ABSTRACT

Tubulin-dependent GTP hydrolysis was evaluated for its potential as a relatively simple screening assay for new antimitotic drugs. Carbamates of aromatic amines were chosen as the test system because of the relatively diverse structures of compounds in this class already known to have antimitotic properties and because of the large number of such compounds in the NSC collection of the National Cancer Institute. Of 162 compounds evaluated, significant alterations in the GTPase reaction were observed with 36 agents. Sixteen of these had substantial inhibitory effects on tubulin polymerization (true positives), while ten did not (false negatives). There were no false negatives. GTP hydrolysis occur with different antimitotic drugs (6, 8-15), but no antitubulin compound has yet been observed that effectively to select new antitubulin compounds with potential as antimitotic agents from a large group of compounds of unknown activity.

INTRODUCTION

Antimitotic agents are well known as potential antineoplastic compounds and act at the microtubule level to inhibit cell division. Thus far the most clinically useful drugs in this class are the Vinca alkaloids, but other agents which have recently entered research trials in human subjects are maytansine (1, 2) and taxol (3, 4). The identification of specific classes of compounds as antimitotic agents has generally been rather haphazard, for the cellular studies required to define this mechanism of action are relatively cumbersome.

Microtubules require hydrolysis of GTP for their assembly (5), and the major component of these organelles, the protein tubulin, is the molecular target of virtually all antimitotic agents. Moreover, purified tubulin will hydrolyze GTP under a variety of reaction conditions (6, 7). Although one might anticipate that antimitotic agents, since most of them inhibit microtubule assembly, would also inhibit GTP hydrolysis, this is not the case. Both inhibition and stimulation of tubulin-dependent GTP hydrolysis occur with different antimitotic drugs (6, 8-15), but no antitubulin compound has yet been observed that does not alter the reaction. This observation led us to propose the tubulin-dependent GTPase reaction as a simple in vitro screening test to rapidly identify new antimitotic compounds (16, 17).

Beginning with methyl carbamates of aminobenzimidazole (18-21), over the last 10 to 15 yr an increasing number of compounds have been described with antimitotic, antileukemic, and/or antitubulin properties (10, 17, 22-26). Six of these are presented in Fig. 1—nocodazole (19), NSC 181928, and the more active NSC 330770 (10, 22, 23), tubulozole (24), NSC 251635 (25), and NSC 215914 (17). The only common feature these molecules appear to share is the carbamate group, although all of them may be viewed as carbamates of aromatic amines, and two of them are bis-carbamates. Because of the heterogeneity of these structures, we decided that it would be worthwhile to perform a computer search of the National Cancer Institute's drug collection for carbamates and use the compounds it provided to evaluate the potential of the GTPase assay as a screen for selecting potential antimitotic drugs from a large collection of agents. This report describes our findings.

MATERIALS AND METHODS

Materials. Electrophoretically homogeneous bovine tubulin and heat-treated MAPs2 were prepared as described previously (26), including removal of unbound nucleotide by gel filtration chromatography. Nonradiolabeled GTP (Sigma Chemical Co., St. Louis, MO) and [8-3H]GTP (Moravek Biochemicals, Brea, CA) were repurified by ion-exchange chromatography (27). Nocodazole was from Aldrich Chemical Co., Milwaukee, WI, and [3H]colchicine from Amersham Corp., Arlington Heights, IL. All other drugs were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute. Drug solutions were prepared in dimethyl sulfoxide, and drug-free control samples contained equivalent amounts of the solvent, which did not alter the reactions being examined. Stock solutions of monosodium glutamate (2 M) and MES (1 M) were adjusted, respectively, to pH 6.6 with HCl and pH 6.9 with NaOH.

Methods. GTP hydrolysis was measured by following the generation of [8-14C]GDP from [8-14C]GTP by thin-layer chromatography and autoradiography on polyethyleneimine-cellulose and autoradiography, tubulin polymerization by turbidimetry (28), binding of [3H]colchicine to tubulin by the DEA-cellulose filter technique (29), and growth of L1210 murine leukemia cells with quantitation of cells arrested in mitosis as described previously (10-13).

Drugs were screened for GTP hydrolysis in 30-¡l reaction mixtures containing 1.0 mg/ml of tubulin, 1 M monosodium glutamate, 1.0 mM MgCl2, 10% (v/v) dimethyl sulfoxide, 50 ¡M [8-14C]GTP, and drugs at 100 ¡M. Incubation was for 20 min at 37°C. Each compound was evaluated in a minimum of two independent assays.

Tubulin polymerization dependent on glutamate (7) was examined in 0.25-m1 reaction mixtures with drugs at 10 and/or 80 ¡M containing 1.0 mg/ml of tubulin, 0.5 mM GTP, and 1.0 mM monosodium glutamate, with the dimethyl sulfoxide concentration 1% (v/v). Both temperature-dependent polymerization at 37°C and cold reversibility of reactions at 0°C were evaluated. ICP values for drugs in this system were determined as described previously (15). The 1.0 M glutamate system was used in most of the studies described here because it not only eliminates the requirement for MAPs in both the GTPase and polymerization reactions (7, 9, 26), but it also markedly stabilizes tubulin (30).

Polymerization dependent on MAPs was also evaluated. Reaction mixtures contained 1.5 mg/ml of tubulin, 0.75 mg/ml of heat-treated MAPs (which have minimal nucleoside diphosphate kinase and nonspecific phosphatase activities—see Ref. 26), 0.1 M MES, 0.5 mM MgCl2, 0.4 mM GTP, 4% dimethyl sulfoxide (v/v), and drugs at various concentrations.

2 The abbreviations used are: MAPs, microtubule-associated proteins; MES, 4-morpholinethanesulfonate; IC50, 50% inhibitory concentration.
The Computer Search for Carbamates in the National Cancer Institute’s Drug Collection. Although highly varied, the carbamates with antimitotic activity (Fig. 1) share structural features. Besides being carbamates of aromatic amines, all possess a second bulky substituent on the aromatic ring. For convenience, we will refer to the central aromatic ring(s) of these compounds as the “nucleus” of the carbamate (e.g., the benzimidazole rings in nocodazole or the benzene ring in NSC 215914). The computer search was therefore based on the following restrictions: (a) only carbamates of aromatic amines were acceptable; (b) a second substituent on the aromatic nucleus was required, provided it was not adjacent to the carbamate function; and (c) selected compounds had to be in stock.

Identification was made of 295 compounds meeting these conditions, but 133 were eliminated based on the following criteria: (a) compounds presented in Fig. 1; (b) compounds differing from those in Fig. 1 only in the carbamate function; (c) analogues of colchicine; (d) analogues with the nucleus of nocodazole (the benzimidazole moiety) or of NSC 181928 and NSC 330770 (the deazahydropteridine nucleus), provided the carbamate and second substituent were attached at the same locations on the nucleus; and (e) compounds subjectively judged to have “uninteresting” structures, generally because the second substituent was small and/or nonorganic.

The remaining 162 compounds were evaluated for effects on tubulin-independent GTP hydrolysis at 100 nM drug (20-min incubation at 37°C). Of these, 136 were judged to be negative, since the hydrolytic reactions were within 15% of the control reactions. Of the remaining 26 agents, 22 stimulated the reaction (hydrolysis > 120% of that in a control reaction), and 3 inhibited it (hydrolysis < 80% of that in the control reaction). These compounds were considered positives in the GTPase assay. One compound (NSC 107390) yielded erratic results, but it reproducibly stimulated GTP hydrolysis relative to the control when reaction conditions were altered (see below). For the purposes of this discussion we will consider it, too, as a positive in the GTPase assay.

We next evaluated the effects of the carbamates on glutamate- and MAP-dependent tubulin polymerization reactions, to determine the degree of correlation between the GTPase and polymerization assays. The 26 positives in the GTPase assay were examined at 80 and/or 10 μM drug in the glutamate system and at 20 μM in the MAPs system; and 78 GTPase-negative compounds were evaluated in the glutamate system.

For the purposes of this report, we have arbitrarily defined as a “true positive” of the GTPase assay any of the 26 agents which inhibited the extent of polymerization by over 50% at 10 μM in the glutamate system or at 20 μM in the MAPs system (drug concentrations approximately stoichiometric with those of tubulin). Sixteen compounds were in this category (structures...
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Fig. 2. Structural formulas of 16 carbamate compounds identified by effects on tubulin-dependent GTP hydrolysis which inhibit tubulin polymerization. See text for further details.

in Fig. 2). Five agents inhibited the glutamate polymerization reaction by at least 50% at 80 μM, and 5 agents inhibited by less than 50% at the higher drug concentration. We consider these latter two groups as “false positives.”

The GTPase-negative compounds were first examined at 80 μM in the glutamate system, and 72 inhibited polymerization less than 50%. None was effective at 10 μM. There were thus no “false negatives,” compounds that strongly inhibited polymerization without altering the GTPase reaction.

Of the “true positives,” 12 inhibited both the glutamate- and MAP-dependent reactions. Two compounds (NSC 346624 and NSC 377071) inhibited polymerization by at least 50% at 10 μM in glutamate but not at 20 μM with MAPs (NSC 377071 did inhibit 50% with MAPs at 25 μM, however). Two compounds (NSC 275954 and NSC 275971) inhibited polymerization by at least 50% only with MAPs. The glutamate reaction was atypical with both agents, for turbidity increased at a linear rate only after a prolonged lag phase.

The 16 “true positive” compounds were further evaluated for cytotoxic effects on L1210 cells. Only four had IC50 values in the micromolar range: NSC 311480 (0.09 μM); NSC 311481 (0.08 μM); NSC 344270 (0.3 μM); and NSC 377071 (2 μM). All four compounds caused cells to accumulate in metaphase arrest at cytotoxic drug concentrations, confirming that their mechanism of cytotoxicity was at the microtubule level.

The “true positives” were also evaluated for inhibition of the binding of radiolabeled colchicine to tubulin. The most inhibitory compounds (approximately 50 to 75% inhibition when present in a 1:1 molar ratio to colchicine) were NSC 107390, NSC 311481, and NSC 344270. While our preliminary data are consistent with a competitive mode of inhibition for most compounds, the pattern obtained with NSC 107390 (as well as NSC 311478 and NSC 311480) was strongly suggestive of noncompetitive inhibition.

Because of the initially ambiguous GTPase results with NSC 107390, we decided to explore the reaction further, examining its time course over a 10-h incubation with 200 μM GTP (Fig. 3). In the main panel GTP hydrolysis is presented as a percentage of the control value with time on a logarithmic scale (late time points are not presented if GTP was exhausted in a reaction mixture). The 10-h incubation was possible because glutamate dramatically stabilizes tubulin (30), as well as induces polymerization and GTP hydrolysis (7, 9). The drugs chosen for this study were nocodazole, colchicine, podophyllotoxin, two new carbamates with substantial antimitotic activity (NSC 311481 and NSC 344270), the agent with equivocal activity in the initial screening study (NSC 107390), and two agents of similar molecular structure but different effects on GTP hy-
drolysis (NSC 346626 and NSC 346661). The inset presents the time course of the control reaction and those with colchicine, podophyllotoxin, NSC 346626, and NSC 346661, with nmol/ml of GDP formed plotted against time on a linear scale. The primary points we wish to make from this experiment involve the use of the GTPase assay as a screening test for potential antitubulin agents. There is no common pattern observed with every active agent. Generally there are time points with each agent when net GTP hydrolysis was either inhibited or stimulated. Consequently, most reactions containing drug will be similar to the control reaction at some point in their time course. Second, changing the GTP concentration had a significant effect on the reaction with NSC 107390 relative to the control reaction, for reproducible stimulation (at 20 min) occurred with 200 μM GTP. A similar situation could arise with other active agents in the future.

In addition, the reactions observed with podophyllotoxin, NSC 346626, and NSC 346661 deserve special mention. Podophyllotoxin has been described as an inhibitor of tubulin-dependent GTP hydrolysis, in contrast to the stimulation obtained with colchicine (8, 9, 12, 14), but this is clearly an oversimplification. Under the reaction conditions used here, podophyllotoxin profoundly inhibited GTP hydrolysis only for about 10 min. At this point a hydrolytic reaction began that caught up with the control reaction at about 90 min, resulting in significant stimulation at long incubation times. Since there was no turbidity change associated with either phase of the GTPase reaction in the presence of podophyllotoxin, the physical basis of the drug's effects on hydrolysis is unknown.

Despite similar structures, NSC 346626 and NSC 346661 affected the GTPase reaction differently. NSC 346661 inhibited GTP hydrolysis over the entire time course of the reaction. NSC 346626 stimulated the reaction for at least 1 h, but then hydrolysis substantially decelerated and almost ceased entirely by about 3 h (see Fig. 3, inset). (Of the other agents in this class, NSC 346624 and NSC 346625 were stimulatory, while NSC 346735 and NSC 346736 were inhibitory.) With both NSC 346626 and NSC 346661 there were delayed, but marked, increases in turbidity under this reaction condition, possibly indicating precipitation or aggregation of the protein and accounting for the limited hydrolysis obtained with NSC 346661 and the decreasing reaction with NSC 346626.

**DISCUSSION**

Our goal in this study was to evaluate the potential of tubulin-dependent GTP hydrolysis as a simple screening assay of compounds of unknown activity as possible antimitotic agents directed against tubulin. The results presented here confirm the prediction (16, 17) that the assay could serve this function. A major advantage of the GTPase assay over polymerization and cytotoxicity/antimitosis studies is economy of materials and its ready adaptation to large-scale screening. We have used a 30-μl reaction mixture, and this can be further reduced to conserve materials if desired. We have also found that a single investigator can screen 50 compounds in a single experiment without undue difficulty. Final confirmation of cytotoxic activity at the microtubule level, of course, requires additional studies, such as those performed here.

A primary requirement for any screening procedure is minimal generation of both false negatives and false positives. The correlation between effects on GTP hydrolysis and on tubulin polymerization was quite satisfactory. There were no false negatives (excluding the borderline case of NSC 107390) in terms of compounds which inhibited polymerization without affecting GTP hydrolysis, and there were only ten false positives (compounds with little effect on polymerization that altered tubulin-dependent GTP hydrolysis under the screening assay reaction conditions).

In terms of correlation with cytotoxicity, the screening assay was less successful: only 4 of 26 GTPase-positive compounds had significant effects on cell growth (no attempt was made to establish a "false negative" rate in this more complex evaluation procedure). Nevertheless, all four "true positive" cytotoxic agents did appear to have as their mechanism of action inhibition of mitosis, as predicted by the GTPase assay. The primary problem with the assay, at least with carboxamides, appears to reside in a greater sensitivity of tubulin, as opposed to cells, to these compounds. Whether this sensitivity reflects differences in permeability, cellular metabolism, or long-term drug stability cannot be determined from the experiments we have performed thus far.

Fig. 3 demonstrates another limitation in the GTPase screening test as we performed it in the studies presented here. The variable effects of different agents on the reaction make it unlikely that all antimitotic compounds can be identified in a single assay procedure. Consequently, as opposed to the duplicate assays, to reduce the possibility of error, which we performed in this study, we would recommend that each agent be evaluated under two different reaction conditions, including differing reaction times, if the GTPase reaction is used to select potential antitubulin compounds.

Carbamate compounds were chosen as a test of the screening assay, because of the large number of available compounds and the likelihood that positive agents might be found. The fact that active carboxamides have highly variable structures is puzzling and makes it difficult to evaluate these compounds in terms of structure-activity relationships. It is probable that optimally active compounds in this class remain to be discovered and make their continued evaluation a worthwhile effort.

While their nuclei vary somewhat, several of the positive agents identified in this study probably represent analogues of benzimidazole carboxamides (NSC 311478, NSC 311480, NSC 311481) or of NSC 181928/330770 (NSC 344270). But no such obvious analogies exist in the other compounds. In one group of agents (NSC 107390, 275954, 275971, 281383, 294307, and 377071), although the nucleus is either benzimidazole itself or structurally analogous to it, the carbamate function and the second substituent bear different relationships to each other than occur in benzimidazole antimitotics such as nocodazole. Similarly, although there is a structural similarity in the nuclei (two fused 6-member rings), the carbamate function and the second substituent are narrowly separated in the family of compounds represented by NSC 346624, as opposed to their wide separation in NSC 181928/330770. These marked structural variations may indicate overlapping binding sites as opposed to a unique site for all compounds.

In reviewing the literature, we found that at least two of the agents positive in the GTPase and polymerization assays have previously been described. (a) The L1210 cytotoxicity and antimitotic activity of NSC 344270 were compared to those of NSC 330770 by Wheeler et al. (31). These workers found the latter compound about 60-fold more cytotoxic, as opposed to

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[3] Over the course of the studies described here, we noted progressive deterioration of inhibitory effects with the solutions and/or dry stock powders of many of the agents we have used: nocodazole; NSC 181928; 330770; 311480; 311481; 346624; 346625; 346626; 346661; and 346735. The instability of NSC 346626, 346661, and 311481 may have contributed to their decreasing stimulation of GTP hydrolysis at longer incubation times in the experiment presented in Fig. 3.
the 3- to 4-fold greater activity we observed. (b) NSC 377071 is the anthelmintic agent cambendazole, which has been noted to cause mitotic arrest in mammalian cells (18, 32). In addition, two groups (20, 33) have described inhibition of microtubule assembly with NSC 377071, although it was less potent in their hands as compared to ours (IC50 value of 70 to 80 μM, rather than 25 μM).

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