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Derivatives of 5,6-Diphenylpyridazin-3-one: Synthetic Antimitotic Agents Which Interact with Plant and Mammalian Tubulin at a New Drug-binding Site

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ABSTRACT

A series of derivatives of 5,6-diphenylpyridazin-3-one (DPP) was examined for interactions with calf brain tubulin following the demonstration that many members of the class caused significant mitotic effects in intact animals, while others had activity against murine P388 leukemia. In L1210 cells several DPP derivatives caused a rise in the mitotic index which correlated well with the cytotoxicity of the drugs. Active DPP derivatives markedly stimulated tubulin-dependent guanosine triphosphate hydrolysis and inhibited tubulin polymerization or induced tubulin oligomer formation, depending on specific reaction conditions. These new agents, however, did not interfere with the binding to tubulin of radiolabeled colchicine, vinblastine, maytansine, or guanosine triphosphate. They thus appear to bind at a previously undescribed site on the tubulin molecule. Some DPP derivatives have significant herbicidal activity, causing mitotic disruption and a rise in the mitotic index in seedling root tissues. Although the DPP derivatives most toxic to plant tissues differ from those most active in inhibiting calf brain tubulin polymerization, virtually all active compounds bear a nitrile substituent at position 4 of the pyridazinone ring. Most active derivatives also bear substituents of varying structure at position 2 of this ring, but no clear structure-function pattern is apparent at this position. The phenyl rings in the most active herbicidal DPP derivatives either are unsubstituted or bear fluorine atoms. Derivatives with chlorine substituents have no detectable herbicidal activity. In contrast, interactions with calf brain tubulin are substantially enhanced if the phenyl rings bear chlorine substituents.

INTRODUCTION

The observation of antihypertensive activity for a DPP[2] derivative led to the synthesis of several analogues to explore structure-activity relationships in this novel class of compounds (1, 2) (see Fig. 1 and Table 1). Although several of these agents were active in reducing blood pressure in a spontaneously hypertensive rat model (1, 2), some DPP derivatives displayed significant herbicidal activity (3). Preliminary toxicological studies of the herbicidal compounds included the observation of bizarre mitotic figures in renal tissue (4, 5). A number of the agents were therefore submitted to the National Cancer Institute for screening for antineoplastic activity, and a significant increase in survival of mice given simultaneous injections of P388 leukemia cells and selected DPP derivatives was observed. Since we have been screening antineoplastic drugs of uncertain mechanism of action for their effects on tubulin-dependent GTP hydrolysis (6-8) to identify new antimitotic agents, we examined these compounds. They were found to stimulate GTP hydrolysis dramatically, and as a consequence we have examined in detail the interactions of this class of drugs with tubulin. In particular, we wanted to establish that they were antimitotic agents in mammalian cells, determine their effects on tubulin polymerization, locate their binding site on tubulin, and determine structure-function correlations among presently available compounds. Our initial findings are reported here, together with a summary of unpublished observations in plant tissues.

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[2] The abbreviations used are: DPP, 5,6-diphenylpyridazin-3-one; MES, 2-(*N*-morpholino)ethanesulfonate; MAPs, microtubule-associated proteins.

MATERIALS AND METHODS

Materials. All derivatives of DPP were prepared as described previously (1). GTP, vinblastine, colchicine, and MES were obtained from Sigma; podophyllotoxin was from Aldrich; [8-^{14}C]GTP was from Moravek Biochemicals; and [^{3}H]colchicine and [^{3}H]vinblastine were from Amersham. Maytansine was provided by the Natural Products Branch, National Cancer Institute, and [^{3}H]maytansine was a generous gift of Dr. D. G. Johns (9). GTP and [8-^{14}C]GTP were repurified by ion-exchange gradient chromatography. Electrophoretically homogeneous calf brain tubulin and MAPs purified by DEAE-cellulose chromatography were prepared as described previously (10). All drugs were dissolved in dimethyl sulfoxide, and control reaction mixtures always contained an equivalent amount of the solvent.

Methods. Measurement of GTP hydrolysis and colchicine binding to tubulin, turbidimetric analysis of microtubule assembly, and electron microscopy of negatively stained specimens were performed as described elsewhere (10, 11). Measurement of the binding of radiolabeled vinblastine and maytansine to tubulin was performed by the centrifugal gel filtration method of Penefsky (12) as described in detail previously (13). Analysis of drug effects on the mitotic index and on cytotoxicity in cell culture was performed with murine L1210 leukemia cells, as described by Wolpert-DeFilippes *et al.* (14).

RESULTS

The basic structure of the DPP derivatives described here is presented in Fig. 1, with structural details presented in Table 1. A variety of substituents at position 2 of the pyridazinone ring and on the two phenyl rings have been observed in active compounds (in all cases the substituents on the phenyl rings have been identical). Virtually all active agents in both plant and mammalian systems have had a nitrile group at position 4.

Summary of Findings in Plant Tissues. The observation of significant herbicidal activity in the DPP derivative NSC 362448 led to efforts to define its mechanism of action. Studies with root tip tissue demonstrated defective cell division, with coalescence of condensed chromosomes in the cell center, and the disappearance of microtubules. Unusual new structures were also observed: macrotubules about 75 nm in diameter, 3 times as wide as normal microtubules. A large number of DPP derivatives were also observed to inhibit flagellar regrowth in *Chlamydomonas* cells (*cf.* Refs. 15 and 16). In this study the primary comparison was between a group of derivatives with varied substituents at position 2 of the pyridazinone ring, a nitrile group at position 4, and unsubstituted phenyl rings. Although some of the compounds inhibited flagellar regrowth by 50% at drug doses as low as 0.4 μM, none of the agents most active in inhibiting flagellar regrowth were among the drugs most active in inhibiting bovine tubulin polymerization *in vitro*.

Demonstration That Tubulin Is the Probable Target of DPP Derivatives in Mammalian Tissues. A group of the DPP derivatives was submitted to the National Cancer Institute for screen-

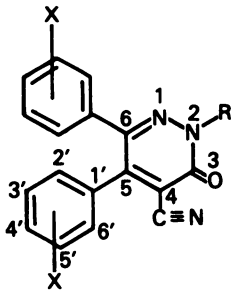

Fig. 1. Structural formula of the DPP derivatives.

Table 1 *Inhibition of microtubule assembly by DPP derivatives*

ID_{50}[a] values were established in 0.25-ml reaction mixtures containing tubulin (1.0 mg/ml), MAPS purified by DEAE-cellulose chromatography (0.5 mg/ml) (10), 0.1 M MES (pH 6.9), 0.5 mM $MgCl_2$, 1.0 mM GTP, 2% (v/v) dimethyl sulfoxide, and varying concentrations of the indicated drugs. ID_{50} values were based on a minimum of two independent determinations.

Drug (NSC no.)	X[b]	R[b]
		ID_{50} 5–10 μM
362449	H	CH_2COOH
362453	Cl at 4′	$(CH_2)_2$-O-$CO(CH_2)_2COOH$
362455	Cl at 3′ and 4′	$(CH_2)_2OH$
602746	Cl at 3′ and 4′	H
		ID_{50} 10–15 μM
351477	Cl at 4′	H
351478	Cl at 4′	$(CH_2)_2OH$
351482	Cl at 4′	$(CH_2)_3COOH$
602744	Cl at 4′	$(CH_2)_2COOH$
		ID_{50} 15–25 μM
362458	H	CH_3
362460	Cl at 4′	$(CH_2)_2$-O-$COCH_2NH_2$·HBr
601680	CH_3 at 4′	H
601683	Br at 4′	H
602747	Cl at 4′	CH_2CO-OCH_3
603334	Cl at 4′	CH_2COCH_3
		ID_{50} 25–50 μM
362448	H	CH_2CO-O-CH_2CH_3
362450	Cl at 2′	$(CH_2)_2OH$
362454	F at 4′	$(CH_2)_2OH$
602745	F at 4′	H
602748	Cl at 2′	H
		ID_{50} 50–100 μM
362444	H	$(CH_2)_2OH$
362445	H	H
362451	H	$(CH_2)_2COOH$
		ID_{50} > 100 μM
362447	OCH_3 at 4′	$(CH_2)_2OH$
601681	H	$(CH_2)_2CO$-O-$(CH_2)_2OCH_3$
601682	Cl at 4′	$(CH_2)_3CO$-OCH_2CH_3
601684	Cl at 4′	$(CH_2)_2Cl$

[a] ID_{50}, concentration of drug resulting in a 50% reduction in the turbidity reading after a 20-min incubation at 37°C.

[b] Locations of X and R in the DPP derivatives is as indicated in Fig. 1.

Table 2 *Stimulation of tubulin-dependent GTP hydrolysis by DPP derivatives*

Each 50-μl reaction mixture contained tubulin (1 mg/ml), 0.1 mM [8-^{14}C]GTP, the indicated drug at 0.1 mM, 1% dimethyl sulfoxide (v/v), 0.1 M MES (pH 6.4), and 0.5 mM $MgCl_2$. Incubation was for 20 min at 37°C. Data are expressed as nmol of [8-^{14}C]GDP formed per ml of reaction. This experiment was performed twice with comparable results. In addition, stimulation of GTP hydrolysis by DPP derivatives was observed in six experiments in 1 M glutamate and in two experiments with heat-treated MAPs (*cf.* Ref. 8).

Drug added	nmol GDP formed
None	1.7
Colchicine	8.9
NSC 362453	44.8

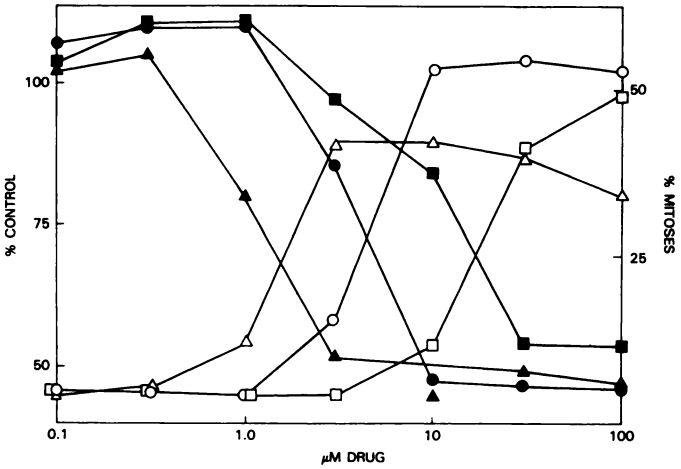

Fig. 2. Cytotoxic and antimitotic effects of DPP derivatives on murine L1210 leukemia cells. These studies were performed as described by Wolpert-DeFilippes *et al.* (14). Dimethyl sulfoxide concentration was 1% (v/v). ○, □, △, mitotic index (control, 2.8% mitoses); ●, ■, ▲, cytotoxic effects of the drugs (control, 3.9 × 10^5 cells/ml). Details of drug structures are presented in Table 1. NSC 362453 (○, ●); NSC 351478 (△, ▲); NSC 602746 (□, ■). This study was performed in its entirety a single time. Similar results were obtained with NSC 362453 in a second experiment. In addition, a high mitotic index was obtained two additional times in a cell culture treated with 100 μM NSC 351478.

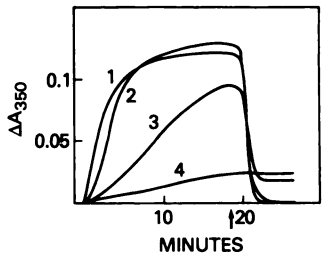

Fig. 3. Inhibition of microtubule assembly by NSC 362449. Each 0.25-ml reaction mixture contained tubulin (0.75 mg/ml), MAPs purified by DEAE-cellulose chromatography (0.38 mg/ml), 1.0 mM GTP, 0.1 M MES (pH 6.9), 0.5 mM $MgCl_2$, 2% (v/v) dimethyl sulfoxide, and NSC 362449 (see Table 1 for details of drug structure) at the following concentrations: *Curve 1*, none; *curve 2*, 5 μM; *Curve 3*, 7.5 μM; *Curve 4*, 10 μM. At zero time the electronic temperature controller (see Ref. 10) was set at 37°C; at the time indicated by the *arrow* on the *abscissa*, the temperature controller was set at 0°C. Similar results were obtained in at least four experiments.

ing for antineoplastic activity. Several compounds proved active against murine P388 leukemia, but these differed from the most active herbicides. (The most promising drugs at this point appear to be NSC 351478 and NSC 362454.) Nevertheless, since tubulin appeared to be a likely target for this class of agents, we examined the effect of DPP derivatives on tubulin-dependent GTP hydrolysis (6–8). Many of these drugs stimulated the reaction under three different conditions (in 1 M glutamate and in 0.1 M MES both with and without heat-treated MAPs). Table 2 compares the potent stimulation of GTP hydrolysis by NSC 362453 in 0.1 M MES without MAPs to the much less marked effect of colchicine, the first drug reported to stimulate tubulin-dependent GTP hydrolysis (17).

The GTPase studies and the observations in plant tissues predict that the DPP derivatives should cause mitotic arrest in mammalian cells in culture. This was confirmed with three drugs studied in murine L1210 leukemia cells (Fig. 2). The rise in mitotic index correlated well with cytotoxic effects of the 3 compounds.

DPP Derivatives Inhibit Microtubule Assembly and/or Induce Tubulin Oligomer Formation. Generally antimitotic agents inhibit microtubule assembly *in vitro*. This was readily demonstrated with many DPP derivatives (Fig. 3; also see Table 1).

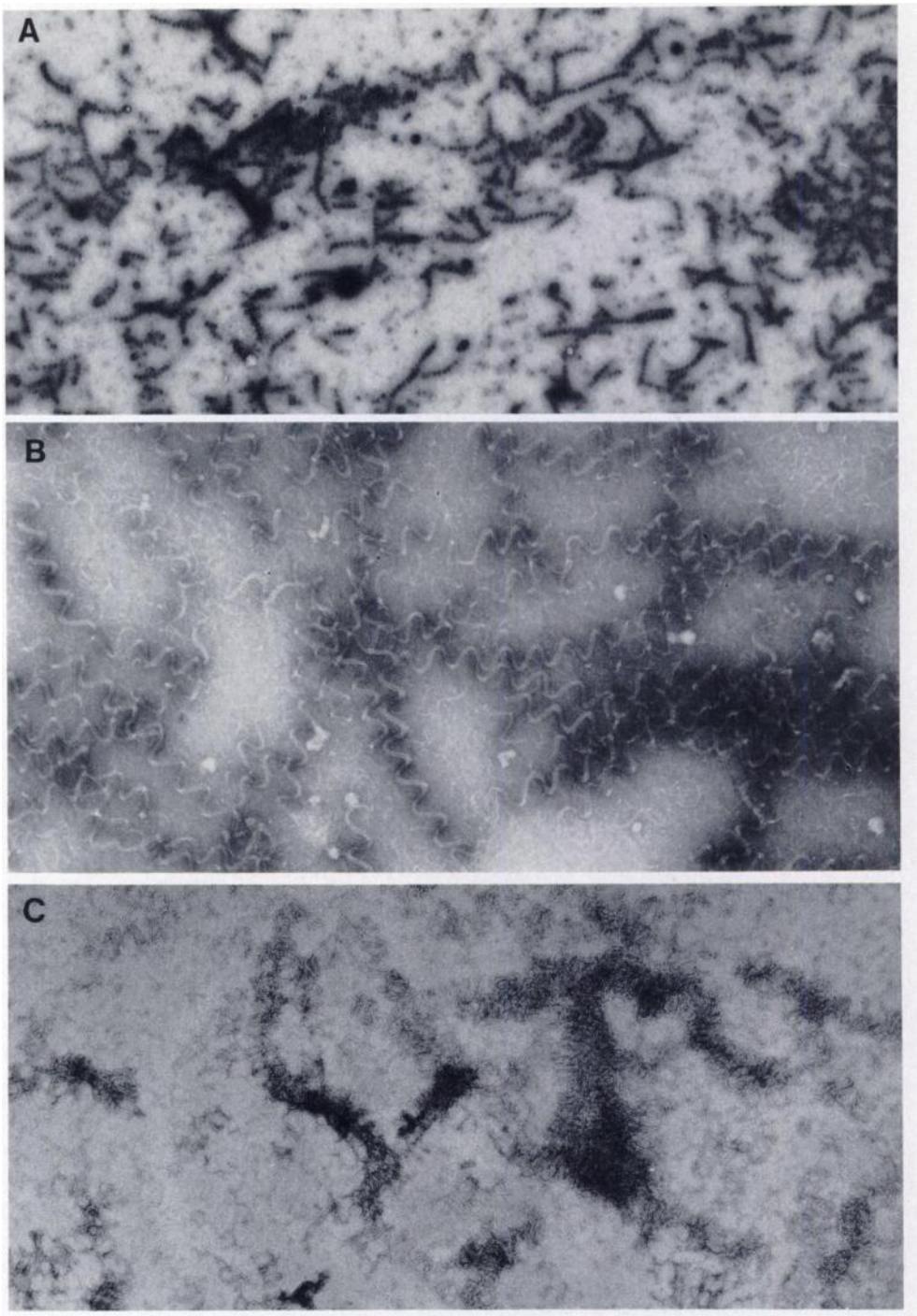

Fig. 4. Morphology of tubulin oligomers induced with NSC 362453 (see Table 1 for details of drug structure) and vinblastine. *A* and *B*, oligomers formed with NSC 362453. The 0.25-ml reaction mixture contained tubulin (1.0 mg/ml), 0.1 mM GTP, 1.0 M monosodium glutamate (pH 6.6), 1.0 mM $MgCl_2$, 4% (v/v) dimethyl sulfoxide, and 0.1 mM NSC 362453. After 20 min at 37°C, a negatively stained specimen was prepared and examined in the electron microscope. *C*, oligomers formed with vinblastine. Reaction mixture as above, except that the drug was 10 μM vinblastine instead of NSC 362453. *A*, × 7,900; *B* and *C*, × 52,700.

One of the most inhibitory members of this class of drugs was NSC 362449. In a system consisting of purified tubulin and MAPs (Fig. 3), progressive inhibition of nucleation, elongation, and extent of polymerization was observed as the drug concentration was raised, with complete inhibition occurring at 10 μM. In a second series of experiments we demonstrated that glycerol-induced polymerization of purified tubulin (18) was inhibited by NSC 362449 (data not presented), confirming that the target of the DPP derivatives is tubulin and not the MAPs (as predicted by the GTPase experiments without MAPs).

In 1.0 M glutamate several of the DPP derivatives were found to induce cold-irreversible turbidity development rather than simply inhibit cold-reversible polymerization, as is the case with most other antimitotic drugs (6–8). Electron microscopic examination of reaction mixtures at 37°C demonstrated the formation of well-defined helical oligomers. Fig. 4*A* presents a low magnification (×7,900) view of these structures; Fig. 4*B* shows a high magnification (×52,700) view. There is a clearly visible filamentous substructure, perhaps representing protofilaments (2 and occasionally 3 filaments can be distinguished). The helical oligomers are relatively short, and the repeat distance in the helix is about 90 nm. Neither turbidity development nor oligomer formation required GTP, and GTP had little effect on this reaction; nor is there turbidity development or oligomer formation at low ionic strength without MAPs. With some preparations of MAPs, DPP derivative-induced turbidity development also occurs, but the structures formed are not as ordered as those in glutamate. We have not yet been able to correlate DPP derivative-induced turbidity development with a specific MAP component (*cf.* Ref. 19).

Table 3 *Failure of DPP derivatives to inhibit the binding of radiolabeled drugs to tubulin*

Second drug added	% of control after following radiolabeled drug added		
	Colchicine[a]	Vinblastine[b]	Maytansine[b]
None	100	100	100
NSC 362449	106	130	103
NSC 362453	100	107	100
Podophyllotoxin	1.3	113	99
Maytansine	103	6.9	
Vinblastine	101		45

[a] Each 0.1-ml reaction mixture contained tubulin (0.1 mg/ml), 5 μM [^{3}H]-colchicine, the competing drug at 0.5 mM, and 5% (v/v) dimethyl sulfoxide. Further reaction components and further experimental details are as described previously (10). Comparable results were obtained in three different experiments.

[b] Each 0.4-ml reaction mixture contained tubulin (1.0 mg/ml), 0.1 M MES (pH 6.9), 0.5 mM $MgCl_2$, 0.1 mM GTP, 2.5% (v/v) dimethyl sulfoxide, and either 25 μM [^{3}H]vinblastine with the competing drugs at 1 mM or 10 μM [^{3}H]maytansine with the competing drugs at 0.25 mM. Incubation was at room temperature for 20 min. Comparable results were obtained in three different experiments with vinblastine. The maytansine experiment was performed once.

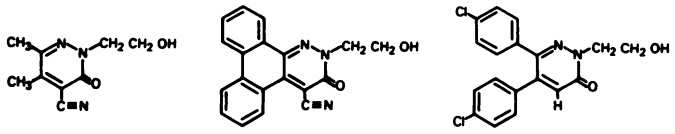

Fig. 5. Formulas of compounds which are structurally related to the DPP derivatives and which do not inhibit *in vitro* microtubule assembly.

The *Vinca* alkaloids have long been known to induce formation of aberrant tubulin structures *in vitro* and *in vivo*, including formation of oligomers of purified tubulin in 1.0 M glutamate. To determine whether there was any relationship between the DPP-induced oligomers and those formed with vinblastine, we examined the morphology of the vinblastine-induced structures (Fig. 4*C*). Although these were also helical, they were coiled much more tightly and appeared to be considerably larger than those formed with the DPP derivatives. The vinblastine-induced structures formed in 1.0 M glutamate are morphologically similar to those described by other workers (19, 20) using different reaction conditions.

Effects of DPP Derivatives on Drug and Nucleotide Binding to Tubulin. To determine whether the DPP derivatives bound at a previously described site on tubulin, we examined the effect of these drugs on the binding of radiolabeled colchicine, vinblastine, and maytansine (Table 3). No inhibitory effect was observed. These new agents also do not affect the binding of GTP to tubulin (data not presented), as predicted by their brisk stimulation of GTP hydrolysis requiring turnover of GTP at the exchangeable site.

Structure-Activity Relationships. The availability of a large number of DPP derivatives permitted a relatively extensive analysis of the structural requirements for interaction of this class of drug with tubulin *in vitro*. Our most important findings are summarized in Table 1, in which the drug concentration required to inhibit the extent of microtubule assembly by 50% after 20 minutes at 37°C was determined. The drug concentrations required to inhibit reaction rate by 50% were considerably lower, but relative activities of the drugs were unaltered.

No clear pattern of activity related to substituents at position 2 of the pyridazinone ring emerged from these experiments. In fact, two of the most active inhibitors of assembly (NSC 351477 and NSC 602746) were unsubstituted at this position. A clear pattern is observed, however, when the symmetrical substituents on the phenyl rings are considered. Activity is progressively enhanced with a fluorine atom at position 4′, a bromine at 4′, a chlorine at 4′, and two chlorines at 3′ and 4′. Available compounds also indicate that a methyl group at position 4′ is equivalent to a bromine at that position and that a compound with a chlorine substituent at position 4′ is about 3–4 times more active than one with a chlorine at position 2′, which is in turn 2–3 times more inhibitory than an unsubstituted compound.

Finally, Fig. 5 presents the structures of three inactive compounds. The two phenyl rings seem to be essential for inhibition of microtubule assembly, and they must have at least some degree of mobility (alternatively, the fused structure presented in Fig. 5 may represent an unfavorable conformation for an interaction with tubulin). All compounds with no substituent or a large substituent at position 4 of the pyridazinone ring have been inactive.

DISCUSSION

The DPP derivatives are a new multiagent class of antimitotic drugs, many of which interact directly with bovine brain tubulin. Although no direct interaction with plant tubulin has been documented, it is likely that their effects in inhibiting flagellar regrowth and inducing mitotic disruption derive from an analogous interaction with plant tubulin. It must be emphasized that specific DPP derivatives have maximum inhibitory activity only with one class of organisms. Those most active in plant tissues interact only weakly with bovine tubulin and *vice versa*. In structure-function studies on herbicidal activity, compounds with unsubstituted phenyl rings were highly active, as were those with fluorine substituents. Chlorine groups on the two phenyl rings eliminated herbicidal activity.[3] The opposite is the case for interactions of DPP derivatives with bovine tubulin.

The interactions of the DPP derivatives with tubulin are reminiscent of colchicine and many colchicine analogues in stimulating tubulin-dependent GTP hydrolysis even as they inhibit the microtubule assembly reaction associated with GTP breakdown (6–8, 17). However, these drugs do not inhibit the binding of colchicine to tubulin.

The DPP derivatives are also reminiscent of the *Vinca* alkaloids in that they inhibit microtubule assembly and induce tubulin oligomer formation. In addition, distinct new structures (macrotubules) were observed in DPP derivative-treated seedling roots. Although it is not yet known whether these macrotubules contain tubulin, they may bear some relationship to the oligomeric structures formed from purified bovine tubulin in glutamate. Perhaps analogously, the tight spirals induced by *Vinca* alkaloids *in vitro* (19, 20) differ greatly morphologically from the tubulin paracrystals observed in cells treated with *Vinca* alkaloids. Nevertheless, the DPP derivative-induced oligomer differs morphologically from the *Vinca* alkaloid-induced oligomer, and DPP derivatives are unable to inhibit the binding of either vinblastine or maytansine to tubulin (9). Moreover, both vinblastine and maytansine inhibit rather than stimulate tubulin-dependent GTP hydrolysis (7, 8, 17).

The DPP derivatives thus seem to bind to a previously undescribed site on tubulin, for they also do not inhibit the binding of guanine nucleotides at the exchangeable GTP site. Definitive proof of this hypothesis requires radiolabeled agents, and we are attempting their synthesis.

Some of the DPP derivatives (particularly NSC 351478) were effective in the treatment of P388 leukemia in mice. The structure-activity studies suggest promising leads for the synthesis of additional compounds. While the substituents at position 2 of the pyridazinone ring do not fall into a clear pattern,

[3] L. J. Powers, unpublished data.

alterations at this position can have major effects on the activity of the resulting compounds. More specific was the effect of chlorinating the phenyl rings. Position 4′ seems most important, but a second chlorine at position 3′ further enhanced *in vitro* inhibition of microtubule assembly.

Position 4 of the pyridazinone ring is also of major importance. Virtually all compounds which inhibit polymerization of bovine tubulin or which have herbicidal activity bear a nitrile group at this position. A large number of inactive compounds have been examined bearing a variety of substituents (or none at all; see Fig. 5). A single compound of this group (analogous to NSC 362449, but bearing a chlorine instead of a nitrile at position 4) has had activity. This compound was about one-third as effective as NSC 362449 in inhibiting microtubule assembly. Thus, while the nitrile group cannot be considered essential for inhibitory activity in the DPP derivative series, it is nonetheless of major importance. It appears that a substituent is required at position 4 and that it must be relatively small.

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