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METHYLENEDIOXY-BENZOPYRAN ANALOGS OF PODOPHYLLOTOXIN, A NEW SYNTHETIC CLASS OF ANTIMITOTIC AGENTS THAT INHIBIT TUBULIN POLYMERIZATION

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Abstract—A new class of compounds was synthesized and, based on structural analogy to podophyllotoxin, examined as potential microtubule inhibitors and evaluated for *in vivo* antineoplastic activity. These agents are derivatives of methylenedioxy-benzopyran bearing a phenyl substituent at position 8. The hydrogen atoms at positions 7 and 8 are in a *trans* configuration, in contrast to the *cis* configuration of analogous hydrogen atoms at positions 1 and 2 in podophyllotoxin. Compounds with a variety of substituents at positions 6 and 7 were examined, as well as compounds with varying methoxy substituent patterns on the phenyl ring attached at position 8. The most active compounds inhibited tubulin polymerization at concentrations approximately stoichiometric with tubulin, competitively inhibited the binding of colchicine to tubulin, and caused mitotic arrest at cytotoxic drug concentrations. No structure–activity correlations were obvious for the substituents at positions 6 and 7, but optimal activity was only observed when the phenyl substituent at position 8 was a trimethoxybenzene ring identical to the analogous ring in podophyllotoxin (i.e. methoxy groups at positions 3′, 4′ and 5′). Despite their structural and functional similarities to podophyllotoxin, however, the methylenedioxy-benzopyran derivatives subtly differ from the natural product in their interaction with tubulin, for they stimulated rather than inhibited tubulin-dependent GTP hydrolysis.

Virtually all antimitotic drugs interfere with the formation of a functioning mitotic spindle by preventing microtubule formation, and their basic mechanism appears to be specific interactions with the protein tubulin, the major component of these organelles. Although several distinct drug binding sites on tubulin have been described, the majority of known antimitotic agents appear to bind in a single region of the tubulin molecule, the colchicine/podophyllotoxin site. The binding of colchicine to tubulin has been studied extensively, and this essentially irreversible reaction was originally exploited in the initial purification of tubulin from mammalian brain [1]. Besides podophyllotoxin [2, 3], other drugs that bind to the same or overlapping sites on tubulin include steganacin [4], combretastatin [5] and combretastatins A-1 and B-1 [6], 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone [7], a number of derivatives of benzyl-benzodioxole [8–10], a large number of benzimidazole carbamates including nocodazole [11–13], the 1-deaza-7,8-dihydropteridine carbamate NSC 181928 [14] and related compounds [15], 3-(1-anilinoethylidene)-5-benzylpyrrolidine-2,4-dione (TN-16) [16], and 2-(2-thenyl)sulfonyl-5-bromopyrimidine (NY 4137) [17].

Extension of the chemistry used in the synthesis of the earlier benzyl-benzodioxole derivatives [10, 18, 19]—which appear to be most similar to podophyllotoxin in their interactions with tubulin [8, 9]—led to the preparation of a new phenyl-substituted tricyclic series of compounds with still closer structural analogy to podophyllotoxin [20]. Figure 1 compares podophyllotoxin to the central nucleus of these new heterocyclic agents, which can be considered derivatives of methylenedioxy-benzopyran. (In Fig. 1 standard position numbers are presented for the tricyclic portion of the molecule, but, for simplicity and ease of comparison to podophyllotoxin, nonstandard "prime" numbers are used for the phenyl ring attached at position 8.) In this paper we report that a number of these "benzopyran compounds" have significant inhibitory effects on the growth of murine L1210 leukemia cells in culture with the accumulation of cells in metaphase arrest, on the *in vitro* polymerization of purified tubulin, and on the binding of colchicine to the protein. In addition, initial screening of some of the benzopyran compounds in the National Cancer Institute's P388

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murine leukemia screen has demonstrated potential antineoplastic activity for these new agents.*

MATERIALS AND METHODS

The synthesis of the benzopyran compounds was described elsewhere [20]. NSC 321567 and 370277 were synthesized as described previously [18, 19]. Colchicine was from Sigma (St. Louis, MO), podophyllotoxin from Aldrich (Milwaukee, WI), and [^{3}H]colchicine from Amersham (Arlington Heights, IL). All nonradiolabeled drugs were dissolved in dimethyl sulfoxide, and control reaction mixtures contained equivalent amounts of the solvent. Heat-treated MAPs† and electrophoretically homogeneous tubulin from bovine brain were prepared as described previously [21]. GTP from Sigma and [8-^{14}C]GTP from Moravek (Brea, CA) were repurified as described previously [8].

Tubulin polymerization was followed turbidimetrically [22], as described previously [9], in 0.25-ml reaction mixtures containing 1.0 mg/ml tubulin, 1.0 M monosodium glutamate (pH 6.6 with HCl), 1.0 mM $MgCl_2$, 0.4 mM GTP, and drugs at various concentrations. To determine IC_{50} values, all components except GTP were preincubated for 15 min at 37° in a 0.24-ml volume. Reaction mixtures were then chilled on ice, 10 μl of 10 mM GTP was added, and polymerization was followed spectrophotometrically [8] for 20 min at 37°. Polymerization was then reversed by chilling reaction mixtures to 0°. The drop in turbidity in drug-treated samples was compared to the drop in turbidity in control reaction mixtures (duplicates, which were always within 5% of each other). The following drug concentrations were evaluated, as appropriate: 2, 3, 4, 5, 7.5, 10, 15, 20, 25, 30, 40, 50, 75, and 100 μM. Each IC_{50} value is expressed as a concentration range, i.e. it is defined as being greater than the drug concentration which caused a change in cold-reversible turbidity greater than 50% of the control value and less than the drug concentration which caused a change in cold-reversible turbidity less than 50% of the control value. When an initial value was obtained, it was confirmed independently on a different day. In a few cases, presumably as a consequence of experimental variation, the second experiment did not confirm the precise IC_{50} value. When this occurred, the determination was repeated two more times, and the result of three out of four experiments is presented. With one compound (NSC 602341), values of 15–20 and of 20–25 μM were both obtained twice. The IC_{50} value for this agent is thus cited as 20 μM. It should also be noted that all drugs were evaluated for inhibition of tubulin polymerization under other experimental conditions in addition to that presented here; these experiments also demonstrated significant effects of the active agents on the polymerization reaction.

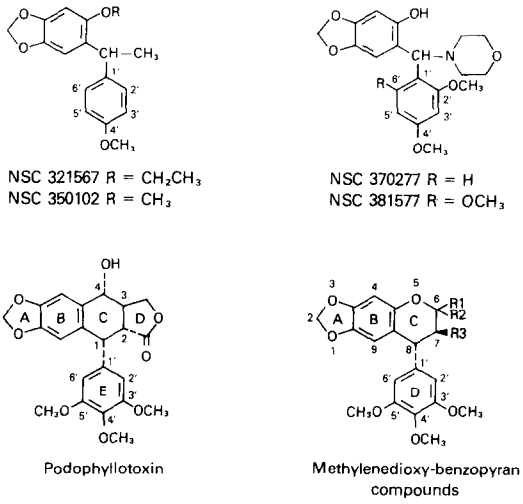

Fig. 1. Structural formulas of podophyllotoxin, the common elements of the methylenedioxy-benzopyran compounds, and selected benzyl-benzodioxole derivatives with strong inhibitory effects on *in vitro* tubulin polymerization.

Colchicine binding to tubulin was measured by the DEAE-cellulose filter assay [23] as described previously [9, 14]. GTP hydrolysis was measured by following the formation of [8-^{14}C]GDP, using thin-layer chromatography on polyethyleneimine-cellulose and autoradiography [24]. Culture of murine L1210 leukemia cells and evaluation of cultured cells for mitotic arrest was performed as described by Wolpert-DeFilippes *et al.* [25], except that before fixation cells were swollen for 5 min in 0.75-strength phosphate-buffered saline solution.

RESULTS AND DISCUSSION

Figure 1 compares the structures of podophyllotoxin and of the previously described active benzyl-benzodioxole derivatives NSC 321567, 350102 [8], 370277, and 381577 [9] to that of the benzopyran compounds. NMR data obtained in the structural identification of the benzopyran compounds [20] indicate that the hydrogen atoms at positions 7 and 8 (when R3 is a non-hydrogen substituent) have a *trans* configuration. Such compounds are diastereomeric with an additional asymmetric center at position 6. The configurations at position 6 are not known at this time.

Table 1 summarizes the activities of compounds with a 3′,4′,5′-trimethoxyphenyl substituent at position 8 (Fig. 1) as inhibitors of glutamate-induced polymerization of purified tubulin. Besides the maximally inhibitory compounds (NSC 371002, 381578,

* Of the fifty-two benzopyran compounds described in this report, thirty-nine were evaluated for *in vivo* activity in murine P388 leukemia. Significant increase in survival time was observed with the following four compounds: NSC 371002, 381578, 381598, and 600023. As with the benzyl-benzodioxole derivatives [10], survival data were similar to those observed in comparable studies with podophyllotoxin, but higher doses of the benzopyran compounds were required. NSC 381578 was also examined in other model murine tumor systems and proved active in two of them, L1210 leukemia and M5076 reticulum cell sarcoma. The authors are indebted to Dr. K. Paull, Drug Synthesis and Chemistry Branch, National Cancer Institute, for providing this information.

† Abbreviations: MAPs, microtubule-associated proteins; and MES, 2-(*N*-morpholino)ethanesulfonate.

Table 1. Relative activities of 3′,4′,5′-trimethoxybenzene benzopyran compounds as inhibitors of tubulin polymerization

Drug (NSC number)	Nonhydrogen substituents at positions 6 and 7	IC_{50} (μM)
371002	6: CH_3; *N*-tetrahydropyrrolyl	10–15
381578	6: OH; CH_3	10–15
381582	6: CH_3; OCH_3	
	7: CH_3	10–15
600023	6: OH; CH_3	
	7: CH_3	10–15
381586	6: OH	
	7: CH_3	15–20
375501	6: *N*-tetrahydropyrrolyl	
	7: CH_3	25–30
600217	6: OCH_3	
	7: CH_3	40–50
381589	6: OH; CH_3	
	7: $COCH_3$	50–75
381598	6: CH_3; OCH_3	50–75
371010	6: CH_3; *N*-tetrahydropyrrolyl	
	7: CH_3	>100
371016	6: CH_3; *N*-tetrahydropyrrolyl	
	7: $COOCH_2CH_3$	>100
375499	6: CH_3	
	7: $COOCH_2CH_3$	
	6–7 bond is double	>100
375500	6: O (ketone)	
	7: $COCH_3$	>100
381583	6: CH_3	
	7: CH_3	
	6–7 bond is double	>100
381587	7: CH_3	
	6–7 bond is double	>100
381588	6: CH_3; OCH_3	
	7: $COOCH_2CH_3$	>100
381591	6: CH_3	
	7: $COCH_3$	
	6–7 bond is double	>100
381595	6: CH_3; OCH_3	
	7: $COCH_3$	>100
602339	6: CH_3; OCH_2CH_3	
	7: CH_3	>100
602343	6: CH_3; OCH_2CH_3	>100
602344	6: CH_3; $OCH_2CH_2CH_3$	
	7: CH_3	>100
602346	6: CH_3; $OCH_2CH_2CH_3$	>100

IC_{50} Values were determined as described in the text in 0.25-ml reaction mixtures containing 1.0 mg/ml tubulin, 1.0 monosodium glutamate (pH 6.6), 1.0 mM $MgCl_2$, 0.4 mM GTP, and drugs at various concentrations, with a drug–tubulin preincubation prior to the addition of GTP. Systematic names of the five most active compounds are as follows: NSC 371002—1-[7,8-dihydro-6-methyl-8-(3,4,5-trimethoxyphenyl)-6*H*-1,3-dioxolo[4,5-*g*][1]benzopyran-6-yl]-pyrrolidine; NSC 381578—7,8-dihydro- 6-methyl- 8-(3,4,5-trimethoxyphenyl)- 6*H*- 1,3-dioxolo[4,5-*g*][1]benzopyran-6-ol; NSC 381582—7,8-dihydro-6,7-dimethyl-6-methoxy-8-(3,4,5-trimethoxyphenyl)-6*H*-1,3-dioxolo[4,5-*g*][1]benzopyran; NSC 600023—7,8-dihydro- 6,7-dimethyl- 8-(3,4,5-trimethoxyphenyl)- 6*H*- 1,3-dioxolo[4,5-*g*]-[1]benzopyran-6-ol; and NSC 381586—7,8-dihydro-7-methyl-8-(3,4,5-trimethoxyphenyl)-6*H*-1,3-dioxolo[4,5-*g*][1]benzopyran-6-ol.

381582 and 600023), three were less active (NSC 381586, 375501, and 600217), while the remainder had negligible inhibitory effects on the reaction. (Included in Table 1 are five inactive compounds with an altered C ring: one a ketone at position 6 [NSC 375500]; four with a 6–7 alkene bond [NSC 375499, 381583, 381587, and 381591] with the single substituents at positions 6 and 7 in *cis* relationship to each other.) The maximally active drugs had IC_{50} values of 10–15 μM, a drug concentration approximately stoichiometric with the amount of tubulin in the polymerization reaction mixture. (Both podophyllotoxin and colchicine had significantly lower IC_{50} values in the system used here—2–3 and 4–5 μM

Table 2. Effect of D (benzene) ring substituents on the activity of benzopyran compounds as inhibitors of tubulin polymerization

	D ring substituents									
	3′: OCH_3 4′: OCH_3 5′: OCH_3	2′: OCH_3	3′: OCH_3	4′: OCH_3	2′: OCH_3 3′: OCH_3	2′: OCH_3 3′: OCH_3 4′: OCH_3	2′: OCH_3 4′: OCH_3 6′: OCH_3	3′: OCH_3 4′: OH 5′: OCH_3	2′: OCH_3 4′: OCH_3	2′: OH 3′: OCH_3
C ring substituents	IC_{50} (μM)									
6: CH_3; *N*-tetrahydropyrrolyl	10–15 (371002)*	—	—	>100 (375508)	—	—	>100 (610796)	—	>100 (610805)	75–100 (375505)
6: OH; CH_3	10–15 (381578)	—	>100 (601100)	>100 (601098)	>100 (614394)	—	>100 (610799)	>100 (602412)	—	>100 (602340)
6: CH_3; OCH_3 7: CH_3	10–15 (381582)	>100 (610808)	>100 (614391)	>100 (601097)	>100 (614393)	>100 (610804)	>100 (614392)	—	>100 (614390)	—
6: OH; CH_3 7: CH_3	10–15 (600023)	>100 (610807)	—	>100 (382989)	>100 (611075)	>100 (610802)	>100 (610800)	—	20–25 (382987)	20 (602341)
6: OH 7: CH_3	15–20 (381586)	—	—	>100 (602345)	—	—	—	—	50–75 (610803)	25–30 (602342)
6: *N*-tetrahydropyrrolyl 7: CH_3	25–30 (375501)	—	—	>100 (375502)	—	—	—	—	>100 (375506)	40–50 (375503)

IC_{50} Values were determined as described in Table 1.
* NSC number.

respectively.) No obvious structure–activity pattern for substituents at the 6 and 7 positions emerged from the data presented in Table 1.

As noted above, with an R3 substituent, the hydrogen atoms at positions 7 and 8 have a *trans* configuration. In podophyllotoxin, however, the analogous hydrogen atoms at positions 1 and 2 are *cis*, while in the inactive picropodophyllotoxin they are *trans*. This may explain why no active compound was obtained in which R3 was larger than a methyl group; but a small R3 alone was not sufficient to yield an active agent (see Table 1).

In the previously reported studies [8, 9] with benzyl-benzodioxole derivatives, we had noted that 4′-methoxy compounds had been more inhibitory of tubulin polymerization than either 3′,4′-dimethoxy or 3′,4′,5′-trimethoxy compounds (i.e. agents with closer apparent structural analogy to podophyllotoxin than the most active drugs). Initially we had prepared only 4′-methoxy and 3′,4′,5′-trimethoxy derivatives of virtually all the benzopyran compounds summarized in Table 1. In no case was a 4′-methoxy benzopyran obtained that had significant activity or that surpassed the activity observed with the cognate trimethoxyphenyl derivative. Thus, adding a fourth ring to the structure—one linked directly to the trimethoxybenzene ring—seems to restore a requirement for greater fidelity to the structure of podophyllotoxin.*

To determine how sensitive tubulin was to structural variations in the phenyl substituent, a number of analogs of the most active 3′,4′,5′-trimethoxyphenyl benzopyran compounds were prepared. These are summarized in Table 2. As in the previously described compounds, spectral data in all cases indicated that the substituted benzene D ring has a *trans* configuration relative to the methyl group at position 7 (if present). Otherwise relative configurations at the optical centers of compounds described in Table 2 are unknown.

2′-, 3′-, and 4′-Methoxy, 2′,3′-dimethoxy and 2′,3′,4′- and 2′,4′,6′-trimethoxy D ring compounds were universally noninhibitory. A single 4′-hydroxy-3′,5′-dimethoxy analog in an active series was inactive. (It has not yet been possible to synthesize a 3′,4′-dimethoxy analog in any series.)

Mixed results, however, were obtained with two additional substituent patterns. Five compounds with a 2′,4′-dimethoxybenzene ring were prepared. While three were not inhibitory, one was weakly active (NSC 610803) and a second (NSC 382987) was only moderately less inhibitory than the analogous 3′,4′,5′-trimethoxybenzene compound. Similarly, five compounds with a 2′-hydroxy-3′-methoxybenzene ring displayed wide divergence in activities relative to the cognate 3′,4′,5′-trimethoxybenzene compounds. Two had little inhibitory activity compared to active analogs (cf. NSC 375505 to 371002 and 602340 to 381578), while three were only moderately less inhibitory than the corresponding trimethoxybenzene structures (cf. NSC 602341 to 600023, 602342 to 381586, and 375503 to 375501).

Thus, the substituents at positions 6 and 7 in the pyran ring may affect the exact pattern of activity resulting from modifications in the attached benzene D ring. Alternatively, however, these differences may actually derive from different relative configurations at the optical centers, as described above. Nonetheless, in all cases maximum inhibition of tubulin polymerization was obtained with a 2′,3′,4′-trimethoxybenzene D ring.

The initial series of benzyl-benzodioxole derivatives included several agents with reasonable cytotoxicity [8], most notably NSC 321567 and 350102 (see Fig. 1), as well as several compounds with *in vivo* antineoplastic activity qualitatively similar to that of podophyllotoxin (higher doses of the benzyl-benzodioxole derivatives were required for comparable therapeutic effects) [10]. The IC_{50} values for these compounds with L1210 murine leukemia cells were about 0.5 μM, with a concomitant rise in the mitotic index of cultured cells at cytotoxic drug concentrations. Initial studies with the second (morpholino) series of benzyl-benzodioxole compounds [9], however, indicated that even the agents most active in inhibiting tubulin polymerization (NSC 370277 and 381577, see Fig. 1) had little cytotoxicity. It therefore was of importance to document the relative effects of the benzopyran compounds on cell growth, particularly since they had activity against murine P388 leukemia *in vivo*. Evaluation of the five benzopyran compounds most effective as inhibitors

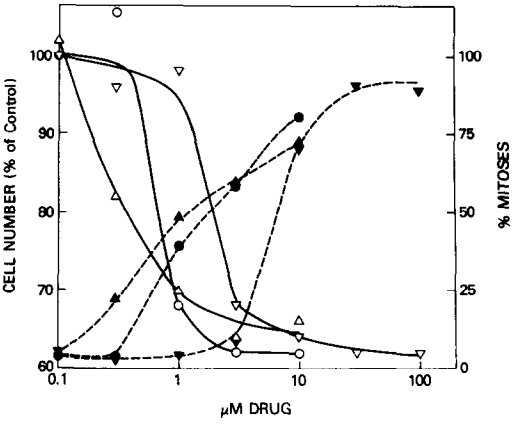

Fig. 2. Comparison of the effects of NSC 381582 (a benzopyran compound), NSC 321567 (a benzyl-benzodioxole derivative), and NSC 370277 (a morpholino benzyl-benzodioxole derivative) on the growth of L1210 murine leukemia cells in culture and on the accumulation of cells arrested in mitosis. Cells were cultured in medium containing the indicated concentration of drug and either counted after 9 hr (open symbols; control cultures contained 5.0×10^5 cells) or harvested, fixed, and stained for determination of mitotic figures after 10 hr (closed symbols; 200 cells counted at each data point with 4% mitoses observed in untreated control cultures). Symbols: (○ and ●) NSC 381582; (△ and ▲) NSC 321567; and (▽ and ▼) NSC 370277.

* There are few reported studies, however, with E ring analogs of podophyllotoxin. 4′-Demethylpodophyllotoxin has been reported to have activity similar to that of podophyllotoxin [2, 3], but a sample of this agent that we obtained from another source than that reported previously [2, 3] was much less potent than podophyllotoxin (an IC_{50} value of 15–20 μM as opposed to 2–3 μM for podophyllotoxin).

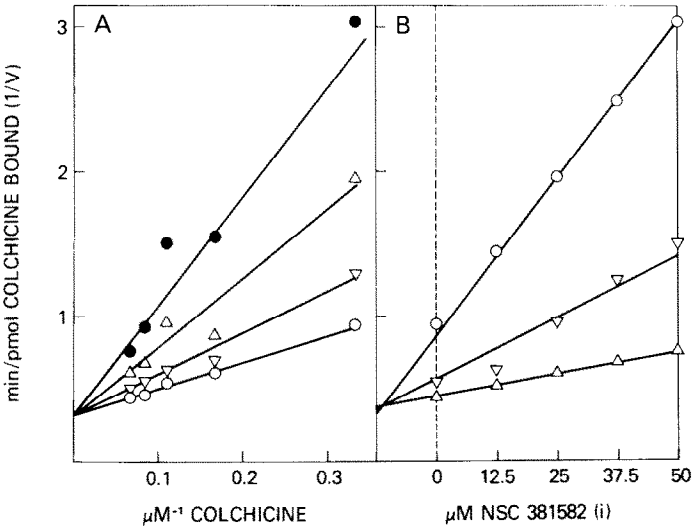

Fig. 3. Competitive inhibition of the binding of colchicine to tubulin by NSC 381582. Reaction conditions were as described in Table 3. In both panels, $1/V$ on the ordinate represents the inverse of the number of picomoles of colchicine retained by the DEAE-cellulose filters. All experimental points 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 of the micromolar concentrations of colchicine used in the experiment. Symbols: (○) no inhibitor; (▽) 12.5 μM NSC 381582; (△) 25 μM NSC 381582; and (●) 50 μM NSC 381582. (B) Dixon analysis of the data. The values on the abscissa (i) are the micromolar concentrations of NSC 381582 used in the experiment. Symbols: (○) 3 μM colchicine; (▽) 9 μM colchicine; and (△) 15 μM colchicine.

of tubulin polymerization (NSC 371002, 381578, 381582, 381586 and 600023) yielded virtually identical cytotoxicity data for the five compounds. Figure 2 compares the effects of NSC 381582 to NSC 321567 and NSC 370277 on both the growth of L1210 cells and the mitotic index of cultured cells. The benzopyran compound had an IC_{50} value modestly greater than that of NSC 321567 (0.7 vs 0.4 μM), whereas that of NSC 370277 was almost 5-fold higher (2 μM).*

* In earlier studies, reported previously [9], NSC 370277 did not appear to inhibit cell growth even at 100 μM. The reason for this difference from the current studies is not known.

With NSC 381582 and NSC 321567 the rise in the mitotic index was concomitant with the inhibition of cell growth, indicating that inhibition of mitosis is the principal cause of the cytotoxicity of these drugs. With NSC 370277 mitotic arrest required drug concentrations somewhat higher than those that inhibited cell growth.

The structure of the benzopyran compounds obviously suggests they are podophyllotoxin analogs. If this is the case, they should bind at the colchicine/podophyllotoxin site on tubulin, for podophyllotoxin is a potent inhibitor of the binding of radiolabeled colchicine to the protein [2, 3]. Examination of the effects of the benzopyran compounds most effective

Table 3. Comparison of the inhibition of colchicine binding to tubulin by benzopyran compounds, podophyllotoxin, and benzyl-benzodioxole derivatives

	Colchicine : Inhibitor		
	1:1	1:5	1:20
Inhibitor	Colchicine bound (% of control)		
NSC 381582 (benzopyran compound)	94	57	22
NSC 600023 (benzopyran compound)	86	52	24
Podophyllotoxin	15		3.0
NSC 321567 (benzyl-benzodioxole)	27		6.9
NSC 370277 (morpholino benzyl-benzodioxole)	104	95	75
NSC 381577 (morpholino benzyl-benzodioxole)	93	40	17

Reaction mixtures (0.1 ml) contained 0.1 mg/ml tubulin, 5 μM [^{3}H]colchicine, and the indicated potential inhibitor at the indicated concentrations. Other reaction components and the DEAE-cellulose paper filtration procedure were as described previously [14]. Incubation was for 10 min at 37°. All values represent the average of duplicate determinations which were within 10% of each other. In the control reaction mixtures, an average of 22.3 pmol of [^{3}H]colchicine was retained by the DEAE-cellulose filters.

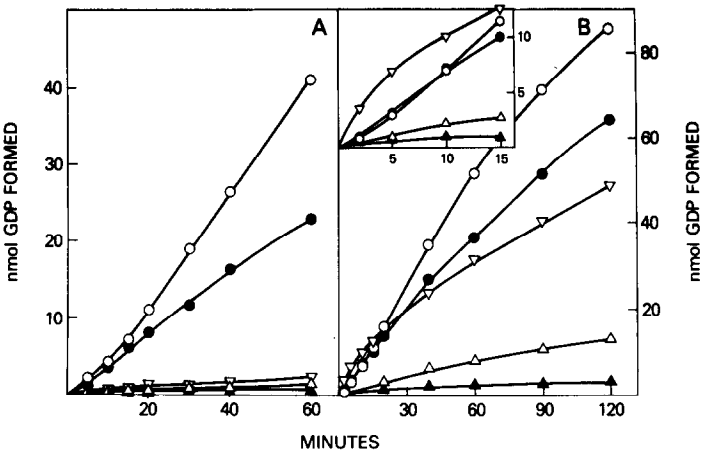

Fig. 4. Comparison of the effects of NSC 381578 on tubulin-dependent GTP hydrolysis to those of colchicine, podophyllotoxin, and NSC 370277 and to reactions without drug. Each 100-μl reaction mixture contained 0.1 M MES (pH 6.4), 0.5 mM $MgCl_2$, 1.0 mg/ml tubulin, 100 μM [8-^{14}C]GTP, no MAPs (panel A) or 0.5 mg/ml heat-treated MAPs (panel B), and 100 μM drug, as indicated by the following symbols: (▽) none; (●) NSC 381578; (○) colchicine; (△) podophyllotoxin; and (▲) NSC 370277. Reaction mixtures were incubated at 37°, and at the indicated times 10-μl aliquots were removed from the reaction mixtures, processed, and analyzed as described previously [24]. Data are expressed as nanomoles GDP formed per milliliter of reaction.

as inhibitors of tubulin polymerization documented similar but weak inhibition of the binding of [^{3}H]colchicine to tubulin. Table 3 compares the strong inhibitory effect of podophyllotoxin, the moderate effect of NSC 321567, and the more feeble effects of NSC 370277, 381577, 381582, and 600023. Nonetheless, the benzopyran compounds are probably competitive inhibitors of the binding of [^{3}H]colchicine to tubulin, for a detailed study with NSC 381582 (Fig. 3) fulfilled kinetic criteria [26] for competitive inhibition and yielded a K_i value of approximately 11 μM for the drug. Previous studies under identical experimental conditions [8, 9] have yielded K_i values of 0.4 μM for podophyllotoxin, 0.6 μM for NSC 350102, 1.0 μM for NSC 321567, and 8 μM for NSC 381577.

Finally, NSC 371002, 381578, 381582, and 600023 were evaluated for their effects on tubulin-dependent GTP hydrolysis. The four compounds had nearly identical effects, and therefore only data obtained with NSC 381578 will be presented. Podophyllotoxin and the benzyl-benzodioxole derivatives inhibit tubulin-dependent GTP hydrolysis, whereas colchicine and steganacin stimulate it under a variety of reaction conditions [8, 9]. Figure 4 presents two studies in 0.1 M MES in which the time course of GTP hydrolysis with NSC 381578 is compared to reactions without drug and to reactions with colchicine, podophyllotoxin, or NSC 370277. The experiment of Fig. 4A was performed without MAPs in the reaction mixtures, whereas that of Fig. 4B contained MAPs (the inset of Fig. 4B presents the early time course of the reaction in greater detail). Under both conditions, rather than the inhibition observed with podophyllotoxin and NSC 370277, the benzopyran compound caused a stimulation of tubulin-dependent GTP hydrolysis, similar to that observed with colchicine. Thus, even though structurally more similar to podophyllotoxin than the benzyl-benzodioxole derivatives, the interaction of the benzopyran compounds with tubulin nevertheless differs in an as yet undefined way from the podophyllotoxin–tubulin interaction that leads to sharply reduced GTP hydrolysis. It should be noted that in the presence of MAPs (without drug) the early burst of GTP hydrolysis is simultaneous with the onset of microtubule assembly and probably stoichiometric with the amount of tubulin polymerized [27]. This phase of the GTPase reaction was inhibited by colchicine and NSC 381578 (Fig. 4B, inset).

In summary, we have described here a new class of synthetic antimitotic agents, some with *in vivo* antineoplastic activity, that inhibit *in vitro* tubulin polymerization. They bind at the colchicine/podophyllotoxin site on tubulin, as demonstrated by the competitive inhibition of colchicine binding with NSC 381582, but this interaction is relatively feeble compared to that of colchicine. Their structures are quite similar to that of podophyllotoxin, with a 2′,3′,4′-trimethoxybenzene ring directly attached to the benzopyran ring required for maximum activity. In particular, analogs with a 4′-monomethoxybenzene ring, similar to active benzyl-benzodioxole derivatives, were inactive. Nonetheless, the benzopyran compounds stimulated tubulin-dependent GTP hydrolysis, whereas podophyllotoxin and the benzyl-benzodioxole derivatives inhibited this reaction. The interaction of these new antimitotic agents with tubulin thus differs subtly from the interaction of podophyllotoxin with the protein.

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