S-Trityl-L-cysteine Is a Reversible, Tight Binding Inhibitor of the Human Kinesin Eg5 That Specifically Blocks Mitotic Progression*

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Human Eg5, responsible for the formation of the bipolar mitotic spindle, has been identified recently as one of the targets of S-trityl-L-cysteine, a potent tumor growth inhibitor in the NCI 60 tumor cell line screen. Here we show that in cell-based assays S-trityl-L-cysteine does not prevent cell cycle progression at the S or G2 phases but inhibits both separation of the duplicated centrosomes and bipolar spindle formation, thereby blocking cells specifically in the M phase of the cell cycle with monoastral spindles. Following removal of S-trityl-L-cysteine, mitotically arrested cells exit mitosis normally. In vitro, S-trityl-L-cysteine targets the catalytic domain of Eg5 and inhibits Eg5 basal and microtubule-activated ATPase activity as well as mant-ADP release. S-Trityl-L-cysteine is a tight binding inhibitor (estimation of \( K_{\text{app}} < 150 \text{ nM} \)) at 300 mM NaCl and 600 nM at 25 mM KCl. S-Trityl-L-cysteine binds more tightly than monastrol because it has both an ~8-fold faster association rate and ~4-fold slower release rate (6.1 \( \mu \text{M}^{-1} \text{s}^{-1} \) and 3.6 \( \text{s}^{-1} \) for S-trityl-L-cysteine versus 0.78 \( \mu \text{M}^{-1} \text{s}^{-1} \) and 15 \( \text{s}^{-1} \) for monastrol). S-Trityl-L-cysteine inhibits Eg5-driven microtubule sliding velocity in a reversible fashion with an \( I_{\text{C50}} \) of 300 nM. The s and d-antiomers of S-tritylcysteine are nearly equally potent, indicating that there is no significant stereospecificity. Among nine different human kinesins tested, S-trityl-L-cysteine is specific for Eg5. The results presented here together with the proven effect on human tumor cell line growth make S-trityl-L-cysteine a very attractive starting point for the development of more potent mitotic inhibitors.

Kinesins form a superfamily of motor proteins with about 14 different subfamilies clearly identified so far. They play important roles in intracellular transport and at different stages of cell division. The driving force behind these processes is ATP hydrolysis.

The roles of different kinesins during cell division make them highly important for understanding fundamental aspects of mitosis and meiosis. In recent years, some of them have appeared as potential targets for anti-cancer drugs (1–3). One of these mitotic kinesins, human Eg5 (HsEg5/KSP), a member of the kinesin-5 family (4), is responsible for the formation and maintenance of the bipolar spindle (5). Eg5 represents an especially attractive target because when inhibited by microinjection with suitable antibodies (5), by RNAi (6), or by treating cells with specific Eg5 inhibitors (7), it displays a very characteristic mitotic arrest phenotype, i.e. a monoastral spindle with an array of microtubules (MTs) emanating from a pair of nonseparated centrosomes surrounded by chromosomes. Cell-based as well as in vitro assays have led to the discovery of a series of inhibitors that target Eg5 and lead to mitotic arrest and cell death. Among these inhibitors are monastrol, the first Eg5 inhibitor discovered (7), terpenendole E, identified from a fungal strain (8), HR22C16, structurally related to monastrol (9), CK0106023, a quinazolinone analogue representing the most potent Eg5 inhibitor identified so far (10), dihydropyrazoles (11), and S-trityl-L-cysteine (STLC) (12). Several of these inhibitors are currently intensively studied as potential anticancer drugs, as tools for studying fundamental processes in mitosis and function of its target (chemical genetics) (13), or simply as a model to understand the mechanisms of inhibition of this important class of proteins (14–17).

By using two small, preselected libraries from the NCI, we have recently identified several new inhibitors of human Eg5 activity, with STLC being the most potent \( \text{in vitro} \) and in cell-based assays (12). STLC was shown to effectively inhibit the \( \text{in vitro} \) Eg5 basal and MT-stimulated ATPase activities (\( I_{\text{C50}} = 1 \mu \text{M} \)) and \( I_{\text{C50}} = 140 \mu \text{M} \), respectively) each at a given protein concentration and to induce mitotic arrest in HeLa cells in the sub-micromolar range (\( I_{\text{C50}} = 0.7 \mu \text{M} \)), without visible effects on the MT network, even at high inhibitor concentration. Additionally, we were able to show that STLC and monastrol share a common binding region on Eg5 (18). STLC has been identified by computer-assisted analysis of cytotoxicity data as a mitotic arrest agent (19), and it has been shown to inhibit tumor growth in the NCI 60 tumor cell line screen, making it a potentially very interesting candidate for drug development.

To better understand its inhibition mechanism, we studied the effect of STLC in HeLa and U2OS cells and \( \text{in vitro} \) by using the recombinantly...
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expressed Eg5 motor domain comprising the first 386 N-terminal residues of the native protein. We describe here the characterization of the interaction of STLC with human Eg5, its mode of inhibition, effects on Hela cells, and the characteristics of both enantiomers. These results provide a basis for understanding the inhibition mechanism of a molecular motor and for the development of more potent inhibitors.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum was bought from Hyclone. Dulbecco’s modified Eagle’s medium was purchased from Invitrogen. Nocodazole, taxol, and aphidicholine were from Sigma, and STLC was from Novabiochem. 96-Well half-area and 96-well nuclease plates were from Greiner Bio-One. The anti-y-tubulin antibody was obtained from Sigma. FITC-conjugated goat anti-rat, FITC-conjugated anti-mouse IgG, and Cy3-conjugated anti-mouse secondary antibodies were bought from Jackson ImmunoResearch, West Grove, PA. The MMP-2 mouse monoclonal antibody was from Dako, Carpinteria, CA. Mant-ADP was purchased from Jena Biosciences, Germany. The chiral chirobiotic T column was from Advanced Separation Technologies Inc., Whippany, NJ.

S-Tritylcysteine Compounds—Stock solutions of STLC (NSC 83262) and S-trityl-p-cysteine (STDC, NSC 124676) were prepared at 50 mM in Me2SO and kept at −20 °C. The purity of all compounds used in this study was checked by liquid chromatography coupled to mass spectrometry. To better judge the small differences observed between STLC and STDC by using the in vitro and cell-based assays, we compared the wavelength scans of both solutions (2 μM of each enantiomer stock solution in 98 μl of ethanol) and found that their concentrations were practically identical with a maximal error of ±4.6%. The enantiomeric purity of STLC and STDC was checked by high performance liquid chromatography (HPLC) on a chiral chirobiotic T column (250 × 4.6 mm) using a mixture of acetonitrile/water (85:15 v/v; flow rate, 1 ml/min). The wavelength for the detection of S-trityl compounds was 235 nm. STLC from either NCI or Novabiochem was eluted at tR 10.9 min, whereas STDC was eluted at tR 13.5 min. Both STLC and STDC used in this study were judged to be at least 97% enantiomerically pure. In addition STLC obtained from the NCI seemed to be enantiomerically slightly more pure than the compound purchased from Novabiochem (supplemental Fig. 1).

Expression and Purification of Recombinant Proteins—Homo sapiens kinesins containing the catalytic domain were purified as follows: Eg5 (construct HsEg52–386 with bound ADP as well as nucleotide-free and X1Eg52–430), uKHC (a member of the kinesin-1 family), MKLP1 and RabK6 (kinesin-6), KIFC1 (kinesin-14), CENP-E (kinesin-8), and Kid (kinesin-10) were expressed Eg5 motor domain comprising the first 386 N-terminal residues of the native protein. We describe here the characterization of the interaction of STLC with human Eg5, its mode of inhibition, effects on HeLa cells, and the characteristics of both enantiomers. These results provide a basis for understanding the inhibition mechanism of a molecular motor and for the development of more potent inhibitors.

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Tubulin Purification from Bovine Brain and Polymerization Assay in Vitro—Tubulin was purified from bovine brain using the polymerization-depolymerization method as described (23). The polymerization assays of tubulin (at 40 μM) in the presence and absence of STLC (50 μM) and two controls (40 μM taxol and 10 μM nocodazole) were performed in 96-well half-area plates in a volume of 100 μl at 37 °C (Sunrise photometer, TECAN, Maennedorf, Switzerland). The buffer used for polymerization of tubulin was 100 mM PIPES, pH 6.75, 1 mM Na-EGTA, 1 mM MgCl2, and 0.7 mM GTP. All concentrations indicated are final after mixing.

Testing the Specificity of STLC Using Other Human Kinesins—The ATPase activity of kinesins was determined by using the enzyme-coupled assay described by Hackney and Jiang (24). The specificity of STLC was determined by measuring the inhibition of the basal or MT-stimulated ATPase activity in the presence of increasing amounts of STLC using NaCl concentrations that had been optimized individually for each kinesin to maximize their activities.

Testing STLC and STDC for Tight Binding—Measurements of inhibition of the basal Eg5 ATPase activity were performed as described by DeBonis et al. (12). To optimize the signal for basal Eg5 activity at low protein concentration, measurements were performed in the presence of 300 mM NaCl. Eg5 at seven different concentrations (0.175, 0.35, 0.7, 1.4, 2.8, 3.5, and 4.2 μM) was incubated at room temperature for 25 min with STLC or STDC from 0 to 5 μM. The final Me2SO concentration in all cases was 2% in a final volume of 30 μl. The mixture containing Eg5 and STLC or STDC was rapidly mixed with 170 μl ATPE buffer previously dispensed into wells of a μclear 96-well plate, and the resulting decrease in absorbance at 340 nm was measured using the 96-well Sunrise photometer. The IC50 values for inhibition of basal Eg5 ATPase activity by increasing amounts of inhibitor were determined in triplicate. In a previous publication (12), we determined initial IC50 values for STLC by fitting the experimental data to Equation 1,

\[
\frac{v}{v_0} = 100 - \left( \frac{[I]}{[I] + IC_{50}} \right)
\]  

(Eq. 1)

where \(v\) is the reaction velocity at different STLC concentrations; \(v_0\) is the control velocity in the absence of inhibitor; \(A\) represents the amplitude; \([I]\) indicates the concentration of STLC, and IC50 indicates the median inhibitory concentration. This led to a nonoptimal fit for tight binding STLC. The experimental data were best fitted now to the Morrison Equation 2 (25) for tight binding inhibitors,

\[
\frac{v}{v_0} = 1 - \left( \frac{[E] + [I] + K_{app}}{[E] + [I] + K_{app}} \right)^2 - 4 \frac{[E][I]}{2[E]}
\]  

(Eq. 2)

where \(v_0\) is the velocity in the absence of inhibitor; \(v_i\) is the measured velocity; \([E]\) is equal to the total enzyme concentration; \([I]\) indicates the added inhibitor concentration, and \(K_{app}\) indicates the apparent equilibrium inhibition constant, which depend on the type of inhibition.

Stopped-flow Analysis—Stopped-flow analysis of the fast phase of STLC binding to the complex of Eg5 with mant-ADP was performed as described previously for monastrol (14). The experiments were performed at high (300 mM NaCl) and low (25 mM KCl) salt concentrations. Excitation was at 285 nm. The data for the fast phase were fitted to Equation 3 at low STLC concentrations,

\[
k_{obs} = k_{-1} + k_{1}[I]
\]  

(Eq. 3)

where \(I\) represents the different STLC concentrations, and \(k_1\) and \(k_{-1}\) are the binding and release rate constants for Reaction 1,

\[E\cdot{}mant-ADP + I \rightleftharpoons E\cdot{}mant-ADP\cdot{}I\]

REACTION 1

and \(k_1\) and \(k_{-1}\) are the rate constants for binding and release of STLC.

Motility Assays—STLC was resuspended at 50 μM in 100% Me2SO and subsequently diluted in Me2SO prior to addition to the motility buffer, to keep a constant 2% Me2SO concentration. Before loading in the chamber, Eg5 was diluted 3-fold to 5 μM using buffer A (80 mM PIPES, pH 6.9, 100 mM NaCl, 2 mM Mg(OAc)2, 1 mM Na-EGTA, 5 mM 1,4-dithiothreitol), supplemented with 1 mM ATP and 0.1 mg/ml casein. The chamber was incubated 5 min at room temperature and then
washed with buffer X containing 1 mM ATP. Finally, in vitro assembled pig brain MTs at 0.075 μM were injected in buffer X containing 1 mM ATP and 20 μM paclitaxel. Both the STLC inhibition and the Me3SO inhibition were confirmed to be fully reversible on washout.

Cell Culture—HeLa and U2OS cells were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and maintained in a humidified incubator at 37 °C in 5% CO2. Stock solutions of 1 mg/ml nocodazole and 50 mM aphidicholine were prepared in Me3SO and kept at −20 °C until use.

Immunofluorescence Microscopy—Cells were left to adhere for at least 36 h on poly-D-lysine-coated glass in 24-well plates before the addition of the drugs. Following incubation with drugs for 8 h, cells were fixed with 1% paraformaldehyde/PBS at 37 °C for 3 min, followed by an incubation in 100% methanol at −20 °C for 5 min and then washing with PBS for 5 min. After two additional 5-min washes, fixed cells were incubated with YL1/2 anti-α-tubulin and anti-γ-tubulin for 1 h and then with FITC-conjugated goat anti-rat and Cy3-conjugated antimouse secondary antibodies for 30 min and counterstained with propidium iodide. Images were collected with a MRC-600 laser scanning confocal apparatus (Bio-Rad) coupled to a Nikon Optiphot microscope.

Flow Cytometric Analysis—Cells treated as above were collected and fixed in 90% methanol at −20 °C for at least 10 min, followed by three washes with PBS. Two-dimensional flow cytometric analysis was carried out using the MPM-2 mouse monoclonal antibody, a specific mitotic marker (26), and propidium iodide, a marker of DNA content. Following incubation with MPM-2, cells were labeled with FITC-conjugated anti-mouse IgG secondary antibodies and propidium iodide as described previously (27). Data were collected using a FACScan flow cytometer (BD Biosciences) using CellQuest software, and only gated cells were taken into account.

Live Cell Imaging and Video Microscopy—For time-lapse microscopy, HeLa cells were plated in glass-bottom dishes covered with a CO2-permeable membrane and then placed inside the video microscopy platform equipped with an incubator enabling the regulation of the temperature and the CO2 level. Time lapse Z series images (Z = 3) were collected with an inverted motorized microscope (Axiovert 200M, Zeiss) controlled by MetaMorph software (Universal Imaging, Downingtown, PA). Tubulin-GFP-marked cells were observed with a plane neofluor objective (63 × 1.4 NA), and fluorescent images were acquired with a CoolSnap HQ charge-coupled device camera (Roper Scientific, Trenton, NJ) every 5 min for 8 h for the control without inhibitor and up to a maximum of 38 h in presence of 5 or 10 μM STLC under low illumination to avoid cell damage. The acquisition time was 100 ms. For each Z series images, the best focus was chosen before the reconstitution of the movie.

RESULTS

Comparison between STLC and STDC—STLC is a non-natural derivative of the α-amino acid cysteine (Fig. 1), which incorporates a single chiral center and consequently exists as two enantiomers (0 and i). The enantiomeric purity of STLC and STDC was determined by HPLC on the chiral chirobiotic T column (supplemental Fig. 1). STLC from either the NCI (Fig. 1A) or Novabiochem (Fig. 1B) was eluted at \( t_r \) 10.9 min, whereas STDC was eluted at \( t_r \) 13.5 min (Fig. 1C). The difference in retention time between the two enantiomers was more evident when the two were mixed and then separated (Fig. 1D). The STLC and STDC used in this study were both judged to be at least 97% enantiomerically pure.

To determine whether one of the enantiomers is more active than the other, we measured the inhibition of basal- and MT-activated ATPase activities using Eg5 concentrations of 0.7 and 0.073 μM, respectively, and quantified the number of monoastral spindles when incubating HeLa cells with STLC (Fig. 2). Both enantiomers inhibit the basal Eg5 ATPase activity with \( IC_{50} \) values of 403 nM for STLC and 611 nM for STDC (Fig. 2A), and the MT-activated ATPase activity with an \( IC_{50} \) of 286 ± 11 nM for STLC and 240 ± 28 nM for STDC (Fig. 2B). Phenotype-based assays with HeLa cells also showed that the two enantiomers are nearly equally potent (Fig. 2C). Within the first 8 h of incubation, both enantiomers gave a concentration-dependent accumulation of mitotic cells with MTs emanating from a single spindle aster (Fig. 2C, upper panels) with \( IC_{50} \) values of 700 and 750 nM for STLC and STDC, respectively (Fig. 2D). Immunofluorescence microscopy using a γ-tubulin antibody as a centrosomal marker revealed the two centrosomes (four γ-tubulin spots) failed to separate in the center of the monoastrons (Fig. 2C, lower panels). This monoastral phenotype has been described previously to result from inhibition of Eg5 activity either after injection of Eg5 antibodies (5) or after inhibition of Eg5 activity by specific inhibitors like monastrol (7), terpendole E (8), HR22C16 (9), or CK0106023 (10). Both enantiomers were also equally potent in inhibiting cell cycle progression at G2/M. Two-dimensional FACScan analysis (using propidium iodide for DNA content and the mitotic marker MPM2 antibody) revealed that addition of STLC and STDC in asynchronously cycling HeLa cells leads to the accumulation of cells, in a concentration-dependent manner, with a 4N DNA content (Fig. 2, E and F). After 24 h of incubation with the drugs at concentrations equal to and above 5 μM, more than 80% of the cells are arrested in G2/M phase (Fig. 2G). Interestingly, after a 24-h incubation period and even at high concentrations the drugs do not appear to be cytoxic. Furthermore, 80% of total cells were mitotic because they were positive for the mitotic marker MPM2 (Fig. 2H). These data suggest that STLC and STDC are potent mitotic inhibitors. Quantitatively similar mitotic arrest data were obtained with the U2OS tumor cell line (data not shown).

Duration and Fate of Mitotic Arrest—Longer incubation times of HeLa cells (Fig. 3A) with STLC showed that the G2/M block persisted for as long as 72 h. FACScan analysis of treated cells revealed that when starting before 48 h there is accumulation of the sub-G1 population of cells, indicative of apoptotic cells, which is more prominent at 72 h. Although they block in mitosis after prolonged incubation with STLC, U2OS cells, in contrast to HeLa cells, experience adaptation and mitotic slippage and proceed to the next cell cycle. After 72 h, there is a peak of 8N cells suggesting that the cells were able to proceed to a second round of DNA replication (Fig. 3B). A sub-G1 population of U2OS cells was also apparent.

STLC as a Specific Inhibitor of Mitotic Progression—We were also interested to know if STLC was able to block cells in a different phase of the cell cycle. U2OS cells were exposed to STLC, following presynchronization with aphidicholine at early G1/S before centrosome duplication (Fig. 3C). U2OS cells, 8 h following aphidicholine release in the presence of 20 μM STLC, were arrested in G2/M, whereas STDC was eluted at \( t_r \) 10.9 min, whereas STDC was eluted at \( t_r \) 13.5 min (Fig. 1C). The difference in retention time between the two enantiomers was more evident when the two were mixed and then separated (Fig. 1D). The STLC and STDC used in this study were both judged to be at least 97% enantiomerically pure.

To determine whether one of the enantiomers is more active than the other, we measured the inhibition of basal- and MT-activated ATPase

Inhibition of Eg5 by STLC

![Image](http://www.jbc.org/)

FIG. 1. Chemical structure of tritylcysteine. The molecule contains a chiral center and exists as \( \alpha \) (left) and \( L \)-enantiomers (right).
**Inhibition of Eg5 by STLC**

**FIG. 2.** Comparison of STLC and STDC. A, inhibition of basal Eg5 ATPase activity by STLC (open squares) and STDC (open circles). B, inhibition of MT-activated Eg5 ATPase activity by STLC (open circles) and STDC (open squares). C, double-immunofluorescence microscopy of cells stained with anti-β-tubulin for MTs and anti-γ-tubulin for centrosomes showing induction of monoastral spindles following treatment with STLC and STDC. D, induction of monoastral spindles in the presence of STLC and STDC. HeLa cells were treated at the indicated concentrations of STLC (solid bars) and STDC (open bars) for 8 h and then were fixed and stained for immunofluorescence microscopy. The percentage of monoastral spindles from the total mitotic cells were then determined. Cell cycle distribution of HeLa cells treated with STLC (E) and STDC (F) by two-dimensional FACScan analysis. 24 h of incubation of cells leads to concentration-dependent accumulation of cells with 4 n DNA content. G, quantitative representation of the histograms in E and F of the % of cells with 4 n DNA. H, quantitative representation of the histograms in E and F of cells positive for the mitotic marker MPM2 indicating a concentration-dependent accumulation of mitotic cells (STLC, solid bars; STDC, open bars).
of STLC, passed through the S phase and arrived at G2/M synchronously where they remained blocked for the next 24 h. Identical behavior was obtained with the MT assembly inhibitor nocodazole (data not shown). These results suggest that STLC does not inhibit cell cycle progression at S or G2 phases and that indeed is a specific inhibitor of mitotic progression.

Reversibility of STLC—We also examined whether the inhibition of Eg5 activity by STLC in mitotically arrested cells is reversible. HeLa cells were blocked in mitosis with STLC or with nocodazole, a well known reversible mitotic/MT assembly inhibitor, or with both. Following release from the drugs, the time course of mitotic exit was determined by FACScan analysis (Fig. 4). HeLa cells readily exited mitosis after STLC washout (Fig. 4A) and with similar kinetics as for nocodazole washout (Fig. 4B). More than 50% of the cells completed mitosis within the first 2 h after drug washout, and practically all cells returned to 2N G1 4 h later (Fig. 4D). Mitotic cells resulting from coincubation with STLC and nocodazole remained arrested after release from nocodazole only (Fig. 4C and D).

STLC Does Not Influence MT Dynamics Either in Vitro or in Cell-based Assays—In order to exclude the possibility that STLC may have additional MT assembling or stabilizing/destabilizing properties, we coincubated HeLa cells with STLC and nocodazole and washed out either both drugs or nocodazole only. We reasoned that if STLC had MT-stabilizing properties then MTs in the presence of STLC should have been resistant to nocodazole. Furthermore, if STLC had MT depolymerization properties, MTs should not polymerize following nocodazole washout in the presence of STLC. The results shown in Fig. 5 indicated that MT stability in mitotic and interphase cells was not sensitive to the presence of STLC. Following a 4-h incubation with nocodazole in the absence or in the presence of STLC, mitotic cells had only very few short MT asters (Fig. 5A). Following nocodazole washout, mitotic cells formed normal bipolar spindles within the first 30 min, and by 60 min cells were exiting mitosis. Under nocodazole washout conditions in the presence of STLC, monoaxial spindles were assembled readily, with a dense MT network nucleating from the center of the cells where the duplicated centrosomes were located. In interphase treated cells, STLC did not affect the interphase MT network and did not affect the presence of few nocodazole-resistant MTs (Fig. 5B). Interphase cells...
reformed a dense MT network within the first 30 min following nocodazole washout even in the presence of STLC, comparable with that of the control untreated cells. These data suggest that STLC at concentrations that induce monoastral spindles does not affect centrosome MT nucleating activity and neither affects MT assembly nor MT stability. These results were confirmed by performing in vitro assays, observing the polymerization of tubulin alone and in the presence of either nocodazole, taxotere, or STLC over a time range of several hours. Even at high STLC concentration (50 μM) no significant influence on MT dynamics was observed in vitro (supplemental Fig. 2).

**STLC Inhibits Bipolar Spindle Formation by Inhibiting Complete Centrosome Separation in Mitosis**—In order to test whether or not STLC is inhibiting the separation of the duplicated centrosomes in early mitosis, we employed live cell imaging of unsynchronized HeLa cells stably expressing GFP-α-tubulin in the absence or in the presence of the inhibitor (Fig. 6). A time lapse of a representative untreated cell showed that from the moment the two centrosomes were visible (Fig. 6A, time 2:50), it took 50 min to arrive into cytokinesis (A, time 3:30), and 35 min to enter anaphase. In contrast, in STLC-treated cells, although the duplicated centrosomes were initially detected as two distinct entities, which were at most separated by 2.9 μm, they finally did not fully separate, and no evidence of bipolar spindle formation was seen (Fig. 6B). The end result in all cells recorded (n = 9) was that the two centrosomes following the short initial separation collapsed into the center of a monoastral spindle. The live imaging results are in full agreement with the data presented in Figs. 1 and 5 and support the hypothesis that inhibition of Eg5 activity by STLC results in the failure to fully separate the duplicated centrosomes resulting in a monoastral spindle leading to a mitotic block.

**STLC and STDC Are Tight Binding Inhibitors of Eg5**—Having investigated the effect of STLC in cell-based assays, we wanted to clarify in more detail the mechanism by which STLC inhibits Eg5. To test the possibility that STLC and STDC are tight binding inhibitors of human Eg5, we preincubated Eg5 at different protein concentrations with increasing amounts of both inhibitors and determined the IC_{50} values. Preincubation is necessary because tight binding inhibitors are often
STLC binding is approximately the same in high and low salt at 6 mM ADP, mant-ADP, and STLC that produces a small ternary complex of Eg5, mant-ADP, and STLC with an STLC-induced conformational change in the initial phase of the reaction. The rate of the fast phase increases with increasing STLC as indicated in Fig. 7A at 25 mM KCl.

**STLC do not produce complete inhibition of ADP release, especially at low salt, as was also observed for inhibition by monastrol (14, 17).** The dose-response plot of fractional ATPase velocities of Eg5 in the absence and presence of STLC indicates that there is no time-dependent component that might characterize STLC as a slow tight binding inhibitor. We therefore measured the progress curve of Eg5 activity (phosphate release) in the absence and presence of STLC initiating the reaction by adding 0.9 mM Eg5 to a mixture of 1 mM ATP and 175 nM inhibitor. Over a period of 10 min, uninhibited as well as inhibited Eg5 displays a linear progress curve (Fig. 8C), confirming that STLC is a tight binding and not a slow tight binding inhibitor of Eg5.

**Specificity of S-Trityl-L-cysteine—**Is the mitotic arrest phenotype observed with these inhibitors uniquely due to the inhibition of human Eg5 activity? Is human Eg5 the only target? The specificity of STLC was tested using other members of the *H. sapiens* kinesin superfamily as follows: conventional kinesin (construct HK379) as the prototype for a plus-end directed molecular motor involved in intracellular transport (29–30); the kinetochore-associated motor CENP-E (31), essential for some aspects of kinetochore MT attachments; MKLP1 and RabK6, essential for cytokinesis (32–33); KIFC1, a C-terminal kinesin-related protein that opposes the activity of Eg5 (34); Kid, required for chromosome congression (35); and KIF2A and KIF2C, two kinesins with an internal motor domain (36), were similarly tested. The results are summarized in Table 1. Among the nine different human kinesins tested, only Eg5 is significantly inhibited by STLC.

**Inhibition of MT Gliding—**Eg5 and other members of the kinesin-5 family have been reported to be involved in the formation and maintenance of the bipolar spindle by moving the antiparallel spindle MTs apart (5). To investigate a possible effect of STLC on the motile characteristics of Eg5, we measured the effect of increasing STLC concentrations on recombinant Eg5 from *Xenopus laevis* (construct XEg51–430). We used *X. laevis* Eg5 because it moves about four times faster than its human counterpart. *H. sapiens* and *X. laevis* Eg5 share 40.4% sequence identity (18.4% highly similar and 9.8% weakly similar) within the motor domain. In the multimotor mode, MTs glide with a velocity of 4.68 μm/min along *Xenopus* Eg5 (Fig. 9). This velocity is inhibited by increasing amounts of STLC with an IC₅₀ of 0.5 μM. Inhibitor washout experiments were performed after successful inhibition, and in all cases, a similar activity was regained indicating that STLC is a reversible inhibitor of Eg5 activity, consistent with the results obtained by FACSscan analysis. We conclude that STLC inhibits both human Eg5 and its homologue from *X. laevis*.

**DISCUSSION**

The mitotic process is highly dynamic, and any drug that alters or inhibits this process is of potential interest as an anticancer agent (1). The secondary effects produced by certain classes of antitumor drugs such as taxanes (taxol and taxotere) and vinca alkaloids (vinblastine, vincristine, and vinorelbine), which have tubulin and MTs as their primary targets, indicate the need to search for new mitotic targets and better drugs. Human Eg5 belongs to a group of mitotic motor proteins with a high interest as potential anticancer drug targets because it is...
involved in establishing and maintaining the bipolar spindle by contributing the forces necessary for the separation of the duplicated centrosomes (5). We have previously identified STLC as an inhibitor of human Eg5 motor protein (12) and report here a more detailed characterization of the mechanism of its inhibitory action.

Our data show that STLC blocks cell cycle progression specifically at mitosis and does not interfere with S and G2 progression. STLC interferes neither with centrosome duplication nor with interphase MT distribution or nucleation of MTs of the centrosomes in mitosis. Instead, STLC inhibits centrosome separation leading to the nucleation of a dense array of MTs emanating from the center of the cell where the two unseparated centrosomes lie. The generation of a monoastral spindle gives a rosette-like distribution of condensed chromosomes leading to mitotic arrest because of the activation of the spindle assembly checkpoint. Sister kinetochores of the rosette-like chromosomes are not properly captured by MTs, and sister kinetochores do not experience equal tension.

Evidence from Drosophila embryos and cells following antibody microinjection and RNAi depletion of the Drosophila Eg5-like protein Klp61F showed that centrosomes initially separate and then collapse together in a kinesin-14-driven process, specifically during prometaphase (37–39). Similar antagonistic behavior between Eg5 and HSET motors were observed in mammalian cells (34). Our live cell imaging results with STLC-treated cells did not show any evidence of a bipolar

FIG. 7. A, dose-response plot of fractional velocities as a function of increasing STLC concentrations at seven different Eg5 concentrations using the inhibition of the basal Eg5 ATPase activity. The Eg5 concentrations used are as follows: ○ = 0.175 μM, □ = 0.35 μM, ■ = 0.7 μM, ■ = 1.4 μM, △ = 2.8 μM, ● = 3.5 μM, and ▼ = 4.2 μM. Each data point is the average of three measurements, taken in parallel and using the same buffer. Error bars represent ± S.D. B, dose-response plot of fractional velocities as a function of increasing STDC concentrations at four different Eg5 concentrations using the inhibition of the basal Eg5 ATPase activity. The Eg5 concentrations used are: ○ = 0.35 μM, ◆ = 0.7 μM, △ = 1.4 μM, □ = 2.8 μM. Each data point is the average of three measurements, taken in parallel and using the same buffer. Error bars represent ± S.D. C, plot of IC50 values for STLC (●) and STDC (◆) obtained from the curves represented in A and B, as a function of Eg5 concentration.
spindle formation and did not show any evidence of full centrosome separation, although an initial but very short distance centrosome separation was detected very early in prophase. One explanation for this discrepancy is that it is possible that there are still some low but undetectable levels of Klp61F following RNAi that may permit the formation of a bipolar spindle, but these low levels are unable to sustain it. One advantage of using specific chemical inhibitors is that depending on the concentration used one can be sure that all the target protein is inhibited. Alternatively, subtle differences in the properties in the motors

![FIG. 8. Mant-ADP release from Eg5 at different salt concentrations. A, slow phase of ADP release. Mant-ADP was chased from Eg5 by rapidly mixing with 200 μM ATP and different STLC concentrations (0 to 16 μM). The Eg5 concentration used was 0.1 μM, and data were fitted with a mutual depletion model. The rate of ADP release is salt-dependent; the presence of 25 mM KCl (filled triangles) gives 90% fractional inhibition at saturating STLC concentration with a Kd of 0.64 μM. In the presence of 300 mM NaCl (open circles), the inhibition is 98% with a KD of 0.13 μM. B, kinetics of fast phase of fluorescence decrease on addition of STLC to Eg5 mant-ADP. A complex of Eg5-mant-ADP was formed by adding a 10-fold excess of mant-ATP to Eg5 and incubating for 10 min at room temperature to allow equilibration of nucleotide at the Eg5-binding site. This complex was then mixed in the stopped-flow apparatus with STLC and ATP (0.2 mM final concentration), and the rate for the initial fast phase was determined. The buffer was A25 with 25 mM KCl (open triangles) or 300 mM NaCl (open circles), and the Eg5 concentration was 0.4 μM. The inset shows results extending the lower STLC concentrations obtained with an Eg5 concentration of 0.125 μM Eg5 in the presence of 25 mM KCl. C, progress curve of Eg5 activity in the absence (filled circles) and presence (filled squares) of STLC followed over a 10-min time period. The STLC concentration was 175 nM. Linear plots are obtained in both cases indicating that STLC is a tight but not slow tight binding inhibitor of Eg5 activity.

![FIG. 9. Inhibition of X. laevis Eg5 motility by STLC. The inhibition of X. laevis Eg5 motility by increasing amounts of STLC is shown as black circles, and the gliding motility after washout of the inhibitor is represented by closed triangles. The IC50 value for this experiment has been determined to be 0.5 μM.

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Inhibition of kinesin superfamily members by STLC</th>
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<tbody>
<tr>
<td>Protein names</td>
<td>Kinesin family</td>
</tr>
<tr>
<td>KIF2A/KIF2b</td>
<td>Kinesin-13</td>
</tr>
<tr>
<td>KIF2C/MCAK</td>
<td>Kinesin-13</td>
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<tr>
<td>KIF5B/HuKHCb</td>
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<td>KIF23/HsMKLP1a</td>
<td>Kinesin-6</td>
</tr>
<tr>
<td>KIFC1/HsKIFC1a</td>
<td>Kinesin-14</td>
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aData are by measuring the basal ATPase activity.
bData are from Ref. 12.
cData are by measuring the MT-stimulated ATPase activity.
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and/or the pathways of spindle assembly between Drosophila and mammals may exist.

Our cell-based data show that the mitotic arrest induced by Eg5 inhibition by STLC is reversible, in agreement with our in vitro MT gliding assays. As soon as STLC is washed out from the cell culture, mitotic arrest is lifted, and the duplicated centrosomes are able to separate and form a bipolar spindle. This in turn permits chromosomes to achieve a bipolar attachment and experience equal tension at the sister kinetochores leading to successful completion of mitosis. The mitotic checkpoint that is triggered by STLC is thus reversible.

Interestingly, our conclusion that STLC is a mitotic inhibitor is supported by previous studies, which evaluated the differential cytotoxicity data of different small molecule inhibitors using the COMPARE program (19). STLC was evaluated to be a mitotic inhibitor even though its target and potential mode of action were completely unknown. Pharmacological data for STLC available from the NCI (40–42) reveal that STLC is a strong cytoplastic drug with a mean inhibition growth concentration $G_{50}$ of 1.31 $\mu$M for the 60 different tumor cell lines. STLC has also been tested in the past by the NCI for potential antitumor activity using in vivo mouse tumor models (43). More than 20 different models have been tested. In addition, data on human lung LX-1 xenografts as well as for toxicity testing on nontumored animals are available. In all cases STLC was found to inhibit tumor growth at doses that were not lethal to the mice. The NCI data taken together with our data show that STLC is a strong mitotic inhibitor because of its ability to specifically inhibit Eg5 motor activity thus making STLC a very interesting candidate for an antitumor agent.

STLC Is a Tight Binding Inhibitor of Eg5—Our analysis of the biochemical data on the mechanism of inhibition of Eg5 motor activity by STLC provides firm evidence that STLC is a tight binding inhibitor of Eg5. In a recent paper (12), we determined the IC$_{50}$ of inhibition of the basal Eg5 ATPase activity to be 1.0 $\mu$M (determined at a Eg5 concentration of 2.6 $\mu$M). The IC$_{50}$ value for the inhibition of MT-stimulated ATPase activity, however, was 0.14 $\mu$M (Eg5 concentration = 0.073 $\mu$M), a factor of about 7 lower. One possible explanation for this effect is that STLC also interacts with MTs thus modifying the MT-stimulated ATPase activity of Eg5. We tested this possibility and dismissed it. In both the earlier and the later work, the IC$_{50}$ values obtained were close to the concentrations of Eg5 used during the tests, indicating tight binding of the inhibitor to Eg5. Further work showed that the IC$_{50}$ values vary as a function of the Eg5 concentration at a fixed substrate concentration, confirming that in fact STLC is a tight binding inhibitor of Eg5. This provides a plausible explanation for the observed differences between the IC$_{50}$ values of basal versus MT-stimulated ATPase activities. The inhibition of the MT-stimulated Eg5 ATPase activity is simply measured at a much lower protein concentration than that for the basal Eg5 ATPase activity, resulting in a much lower IC$_{50}$ value (1 $\mu$M versus 140 nM). STLC also effectively inhibits ADP release from Eg5 alone. The results in Fig. 8 indicate that STLC forms a ternary complex with Eg5-ADP and that the tighter binding of STLC to the ADP complex with Eg5, compared to monastral, is due both to faster association and slower release (6.1 $\mu$M$^{-1}$ s$^{-1}$ and 3.6 s$^{-1}$ for STLC versus 0.78 $\mu$M$^{-1}$ s$^{-1}$ and 15 s$^{-1}$ for monastral) (14). STLC thus appears to work by a similar mechanism as monastral but to bind much tighter.

Both STLC and STDC appear to be tight binding inhibitors. The two enantiomers are almost equally potent inhibitors of basal and MT-stimulated Eg5 ATPase activity. Cell-based assays showed that both enantiomers were nearly equally potent in inhibiting bipolar spindle assembly and that both blocked cells reversibly in the M phase of the cell cycle. In contrast to monastral, whose two enantiomers display different activity profiles, it appears that there is no strong selectivity for the two enantiomers in inhibiting Eg5 motor activity. Because both enantiomers are nearly equally potent, STLC analogues with no chiral center should be potent as well, making the synthesis of such inhibitors easier, because no enantioselective synthesis or enantiomer separation is required. This is currently under investigation.

STLC Reversibly Inhibits MT Gliding—STLC also inhibits X. laevis Eg5 gliding activity with an IC$_{50}$ value of 0.5 $\mu$M, which is very close to the IC$_{50}$ for inducing mitotic arrest in the HeLa cell-based assay (0.7 $\mu$M). Washout experiments after motility assays and FACScan analysis have revealed that STLC is a reversible inhibitor of Eg5 activity, comparable to monastral (7, 12), quinazolinone (10), and terpendole E (8).

Specificity of STLC—Zhu et al. (44) performed a functional analysis of all human kinesins using RNA interference. Among the 41 different kinesins investigated, at least 12 were shown to be involved in mitosis and cytokinesis. Three of them, Eg5, KIF2A, and KIFC1, were shown to be important for bipolar spindle formation. Only Eg5 and KIF2A, when depleted, displayed a similar phenotype (monoastral spindles for Eg5 and monopolar or asymmetric bipolar spindles for KIF2A) and subsequently mitotic arrest, confirming previous results for KIF2A (45). The other 10 mitotic kinesins displayed other phenotypes related to their involvement in chromosome segregation, anaphase spindle dynamics, and cytokinesis. Therefore, we investigated whether monoastral spindles could be due to KIF2A inhibition by measuring its ATPase activity in the presence of STLC. However, in vitro STLC did not inhibit KIF2A activity. In addition, we always observed monopolar but never asymmetric bipolar spindles. STLC did not inhibit any of the other kinesins tested. We therefore conclude that STLC induces mitotic arrest by specifically inhibiting human Eg5 and that it does not inhibit other human mitotic kinesins tested to date. Whether STLC inhibits kinesins involved in intracellular transport remains to be shown.

Antagonistic Effects of Eg5 Inhibitors with Other Agents—Marcus et al. (46) recently reported an antagonistic effect when treating cells with a combination of taxol and HR22C16 (9), leading to a significant decrease of cells in mitotic arrest compared with either drug alone. They argued that a combination of drugs targeting different proteins may not be favorable. An antagonistic effect has also been observed when combining irofulven, a molecule that inhibits DNA synthesis, with STLC or other antimitotic agents (47). It will be interesting to test whether a combination of STLC with taxol or taxotere leads to similar results.

Organosulfur Compounds and Mitotic Arrest—A recent publication investigated the effects of a series of garlic-derived as well as synthetic organosulfur compounds, including STLC, on colon cancer cells (48). They show that STLC specifically induces $G_2$-$M$ cell cycle arrest and subsequently apoptosis through spindle impairment but without disrupting MT structure or dynamics, excluding the possibility that its binds directly to tubulin, in agreement with our study. Apoptotic cell death induced by STLC occurs after 48 h and involves a detectable but weak caspase-3 activation and poly(ADP-ribose) polymerase cleavage, a moderate loss of mitochondrial membrane potential but not JNK1 activation, or release of cytochrome c. Xiao et al. (48) therefore argue that STLC might induce apoptosis through a caspase-independent pathway.

In conclusion, the accumulated cell-based and in vitro data suggest that STLC is a small cell-permeable inhibitor, which tightly and specifically binds to Eg5, a mitotic motor of the kinesin superfamily, but does not interfere with MT dynamics. STLC binds to the Eg5-ADP complex forming an ADP-Eg5-STLC ternary complex that leads to the inhibition of ADP release from Eg5. Consequently, it cannot undergo further ATP-driven conformational changes resulting in the inhibition of Eg5-induced gliding apart of antiparallel spindle MTs. Finally, the duplicated
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centrosomes cannot fully separate, and cells arrest in mitosis with the characteristic monasteral spindle phenotype. Prolonged mitotic arrest eventually leads to apoptotic cell death, probably also through the involvement of a caspase-independent pathway.

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S-Trityl-L-cysteine Is a Reversible, Tight Binding Inhibitor of the Human Kinesin Eg5 That Specifically Blocks Mitotic Progression

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