicine and steganacin enhanced the GTPase reaction.

If the morpholino compounds are indeed podophyllotoxin analogs, as indicated by their structure and their effects on tubulin-dependent GTP hydrolysis, they should competitively inhibit the binding of radiolabeled colchicine to tubulin as does podophyllotoxin. In the study presented in Table 3, the four morpholino compounds which were most potent in inhibiting polymerization were compared to podophyllotoxin, steganacin and NSC 321567 for inhibition of the binding of colchicine to tubulin (the molar ratio of inhibitor to colchicine was 5). None of these morpholino compounds was nearly as potent as either podophyllotoxin or NSC 321567 as inhibitors of colchicine binding (the inhibitory effect of steganacin was intermediate between that of NSC 321567 and that of NSC 600216), and there was no significant inhibition of colchicine binding with NSC 370277. All the compounds listed in Table 1 were also examined for inhibition at a molar ratio of 20 to 1 to radiolabeled colchicine, and feeble inhibition of colchicine binding was observed with nearly all of them (at the higher drug concentration greater than 50% inhibition was observed only with NSC 370277 and NSC 381575, in addition to the three compounds which were inhibitory at the lower concentration).

Despite their limited inhibition of colchicine binding, however, the morpholino compounds are probably competitive inhibitors of this reaction. The 2′,4′,6′-trimethoxy compound NSC 381577 was examined in detail. Lineweaver–Burk analysis (Fig. 2A) of the data of this experiment demonstrates that the compound inhibited the binding of colchicine to tubulin in a competitive fashion, whereas a Dixon plot (Fig. 2B) of the same data yielded a K_i value of approximately 8 μM [20]. More abbreviated studies under the same experimental conditions yielded K_i values of about 0.4 μM for podophyllotoxin, 1 μM for NSC 321567, and 3 μM for steganacin. A previous detailed study with the benzyl-benzodioxole derivative NSC 350102 yielded a K_i value of 0.6 μM [11].

DISCUSSION

We have presented here a structure–function analysis of effects on tubulin polymerization of a series of benzyl-benzodioxole derivatives modified in the benzyl moiety (the C ring, analogous to the E ring of podophyllotoxin). Because the morpholino group at the benzylic bridge carbon between the phenyl and benzodioxole ring systems facilitates synthesis of compounds with various substituents on the phenyl ring [12], we prepared a fairly complete series with methoxy substituents in order to examine the importance of this group as a function of position in the C ring. In addition, we observed several compounds with unexpected activity bearing other substituents (amines or halides) at position 4′. It should be noted that the morpholino compounds (unlike the previously described NSC 321567 and NSC 350102 [11]) have no significant antileukemic activity *in vivo*; and, in addition, with the four agents most effective in inhibiting tubulin polymerization *in vitro*, we were unable to demonstrate cytotoxicity in cell culture or any effect on the mitotic index of cultured cells. The chief interest of these compounds would therefore seem to be in the structure–activity information they can provide to serve as clues for the chemical modification of analogous structures that do have activity *in vivo* and in intact cells.

The benzyl-benzodioxole derivatives seem to be most comparable to podophyllotoxin in their interactions with tubulin and in their molecular structure. The A and B rings of podophyllotoxin are identical to the benzodioxole ring system, whereas the trimethoxybenzene ring (ring E) of podophyllotoxin is comparable to the phenyl ring of the benzyl-benzodioxole derivatives. The carbon atom at the 1 position of the C ring of podophyllotoxin may be the

* The heat-treated MAPs used here, in the absence of tubulin, have no GTPase activity [13].

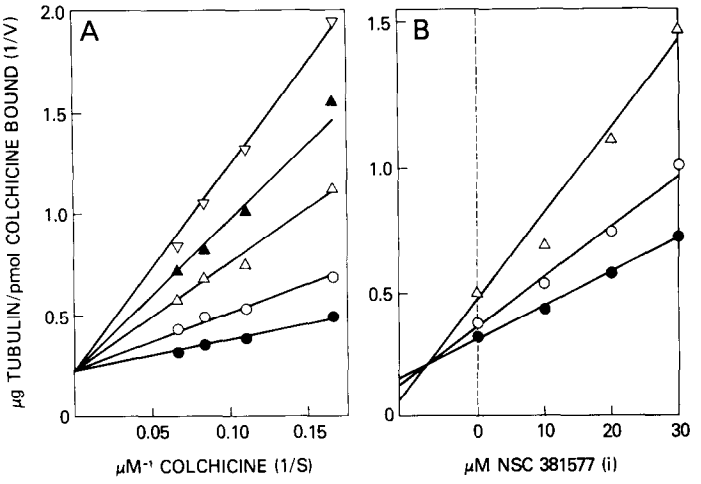


Fig. 2. Competitive inhibition of the binding of colchicine to tubulin by NSC 381577. Reaction conditions were as described in Table 1. In both panels, $1/V$ on the ordinate represents the inverse of the number of pmoles of colchicine retained by the DEAE-cellulose filters. All experimental points represent the average of triplicate determinations which were within 10% of each other. (A) Lineweaver–Burk analysis of the data. The values on the abscissa ($1/S$) are the inverse values of the micromolar concentrations of colchicine used in the experiment. Symbols are as follows: (●) no inhibitor; (○) 10 μM NSC 381577; (△) 20 μM NSC 381577; (▲) 30 μM NSC 381577; and (▽) 40 μM NSC 381577. (B) Dixon analysis of the data. The values on the abscissa (i) are the micromolar concentrations of NSC 381577 used in the experiment. Symbols are as follows: (△) 6 μM colchicine; (○) 9 μM colchicine; and (●) 15 μM colchicine.

structural analog of the benzylic bridge carbon in the benzyl-benzodixole derivatives. The benzyl-benzodioxole compounds, like podophyllotoxin, were competitive inhibitors of the binding of colchicine to tubulin, although none was as potent as podophyllotoxin itself. The morpholino compounds were especially poor competitors of colchicine binding, although their weak inhibitory effect (at least in the case of NSC 381577—the other methoxy-substituted morpholino compounds were too feeble as inhibitors for adequate quantitative studies) fulfills kinetic criteria [20] for competitive inhibition. Finally, the morpholino compounds, like the cytotoxic benzyl-benzodioxole derivatives NSC 321567 and NSC 350102, were most similar to podophyllotoxin in their effects on tubulin-dependent GTP hydrolysis, both under reaction conditions in which polymerization occurred (1.0 M glutamate; 0.1 M Mes + MAPs) or failed to occur (0.1 M Mes without MAPs). The benzyl-benzodioxole derivatives and podophyllotoxin inhibited GTP hydrolysis, but not as profoundly as maytansine and vinblastine [11]. Colchicine, steganacin and the bicyclic colchicine analogs combretastatin and MTPT, on the other hand, stimulate tubulin-dependent GTP hydrolysis [5, 11, 21].

The current structure–activity studies with the morpholino compounds are in agreement with those presented previously [11] with NSC 321567, NSC 350102 and related compounds: a single methoxy group on the C ring yielded maximum activity if at position 4′, while additional methoxy groups at positions 3′ and 5′ lead to progressively reduced activity despite the apparent increase in structural analogy to podophyllotoxin. To explore this seeming paradox the additional morpholino compounds described in Table 1 were synthesized. The members of this series with methoxy substituents exhibited reduced inhibitory activity with a 3′ substituent and enhanced inhibitory activity with a 2′ substituent (in the presence of a 4′-methoxy group). Of particular note was the greater inhibition of polymerization with the 2′,4′-dimethoxy compound (NSC 370277) as compared to the 2′,3′,4′-trimethoxy compound (NSC 381575), as well as the equivalent inhibitory effects on polymerization of the 2′,4′-dimethoxy compound (NSC 370277) and the 2′,4′,6′-trimethoxy compound (NSC 381577). Moreover, the latter compound seems to have the greatest affinity for tubulin of all the C ring methoxy-substituted morpholino compounds, as judged by its IC_{50} value for the inhibition of polymerization and, particularly, by its inhibitory effects on colchicine binding.

In our earlier report on benzyl-benzodioxole derivatives, we noted a number of observations in the literature on the biological activity of podophyllotoxin and colchicine: (i) based on molecular models, Gensler and Gatsonis [22] observed that the trimethoxy benzene ring of podophyllotoxin has limited rotational mobility, while that of the nearly inert picropodophyllotoxin (with reversed configuration at position 2) freely rotates; (ii) also based on molecular models, Margulis [23] proposed that the 1-methoxy group of colchicine corresponds to the 5-methoxy group of podophyllotoxin; (iii) Brossi and his colleagues [24, 25] have reported that, although removal of a single methyl group from colchicine reduces its activity (with a still further drop in activity if two methyl groups are removed), demethylation at position 1 results in a larger drop in activity than demethylation at position 2 or 3; and (iv) Kelleher [2] and Loike *et al.* [3] have reported that interactions of podophyllotoxin (as well as epipodophyllotoxin and deoxypodophyllotoxin) with tubulin are unaltered, or even enhanced, by removal of the 4′-methyl group. Based on these findings, we speculated [11] that the required 4′-methoxy group in the

benzyl-benzodioxole derivatives probably corresponded to the 5′-methoxy group of podophyllotoxin, and that the feeble activity of 3′,4′,5′-trimethoxybenzene benzyl-benzodioxole compounds probably resulted from the freely mobile C ring.

The results presented here with the morpholino compounds validate these speculations. Of particular note are the much greater inhibitory effect of the 2′,3′,4′-trimethoxybenzene compound (NSC 381575) as compared to the 3′,4′,5′-trimethoxybenzene compound (NSC 368260); the still greater inhibitory effect of the 2′,4′-dimethoxybenzene compound (NSC 370277) as compared to NSC 381575 (cf. the greater inhibitory effect on microtubule assembly of 4′-demethylpodophyllotoxin as compared to podophyllotoxin reported by Loike *et al.* [3]); and the maximum effect, if both inhibition of polymerization and of colchicine binding are considered, of the 2′,4′,6′-trimethoxybenzene compound (NSC 381577). The activity of the latter agent may indicate that the side of the trimethoxybenzene ring bearing the substituents fits in a hydrophobic pocket of tubulin while the other side is exposed to the solvent. NSC 381577, with its relatively uniform distribution of methoxy groups, may thus have an increased number of rotational conformations that favor an interaction with tubulin.

We are currently attempting to synthesize agents similar to those described earlier [11], i.e. with a 1-3 carbon substituent at position 5 and a methyl group at the benzylic bridge carbon, bearing substituents on the C ring which have given maximal activity in the morpholino compounds. We hope that such drugs will have enhanced affinity for tubulin and significant antineoplastic activity.

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