In vitro screening for inhibitors of the human mitotic kinesin Eg5 with antimitotic and antitumor activities

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Abstract

Human Eg5, a member of the kinesin superfamily, plays a key role in mitosis, as it is required for the formation of a bipolar spindle. We describe here the first *in vitro* microtubule-activated ATPase-based assay for the identification of small-molecule inhibitors of Eg5. We screened preselected libraries obtained from the National Cancer Institute and identified *S*-trityl-L-cysteine as the most effective Eg5 inhibitor with an IC_{50} of 1.0 μmol/L for the inhibition of basal ATPase activity and 140 nmol/L for the microtubule-activated ATPase activity. Subsequent cell-based assays revealed that *S*-trityl-L-cysteine induced mitotic arrest in HeLa cells (IC_{50}, 700 nmol/L) with characteristic monoastral spindles. *S*-trityl-L-cysteine is 36 times more potent for inducing mitotic arrest than the well-studied inhibitor, monastrol. Gossypol, flexeril, and two phenothiazine analogues were also identified as Eg5 inhibitors, and we found that they all result in monoastral spindles in HeLa cells. It is notable that all the Eg5 inhibitors identified here have been shown previously to inhibit tumor cell line growth in the NCI 60 tumor cell line screen, and we conclude that their antitumor activity may at least in part be explained by their ability to inhibit Eg5 activity. [Mol Cancer Ther 2004;3(9):1079–90]

Introduction

Standard antimitotic natural products used for cancer chemotherapy specifically target tubulin, the microtubule building block (for review, see ref. 1). The most effective agents were mainly first isolated from plants. The *Vinca* alkaloids, vincristine and vinblastine (isolated from leaves of the Madagascar periwinkle plant), are now used to treat leukemia and Hodgkin's lymphoma, whereas paclitaxel (Taxol) [originally extracted from the bark of the western yew tree (2)] and its semisynthetic analogue docetaxel (Taxotere) are approved for the treatment of metastatic breast and ovarian carcinomas. The success of these natural products has initiated the development of ~30 second-generation tubulin drugs currently in preclinical or clinical development (reviewed in ref. 3).

All antimitotic tubulin agents interfere with the assembly and/or disassembly of microtubules and produce a characteristic mitotic arrest phenotype. Even low paclitaxel concentrations (~10 nmol/L), with no obvious effect on microtubule dynamics, are sufficient to block cells in mitosis at the metaphase/anaphase transition (4). Eventually cell death occurs through sustained mitotic arrest or by abnormal exit. Microtubule drugs have several undesired side effects, including neurotoxicity (5), and cells may develop resistance during prolonged treatment (6). Neurotoxic side effects related to tubulin drugs are not surprising because tubulin is a major player not only in cell division but also in mitosis-independent cytoskeletal functions (reviewed in ref. 3).

Several strategies have been proposed for the development of potentially more effective and less toxic anticancer drugs. One is to improve existing drugs or to find new ones that target tubulin (7). Another approach is to target other proteins, such as microtubule-associated proteins or mitotic checkpoint proteins, with inhibition also leading to mitotic arrest and cell death. Members of different protein families (kinase families and kinesin superfamily members) are currently under investigation. Because many of these proteins are thought to have very specialized and specific functions at discrete phases of mitosis, inhibition may hopefully produce fewer side effects than known tubulin drugs.

Members of the kinesin superfamily play important roles in intracellular transport and cell division (8). They participate in different stages of cell division such as spindle pole separation and kinetochore attachment. There are at least nine different human kinesins known to be involved in mitosis, and some of these might be potential targets for drug development (for more information about molecular motors as potential targets in cancer research, see refs. 3, 9). The plus-end directed NH_2-terminal motor Eg5, a member of the BimC (blocked in mitosis) subfamily, is responsible for establishing and maintaining the bipolar spindle (10), whereas its counterpart, the COOH-terminal motor human spleen embryonic tissue and testes (HSET), has been proposed to oppose the force developed by Eg5 (11, 12). Mitotic kinesin-like protein 1 (MKLP1) is required

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for mitotic progression (13). Human KIF4 is associated with chromosomes during mitosis (14). The kinetochore-associated motor CENP-E is essential for chromosome alignment (15). The DNA binding protein Kid is involved in chromosome movement during mitosis (16–18). Mammalian centromere-associated kinesin (MCAK) is associated with the centromere region during mitosis (19). M-phase phosphoprotein 1 (MPP1) is required for completion late in cytokinesis (20, 21). Human RB6K (22) is essential for cytokinesis (23). Other kinesins may turn out to be involved in mitosis when more of the 40 different kinesins reported in the human genome are investigated in detail (24).

Microinjection of antibodies against human Eg5 leads to a stable mitotic block with monoastral microtubule arrays (10, 25), similar to the mitotic arrest observed with drugs that interfere with microtubule assembly. Subsequently, and not unexpectedly, Mayer et al. (26) showed very elegantly that it is possible to specifically target cell division proteins other than tubulin by using a small molecule, monastrol, which targets Eg5 and leads to mitotic arrest. This work on monastrol subsequently led to considerable activity in several fields such as in chemistry for the improved synthesis of monastrol, enantioseparation, and synthesis of monastrol analogues (27–29); in biochemistry for the in-depth characterization of the monastrol-Eg5 interaction (30, 31); in structural biology for the crystallographic structure of human Eg5 in the native form (32) and complexed with monastrol (33); and in cell biology for the detailed characterization of the inhibition of Eg5 during the cell cycle (34, 35). Recently, a new natural product has been described that is extracted from a soil-isolated fungus and named terpendole E, which also targets human Eg5 leading to mitotic arrest (36). A series of 2-(aminomethyl) quinazolinone derivatives has been identified that leads to mitotic arrest and cell death by targeting Eg5 (37); among these, SB-715992 is in phase II for evaluation in anticancer therapy (38). The same approach can in principle be applied to other kinesin motors essential for mitotic spindle function (39).

Kinesins possess an intrinsic basal ATPase activity, which is stimulated in the presence of microtubules by a factor varying from several hundred up to 10,000 times. This characteristic feature can be exploited for high throughput screening. Using different preselected small-molecule libraries from the National Cancer Institute (NCI, Bethesda, MD), we applied a conventional *in vitro* screening procedure to search for compounds inhibiting the ATPase activity of mitotic Eg5. We show that several of the detected inhibitors induce the formation of monoastral spindles and arrest HeLa cells in mitosis. The observed monoastral spindle phenotype is similar to that induced by monastrol and is consistent with an inhibition of Eg5 function. By comparing these results to the NCI database, Eg5 is shown to be the target for several of the tumor growth inhibitors listed in the NCI 60 cell tumor screen. Some of these molecules might serve as starting points for the development of new anticancer drugs with improved specificity and efficacy.

Experimental Procedures

Materials

The 96-well clear plate for screening is from Greiner Bio-One (Frickenhausen, Germany). Chromatographic materials (High-Trap SP and Q-Sepharose, Superose 12) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Chemicals for ATPase assays were from sources indicated by Hackney and Jiang (40). Paclitaxel was from Sigma Chemical Co. (St. Louis, MO) and docetaxel was a gift from Rhone-Poulenc (now Aventis, Paris, France). Small-molecule libraries, potential inhibitors, and inhibitor derivatives are from the NCI at the NIH. All other chemicals were bought from Sigma Chemical.

Purification of Kinesin Motors

The purification of human Eg5 (monomeric construct $Eg5_{2\text{-}386}$), conventional kinesin, *Drosophila melanogaster* nonclaret disjunctional (Ncd), and *Aspergillus nidulans* BimC has been described previously (31).

Purification of Tubulin

Tubulin was purified from bovine brain as described earlier (41), aliquoted at 15 mg/mL, frozen in liquid nitrogen, and stored at −80°C. For the microtubule-activated ATPase activity of Eg5 used in the initial screening procedure, tubulin was polymerized into microtubules overnight at 37°C at 50 μmol/L (5 mg/mL) in the presence of 10 μmol/L paclitaxel.

Measurement of ATPase Rates

All experiments were done at room temperature using the 96-well Sunrise photometer (Tecan, Maennedorf, Switzerland) at a final volume of 200 to 250 μL per well. Steady-state microtubule-activated ATPase rates were measured using the pyruvate kinase/lactate dehydrogenase–linked assay in buffer A25A [25 mmol/L potassium ACES (pH 6.9), 2 mmol/L magnesium acetate, 2 mmol/L potassium EGTA, 0.1 mmol/L potassium EDTA, 1 mmol/L β-mercaptoethanol (40)]. In the presence of paclitaxel stabilized microtubules, 300 nmol/L $Eg5_{2\text{-}386}$ was used for the assay, and in the absence of microtubules, the basal ATPase activity was measured using 4 μmol/L $Eg5_{2\text{-}386}$ for either the above assay or the malachite green assay. For optimal inhibitor solubility, the assays were done in the presence of up to 2.2% DMSO. A control experiment at this DMSO concentration showed no effect on the microtubule-activated ATPase activity. The data were analyzed using Kaleidagraph 3.0 (Synergy Software, Reading, PA) and Microsoft Excel to obtain the kinetic variables k_{cat}, V_m, and $K_{50\%,\ microtubules}$.

Small-Molecule Libraries

Small-molecule libraries used in this study are from the NCI/NIH. The structural diversity set (1,990 molecules) represents the structural diversity of 140,000 molecules kept on 96-well plates. Detailed information about the composition is available elsewhere.[4] The mechanistic set (879 molecules) is unique because the molecules from this screen represent a broad range of growth inhibition

[4] http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html.

patterns in the NCI 60 tumor cell line screen based on the GI_{50} activity of the compounds.[5] Inhibitor analogues tested in this study are from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis (NCI) database. The quality of small organic molecules that have been found to inhibit human Eg5 activity was tested using liquid chromatography-mass spectrometry.

NCI 60 Tumor Cell Line Screen and Definitions of Antitumor Activity

A detailed description of the DTP human tumor cell line screen can be found elsewhere[6] (42–44). When comparing our screening data with the data from the NCI 60 tumor cell line screen, we used the NCI Cancer Screen Current Data DTP 60 Cell/5 Dose (September 2003). GI_{50} is 50% inhibition of growth. IC_{50} is the median inhibitory concentration.

In vitro Inhibitor Screening of Human Eg5

A mixture (244 μL) of paclitaxel stabilized microtubules in buffer A25A and enzymes was aliquoted onto a 96-well clear plate. For the structural diversity set, small molecules (2.5 μL) were added to a final concentration of 100 μmol/L. Small molecules of the mechanistic set were measured at a final concentration of 50 μmol/L. The first (A1-H1) and last (A12-H12) column of each 96-well plate were used for negative (the activity of Eg5 in the absence of any inhibitor) and positive (inhibition of Eg5 activity by monastrol) controls. After adding 4 μL Eg5 to all 96 wells, using either 8-channel or 12-channel Pipetman, the solutions were mixed and the absorbance at 340 nm was measured for 5 to 10 minutes, taking measurements every 5 seconds for each well. Data were imported into Microsoft Excel and treated automatically. The *Z* factor (45) was used as a criterion for judging the quality of the collected data. Molecules for which the measured ATPase activity was reduced by more than three times the SD of the mean of the uninhibited ATPase activity for each plate (eight data points) were considered as potential inhibitors of Eg5 activity and aliquoted into two new 96-well plates (154 molecules). As a second step, the basal ATPase activity of these selected molecules was measured at 50 μmol/L using Eg5 at 3.4 μmol/L. The inhibition of the final 15 molecules was tested using the malachite green assay.

Determination of IC_{50} Values by Inhibiting the *In vitro* ATPase Activity

The IC_{50} values for the inhibition of *in vitro* basal and microtubule-activated ATPase activities of kinesin motors were determined as described recently (31). Monastrol was used as a positive control. When necessary, the inhibitory concentrations were adapted depending on the initial IC_{50} value. Each inhibitory concentration was measured three times and averaged data points are shown with error bars ± SD.

Cell Culture Immunofluorescence Microscopy

HeLa cells were grown on DMEM (Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and maintained in a humid incubator at 37°C in 5% CO_2. Cells were left to adhere for at least 36 hours on poly-D-lysine–coated glass in 24-well plates before addition of the drugs. Following incubation with drugs for 8 hours, cells were fixed with 1% paraformaldehyde-PBS at 37°C for 3 minutes followed by an incubation in 100% methanol at −20°C for 5 minutes and washed with PBS for 5 minutes. After two additional 5-minute washes, fixed cells were incubated with YL1/2 anti-α-tubulin (a generous gift from Dr. J.V. Kilmartin, Medical Research Council, Cambridge, United Kingdom) for 1 hour and with a FITC-conjugated goat anti-rat secondary antibody (The Jackson Laboratory, West Grove, PA) for 30 minutes and counterstained with propidium iodide. Images were collected with a MRC-600 laser scanning confocal apparatus (Bio-Rad Laboratories, Hercules, CA) coupled to a Nikon Optiphot microscope (Melville, NY).

Results

To use the inhibition of the microtubule-activated ATPase activity of human Eg5 as a selection criterion, we first needed to find a stable and active Eg5 construct that could be easily purified in large amounts. Cloning, expression, and purification of several human Eg5 constructs have been reported previously (31). Monomeric Eg5 (i.e., the construct $Eg5_{2\text{-}386}$) was found to be the most stable construct and could be purified in the fully active state in large enough quantities to develop and perform the high throughput screen. Stability was examined by measuring ATPase activity over a 5-day period at 4°C and at room temperature. At 4°C, the Eg5 protein is stable for at least 5 days, losing only 20% of its initial activity (data not shown).

Identification of Potential Eg5 Inhibitors by *In vitro* Screening

The inhibition of Eg5 activity by small molecules was measured using the microtubule-activated ATPase assay at 100 μmol/L for molecules from the structural diversity set and 50 μmol/L for molecules from the mechanistic set. The overall *Z* factor achieved for this test was 0.7, calculated from control data as described in Experimental Procedures. Of the 2,869 molecules tested, 154 molecules inhibited microtubule-activated Eg5 activity. These molecules were regrouped into two 96-well plates and their effect on the basal Eg5 ATPase activity (without microtubules) was measured to exclude molecules that inhibit the microtubule-activated Eg5 activity by influencing, for example, microtubule polymerization and depolymerization. Of the initial 154 molecules, 15 inhibited the basal Eg5 ATPase activity. The overall *Z* factor for this assay was 0.8. Therefore, 139 molecules inhibited the microtubule-activated Eg5 ATPase activity without directly targeting Eg5 probably by influencing microtubule depolymerization or some other component of the assay (lactate

[5] http://dtp.nci.nih.gov/branches/dscb/mechanistic_explanation.html.

[6] http://dtp.nci.nih.gov/branches/btb/ivclsp.html.

dehydrogenase or pyruvate kinase) or by preventing Eg5 from binding to microtubules. To identify molecules, which act on enzymes included in the coupled test (lactate dehydrogenase or pyruvate kinase) and not Eg5 itself, we measured the basal Eg5 ATPase in the presence of the 15 inhibitors using the malachite green ATPase activity test (40). Ten molecules significantly inhibited Eg5 activity. The screening strategy and example of the results obtained by measuring in 96-well plates are summarized in Fig. 1. These molecules were further characterized. The chemical names (and when available trivial names), National Service Center (NSC) numbers referred to as in the text, and chemical structures of Eg5 inhibitors are shown in Table 1.

Determination of IC_{50} Values for Basal ATPase Activity

To compare the efficiency of these 10 compounds, we determined the IC_{50} values by measuring the inhibition of basal ATPase activity in the absence of microtubules using increasing amounts of inhibitors and included racemic monastrol as a positive control. The results are shown in Table 2. The IC_{50} values cover a broad range from very low micromolar (1.0 μmol/L) concentrations for NSC 83265 up to ~145 μmol/L for NSC 270718. This clearly proves the usefulness of the *in vitro* screening procedure because it shows that the assay is sensitive enough to pick up even weakly inhibiting molecules. The inhibition curves of a selected set of inhibitors are shown in Fig. 2.

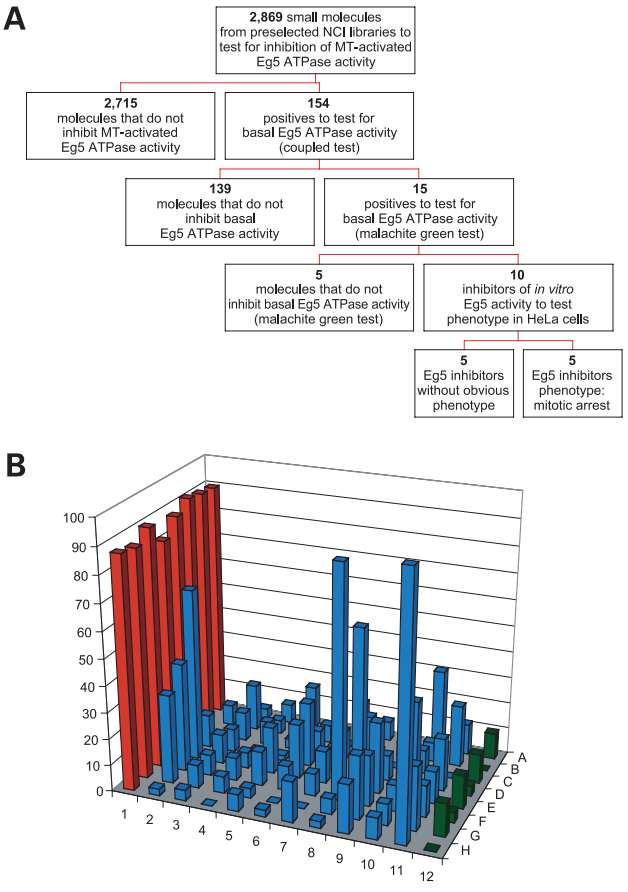


Figure 1. *In vitro* screening of human Eg5. **A,** summary of the complete screening procedure employed to identify inhibitors producing a mitotic arrest phenotype in HeLa cells. Of the 2,869 molecules tested, five small molecules significantly provoked monoastral spindles when incubated with HeLa cells. **B,** example of inhibition of the basal ATPase activity of Eg5 using a 96-well screening procedure. *Column 1,* inhibition of the Eg5 ATPase activity by monastrol used as a control inhibitor. *Column 12,* uninhibited basal ATPase activity. *Columns 2–11,* results for small-molecule inhibitors of microtubule-activated Eg5 ATPase activity (at 50 μmol/L).

Specificity of Eg5 Inhibitors Tested on Other Kinesins

The specificity of the Eg5 inhibitors was tested using three other members of the kinesin superfamily: human conventional kinesin (construct HK379) as the prototype for a plus-end directed molecular motor involved in intracellular transport (46, 47); *A. nidulans* construct Trx_1-428, a member of the BimC kinesin subfamily (48); and dimeric Ncd (construct MC5) as a minus-end directed prototype kinesin (49). The results are summarized in the last three columns of Table 2. Two compounds (NSC 56817 and NSC 119889) weakly inhibit BimC, whereas NSC 622124 inhibits Ncd. The other seven compounds do not significantly inhibit the three kinesins.

Phenotype Description of Eg5 Inhibitors

Two of 10 inhibitors were outstanding. At inhibitory concentrations of 100 μmol/L, nearly 100% of mitotic cells treated with NSC 83265 showed monoastral spindles (Fig. 3A). It is noteworthy that interphase HeLa cells seemed to be unaffected and exhibited an apparent normal interphase radial microtubule network even at a *S*-trityl-L-cysteine concentration of 100 μmol/L (Fig. 3B). At 100 μmol/L NSC 78206, 90% of the mitotic cells had monoastral spindles (Fig. 3C). At 100 μmol/L, there was also an accumulation of binucleate cells, indicating that flexeril may inhibit post-metaphase processes, with cells failing in cytokinesis (Fig. 3D). At 100 μmol/L inhibitory concentration, cells treated with NSC 56817, NSC 169676, or NSC 59349 were already dead, indicating high toxicity at this concentration. The experiment was repeated for the three compounds at 10 μmol/L and all gave the typical Eg5 phenotype with 20%, 40%, and 40% of all mitotic cells showing mitotic arrest for compounds NSC 59349 (Fig. 3E), NSC 169676, and NSC 56817 (Fig. 3F), respectively. For five compounds (NSC 622124, NSC 119889, NSC 172033, NSC 270718, and NSC 125034), 5% to 50% of the mitotic cells were in mitotic arrest. Based on these preliminary results, we decided to study the following compounds, *S*-trityl-L-cysteine (NSC 83265), flexeril (NSC 78206), phenothiazine analogues (NSC 169676 and NSC 59349) and gossypol (NSC 56817), in more detail.

Inhibition of Microtubule-Activated ATPase Activity

We determined IC_{50} values of the microtubule-activated Eg5 ATPase activity in the presence of the five inhibitors (Table 2). Again, NSC 83265 was the most effective inhibitor with an IC_{50} of 140 nmol/L. The two phenothiazine analogues (NSC 59349 and NSC 169676) inhibited Eg5 activity in the low micromolar range with an IC_{50} of 7 and

Table 1. New inhibitors of the human Eg5 ATPase activity

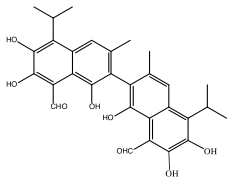
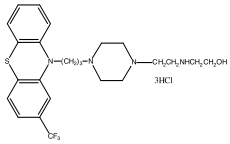
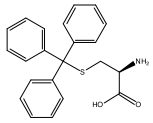
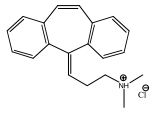
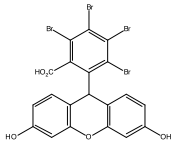
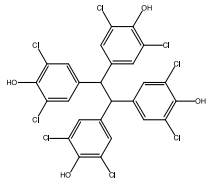
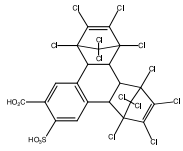
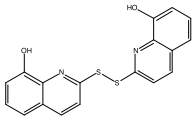
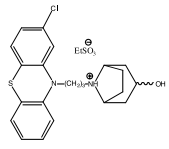
Name	NSC No.	Structure
1,6,7,1′,6′,7′-Hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-[2,2′-binaphthalene]-8,8′-dicarboxaldehyde (gossypol)	56817	
2-{[2-(4-{3-[2-(trifluoromethyl)-10H-phenothiazin-10-yl]propyl}-1-piperazinyl)ethyl]amino}-ethanol trihydrochloride	169676	
S-trityl-L-cysteine	83265	
No structure no name available in chemical databases ($K_6Mo_{18}O_{62}P_2$)	622124	$K_6Mo_{18}O_{62}P_2$
3-(5H-dibenzo[*a*,*d*]cyclohepten-5-ylidene)-*N*,*N*-dimethyl-1-propanamine hydrochloride (flexeril)	78206	$K_6Mo_{18}0_{62}P_2$ 
2,3,4,5-Tetrabromo-6-(3,6-dihydroxy-9H-xanthen-9-yl)-benzoic acid	119889	
1,1,2,2-Tetra(3,5-dichloro-4-hydroxyphenyl)ethane	172033	
10-Carboxy-1,2,3,4,5,6,7,8,13,13,14,14-dodecachloro-1,4,4a,4b,5,8,8a,8b-octahydro-11-sulfo-1,4:5,8-dimethanotriphenylene	270718	
2,2′-Dithiobis-(8-quinolinol)	125034	
8-[3-(2-Chloro-10H-phenothiazin-10-yl)propyl]-8-azabicyclo[3.2.1]octane-3-ol, compound with ethanesulfonic acid	59349	

Table 2. Specificity of Eg5 inhibitors tested on human Eg5 and other kinesins by measuring the inhibition of basal ATPase activity

NSC No.	HsEg5* $Eg5_{2-386}$		Human Conventional Kinesin HK379	*A. nidulans* BimC Trx_1-428	Dimeric Ncd MC5
	−Microtubules	+Microtubules			
56817	25 ± 3.6	10.8 ± 3	NI	140 ± 30	NI
169676	6.6 ± 0.7	9.0 ± 1.4	NI	NI	NI
83265	1.0 ± 0.2	0.14 ± 0.01	NI	NI	NI
622124	13 ± 3.5		NI	NI	28 ± 9
78206	14 ± 1	36 ± 12	NI	NI	NI
119889	88 ± 20		NI	115 ± 14	NI
172033	26.6 ± 6		NI	NI	NI
270718	22 ± 7		NI	NI	NI
125034	145 ± 50		NI	NI	NI
59349	5.6 ± 0.8	7.0 ± 2.2	NI	NI	NI

NOTE: The IC_{50} values are indicated in μmol/L. NI, not indicated.
*For human Eg5, the inhibition of the microtubule-activated ATPase activity is also shown.

9 μmol/L, whereas gossypol inhibited Eg5 activity with an IC_{50} of 10.8 μmol/L. Flexeril inhibited Eg5 less effectively with an IC_{50} of 36 μmol/L. The results are summarized in Fig. 4.

Quantification of Mitotic Arrest

Inhibition of human Eg5 by either specific antibodies (10) or small-molecule inhibitors such as monastrol (26) or terpendole E (36) leads to a mitotic arrest phenotype with the characteristic monoastral spindle. The percentage of mitotic cells with monoastral spindles was calculated for the total number of cells in mitosis after 8-hour incubation with each of the five compounds.

S-trityl-L-cysteine (NSC 83265) was by far the most potent inhibitor with an IC_{50} of 700 nmol/L (Fig. 5A). Mitotic cells with the monoastral spindle phenotype appear at submicromolar inhibitory concentrations. At

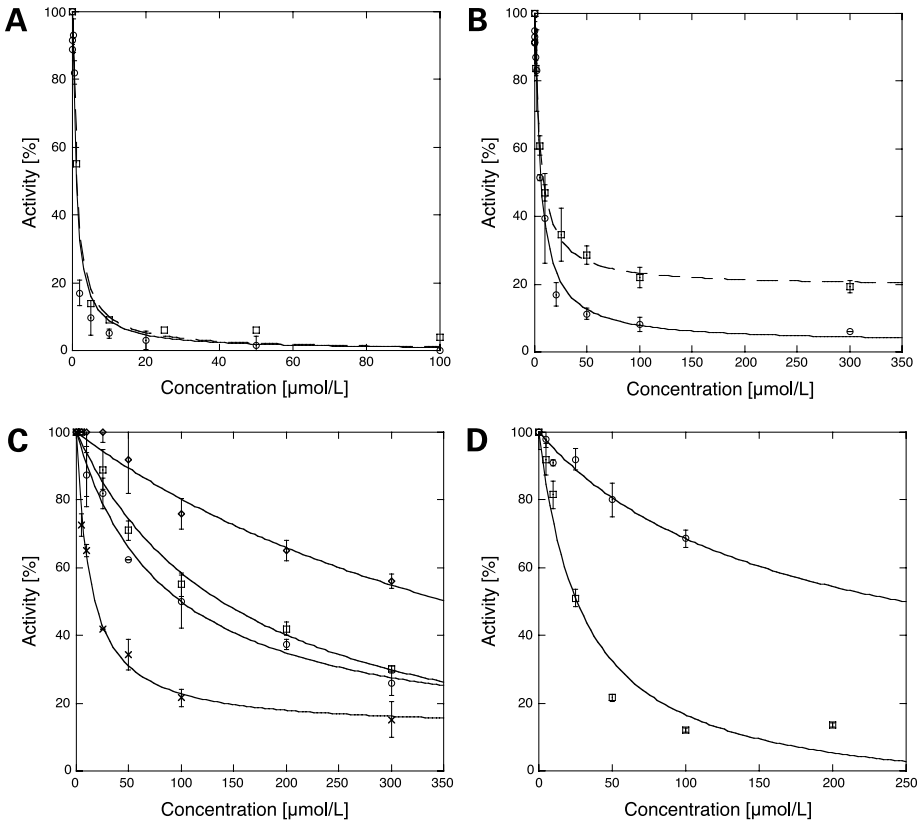


Figure 2. Inhibition of basal Eg5 ATPase activity. **A,** NSC 83265 (○) and NSC 127736 (□); **B,** NSC 169676 (□) and NSC 59349 (○); **C,** flexeril (x) and its analogues [NSC 78248 (◆), NSC 104210 (○), NSC 176555 (□)]; **D,** NSC 56817 (□) and NSC 11979 (○). NSC 11979 is insoluble at >100 μmol/L.

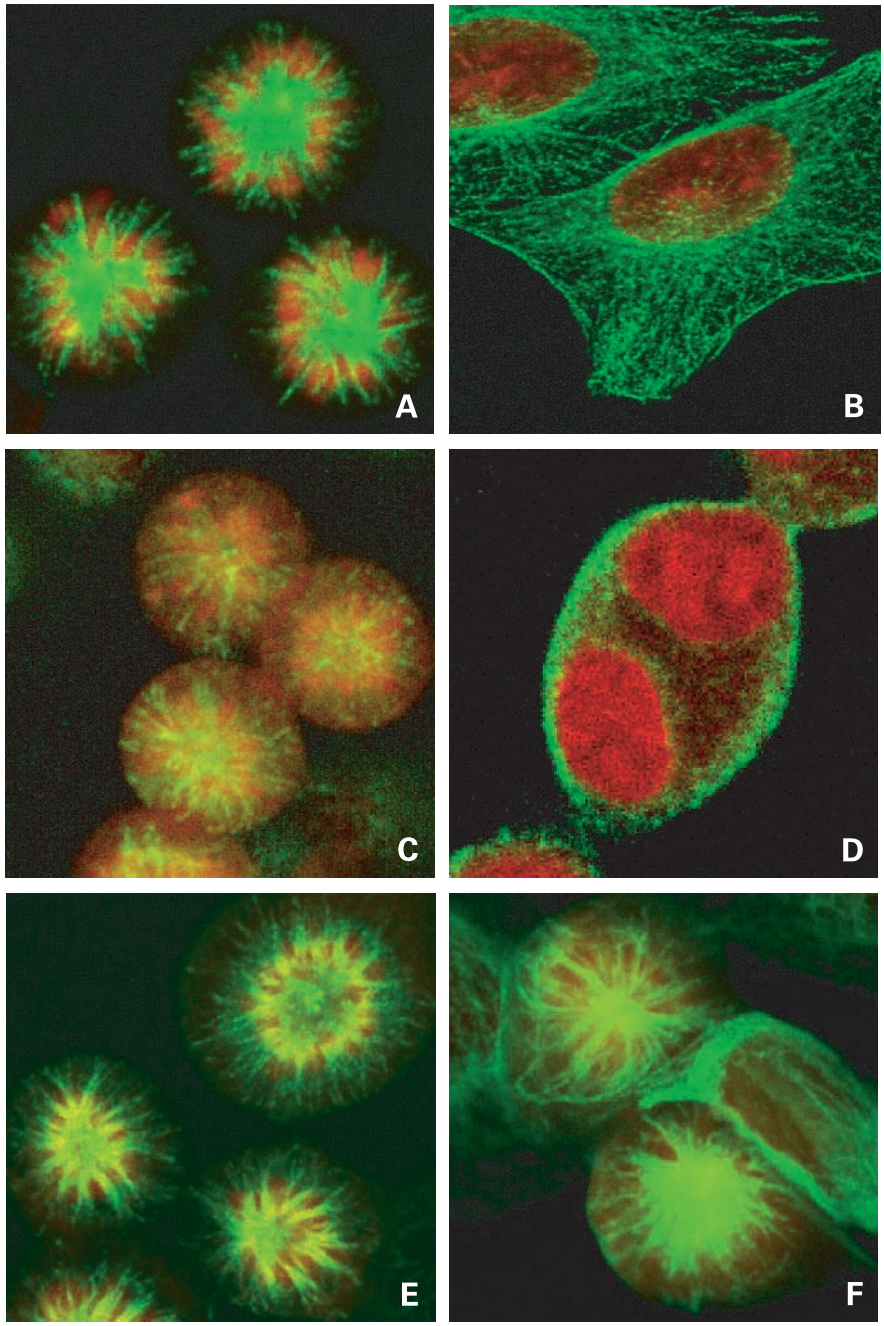


Figure 3. Observed phenotype for new Eg5 inhibitors. **A,** monoastral spindles of mitotic cells treated with *S*-trityl-L-cysteine; **B,** normal interphase microtubule network even at 100 μmol/L *S*-trityl-L-cysteine; **C,** induction of monoastral spindles in the presence of 100 μmol/L flexeril; **D,** induction of binucleated cells in the presence of 100 μmol/L flexeril; **E,** monoastral spindles in the presence of 20 μmol/L NSC 59349; **F,** monoastral spindles in the presence of 20 μmol/L gossypol.

≥2.5 μmol/L, practically all cells in mitosis appeared to be monoastral. An analogue of *S*-trityl-L-cysteine (NSC 127736) inhibits basal and microtubule-activated Eg5 activity equally well (Figs. 2A and 4A) but induces monoastral spindles with an IC_{50} of 13 μmol/L, suggesting that this compound is probably less cell permeable than NSC 83265.

Two different phenothiazine analogues, NSC 169676 and NSC 59349, inhibited both basal and microtubule-activated ATPase activities in a concentration-dependent manner with similar IC_{50} values (Figs. 2B and 4B; Table 2). HeLa cells incubated in the presence of NSC 59349 at >30 μmol/L were all dead. At lower concentrations, there was a dose-dependent appearance (calculated IC_{50}, 12 μmol/L) of monoastral spindles consistent with a loss of function of Eg5 activity in mitotic cells (Fig. 5B). HeLa cells treated with phenothiazine analogue NSC 169676 at >10 μmol/L were already dead, indicating higher cytotoxicity. Thus, although this compound is a potent Eg5 inhibitor *in vitro*, its activity on Eg5 in cells cannot be directly addressed due to its high cytotoxicity unrelated to Eg5 inhibition (Fig. 5B).

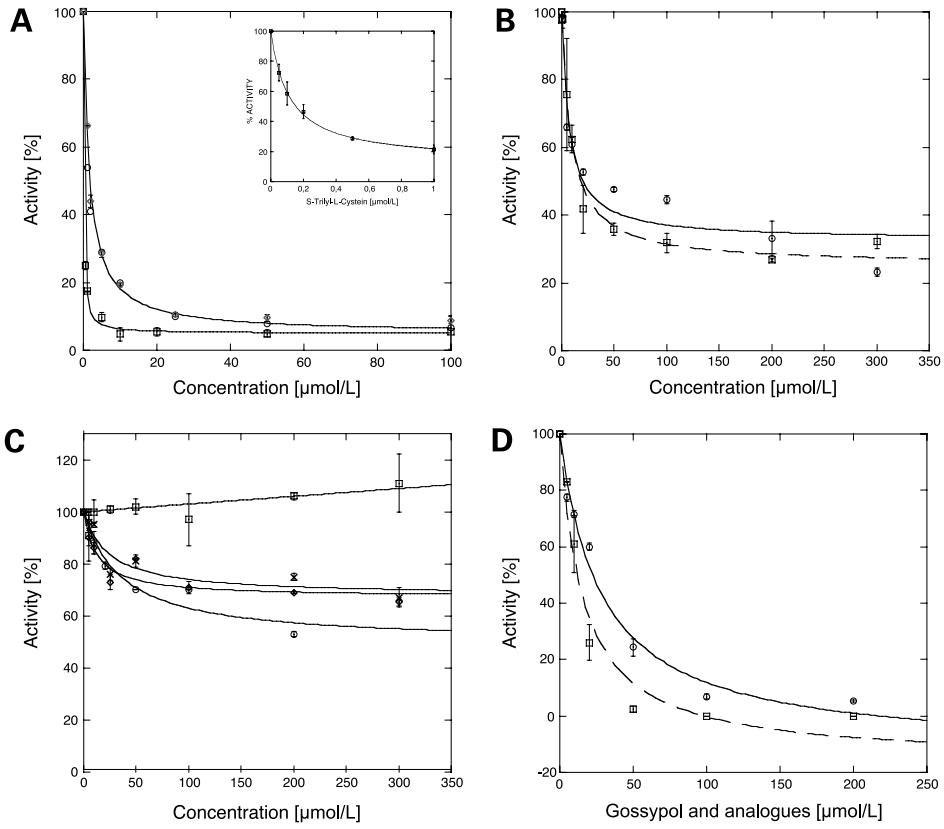


Figure 4. Inhibition of microtubule-activated Eg5 ATPase activity. **A,** *S*-trityl-L-cysteine (□) and its analogue NSC 127736 (○); **B,** phenothiazine analogues: NSC 169676 (□) and NSC 59349 (○); **C,** flexeril and its analogues; **D,** gossypol (□) and its analogue NSC 11979 (○).

Flexeril (NSC 78206) is a strong inhibitor of the basal ATPase activity in a concentration-dependent manner with an IC_{50} of 14 μmol/L (Fig. 2C; Table 2). The inhibition of the microtubule-activated ATPase activity is less pronounced (Fig. 4C), with a maximal inhibition of 40% at high inhibitory concentrations. HeLa cells treated with flexeril also showed a concentration-dependent accumulation of monoastral spindles within 8 hours of incubation. Three flexeril-related compounds (NSC 104210, NSC 176555, and NSC 78248) were also tested. NSC 104210 and NSC 176555 inhibit to a lesser extent than flexeril, Eg5 basal and microtubule-activated ATPase in a concentration-dependent manner, with IC_{50} values of 20 and 27 μmol/L, respectively. NSC 176555 did not significantly inhibit Eg5 activity and, consistent with the *in vitro* results, did not appreciably induce monoastral spindles in HeLa cells. NSC 104210 and NSC 78248 induced the monoastral spindle phenotype but to a lesser extent than flexeril (Fig. 5C).

Gossypol (NSC 56817) and its analogue NSC 11979 (Fig. 5D) are both effective inhibitors of the microtubule-activated Eg5 ATPase activity (Fig. 4D). However, gossypol analogue NSC 11979 in contrast to NSC 56817 does not significantly inhibit the basal ATPase activity of Eg5 (Fig. 2D), suggesting that the observed inhibition of the microtubule-activated ATPase activity is due to another component of the reaction mixture. Both compounds are cytotoxic to HeLa cells at 100 μmol/L. However, consistent with the *in vitro* data, there was an increasing percentage of HeLa cells with monoastral spindles after 8-hour incubation with increasing concentrations of gossypol (NSC 56817) and close to 60% of mitotic cells had monoastral spindles in the presence of the drug. In contrast, cells exposed to NSC 11979 at 50 μmol/L had only 22% mitotic cells with the characteristic monoastral spindles (Fig. 5D).

Discussion

To identify novel Eg5 inhibitors, we have used a simple and rapid *in vitro* assay to screen two small-molecule libraries from the NCI for the inhibition of the basal and microtubule-activated ATPase activities of human Eg5. Of the total 2,869 compounds screened, 10 were identified as inhibitors of basal Eg5 ATPase activity. We found that five of these also caused significant mitotic defects characterized by the presence of monoastral spindles, a phenotype expected for the loss of Eg5 function. Thus, the *in vitro* screening assay used in this study is a very powerful tool and excellent alternative to previous phenotype-based assays for the discovery of new Eg5 inhibitors (26, 36), keeping in mind that recombinant human Eg5 can be easily produced in large amounts in active form. We estimate that >10,000 tests can be done with recombinant Eg5 purified from a 3 L *E. coli* culture.

The two small-molecule libraries from the NCI (structural and mechanistic sets) have the advantage that they contain a set of ''preselected'' molecules. The structural set reflects the structural diversity of chemical compounds available, whereas the mechanistic set contains molecules that display an inhibitory effect on tumor growth in the NCI 60 tumor cell line screen (42–44). Additionally, the NCI maintains a database for the compounds including the results obtained with the NCI 60 tumor cell line screen as well as the effect of these compounds on mouse tumor models. This unique set of data allows us to compare the NCI results with those of our *in vitro* and cell-based assays. Data on previously identified Eg5 inhibitors as well as averaged GI_{50} values of the NCI 60 tumor cell line screen for our selected set of molecules are summarized in Table 3.

Comparison with Other Eg5 Inhibitors

Thus far, monastrol (26), terpendole E (36), and CK0106023, a quinazolinone analogue (50), have been identified in the literature as mitotic inhibitors targeting human Eg5 (Table 3). CK0106023 is the most effective inhibitor of Eg5 activity known thus far. The *R*-enantiomer of CK0106023, which was obtained after synthetic chemical optimization, is the effective molecule in cell-based and *in vitro* assays. This inhibitor arrests cell in mitosis and leads to growth inhibition of human tumor cell lines with a mean GI_{50} of 364 nmol/L of the cell lines tested. Thus, CK0106023 is about a factor 4 more potent than *S*-trityl-L-cysteine (GI_{50}, 1.3 μmol/L, NCI 60 tumor cell line screen). *In vitro* CK0106023 is an allosteric inhibitor of human Eg5 with a K_i of 12 nmol/L compared with 140 nmol/L for *S*-trityl-L-cysteine.

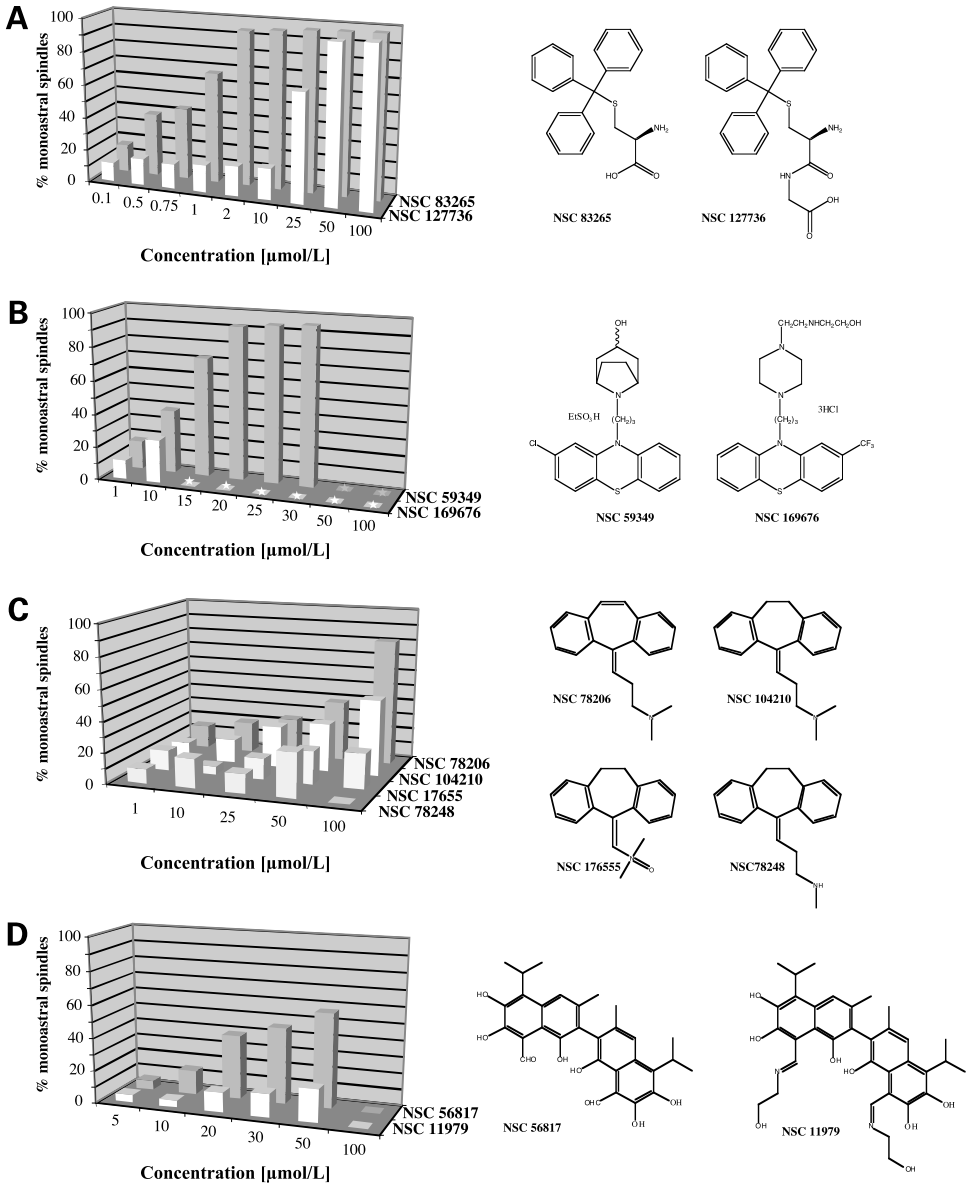
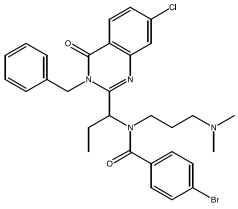
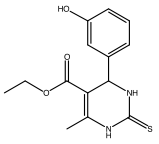
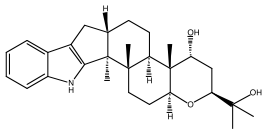


Figure 5. Concentration dependence of induction of monoastral spindles. **A,** NSC 83265 and NSC 127736. HeLa cells were incubated with increasing concentrations of the drug for 8 hours, and following fixation, cells were stained for immunofluorescence microscopy with an anti-tubulin antibody (*green*) and propidium iodide (*red*). Cells with monoastral spindles were scored as a percentage of total mitotic cells. **B,** phenothiazine-based compounds NSC 169676 and NSC 59349. NSC 59349 (*dark bars* and *stars*) induces monoastral spindles, whereas NSC 169676 (*open bars* and *stars*) is cytotoxic at >10 μmol/L. *Stars,* cell death. **C,** flexeril and its analogues (NSC 104210, NSC 176555, and NSC 78248). **D,** gossypol induces monoastral spindles more efficiently than its analogue. *Right,* in all cases, chemical structures of inhibitors and related analogues.

Table 3. Comparison of known human Eg5 mitotic arrest inhibitors

NSC No./Name	Structure	Mitotic Arrest IC_{50}, μmol/L	NCI 60 Tumor Cell Line Screen GI_{50}, mol/L*
CK0106023†		ND	364 nmol/L
Monastrol		25 (50)	ND
Terpendole E		ND	ND
56817		~40	2.92e−6
169676		12	2.97e−5
83265		0.7	1.31e−6
78206		~50	1.42e−5
59349		ND‡	2.19e−6

*Data taken from the NCI database. GI_{50} is 50% inhibition of growth averaged over 60 tumor cell lines.
†Mean growth inhibitory activity of compound CK0106023 toward 12 human cell lines. Data taken from ref. 50.
‡HeLa cells treated with 15 μmol/L NSC 59349 were already dead after 8-hour incubation.

Because we have the active monastrol enantiomer (*S*-isomer) in our laboratory, we can directly compare it with the newly discovered Eg5 inhibitors described in this article using the same Eg5 construct for *in vitro* assays and HeLa cells for the determination of IC_{50} for mitotic arrest. Compared with *S*-monastrol, *S*-trityl-L-cysteine is a more potent inhibitor of microtubule-activated ATPase activity by a factor of 36. We observe a factor of 42 when we compare the IC_{50} of HeLa cells in mitotic arrest when treated with both inhibitors. Interestingly, neither inhibitor is toxic to HeLa cells even at inhibitory concentrations as high as 100 μmol/L.

An IC_{50} for mitotic cells with monoastral spindles treated with terpendole E has not yet been reported. However, the IC_{50} for the inhibition of microtubule-activated ATPase activity for this inhibitor has been determined to be 23 μmol/L (36). Terpendole E inhibited the basal ATPase activity of Eg5 only weakly. Therefore, *S*-trityl-L-cysteine is considerably more efficient than either *S*-monastrol or terpendole E.

S-Trityl-L-Cysteine

To our knowledge, this work describes the first identification of a potential protein target for *S*-trityl-L-cysteine, which might lead to tumor growth inhibition. This non-natural amino acid is listed in the NCI standard agent database as one of 171 molecules with a ''particular high stage of interest at the NCI'' (44). The molecule is enantiomerically pure, is cheap, and can be bought in large amounts. Surprisingly little can be found in the literature, except the study of a few analogues (51, 52). Recently, the antitumor activity of irofulven in the presence of *S*-trityl-L-cysteine and other antimitotic compounds has been studied (53). Of all molecules tested, we found *S*-trityl-L-cysteine to be the most effective for inhibition of the *in vitro* Eg5 activity (IC_{50}, 1.0 μmol/L) and for inducing mitotic arrest in dividing cells (IC_{50}, 700 nmol/L). Evidence that human mitotic Eg5 is the target of *S*-trityl-L-cysteine *in vitro* and cell-based assays is supported by comparing our data with the data from the NCI 60 tumor cell line screen. The inhibition data from all sources are in very good agreement (inhibition of *in vitro* Eg5 basal ATPase activity: IC_{50}, 1.0 μmol/L; HeLa cells in mitotic arrest after treatment with *S*-trityl-L-cysteine: IC_{50}, 0.7 μmol/L; average of inhibition of growth in 60 different tumor cell lines: IC_{50}, 1.31 μmol/L). Additionally, *S*-trityl-L-cysteine is not toxic for interphase cells at 100 μmol/L. Interphase cells show no obvious visual

defect and only mitotic cells display a phenotype. We therefore conclude that human mitotic Eg5 is a target of *S*-trityl-L-cysteine and its inhibition is probably responsible for tumor growth inhibition through induction of mitotic arrest and subsequent death of proliferating cells.

Phenothiazine Analogues

Phenothiazines have a variety of biological activities: different phenothiazine analogues may show antimicrobial, antifungal, psychotropic, and antitumor activities (54) and seem to target different proteins with different mechanisms of action. The antitumor effects of several phenothiazine analogues have been investigated, but the protein target(s) responsible for the antitumor activity has remained unidentified. Using the two small libraries from the NCI, we have found two different phenothiazine analogues (NSC 169676 and NSC 59349) that inhibit the basal and microtubule-activated ATPase activities of human Eg5 and lead to mitotic arrest in HeLa cells. Both molecules inhibit tumor growth in the NCI 60 tumor cell line screen (Table 3). Thus, mitotic Eg5 might be one of the targets of phenothiazine derivatives with antitumor activity. A large number of different phenothiazine analogues from the NCI collection are currently being investigated to identify potentially more effective analogues.

Gossypol

Gossypol is a natural small molecule isolated from cotton seeds. Two well-known effects are potential use as a male antifertility agent and the inhibition of tumor growth (55, 56). Gossypol, which possesses axial chirality, displays atropisomery, so that the molecule exists as two enantiomers. (−)Gossypol possesses higher anticancer potency than (±)gossypol (57). The molecule is highly cell permeable. Gossypol is known to target several proteins such as dehydrogenases (58), cathepsin L (59), protein kinase C (60), topoisomerase II (61), protein kinase A (62), and the serine/threonine protein phosphatase calcineurin (63). To the best of our knowledge, we describe for the first time the inhibition of a member of the kinesin superfamily by gossypol leading to a mitotic arrest phenotype.

Flexeril

Flexeril (NSC 78206) is a commonly prescribed muscle relaxant that we have identified for the first time as inducing mitotic arrest in HeLa cells (Fig. 3E). Like the phenothiazine analogues, it is a tricyclic molecule but with a central seven-membered ring. Amitriptyline (NSC 104210) differs from flexeril by only one double bond, but the *in vitro* and mitotic arrest activity is reduced by a factor of 2.

Specificity of Inhibitors

Is the mitotic arrest phenotype observed with these inhibitors uniquely due to the inhibition of human Eg5 activity? We have tested the effect of the inhibitors identified in the *in vitro* screen on different members of the kinesin superfamily. Three inhibitors (NSC 56817, NSC 622124, and NSC 119889) seem to inhibit other kinesins as well. The remaining seven compounds do not inhibit the other kinesins tested. However, the final proof for the specificity of these inhibitors can only be given when several or, in the best case, all *Homo sapiens* kinesin superfamily members have been tested for inhibition. Therefore, an important step is to identify all the kinesins that are responsible for a mitotic arrest phenotype, as shown recently for 25 kinesins from *D. melanogaster* (64). Human CENP-E and MKLP1 are also known to induce a mitotic arrest phenotype (3) and should be tested for *in vitro* inhibition.

Experiments are currently under way to study in more detail the interaction between this new set of inhibitors and human Eg5 as well as the effect of inhibitor analogues, which will hopefully lead to more efficient Eg5 inhibitors.

Acknowledgments

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