Chemistry & Biology, Vol. 10, 131–137, February, 2003, ©2003 Elsevier Science Ltd. All rights reserved. DOI 10.1016/S1074-5521(03)00020-6

A Novel Action of Terpendole E on the Motor Activity of Mitotic Kinesin Eg5

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Summary

To reveal the mechanism of mitosis, the development of M phase-specific inhibitors is an important strategy. We have been screening microbial products to find specific M phase inhibitors that do not directly target tubulins, and rediscovered terpendole E (TerE) as a novel Eg5 inhibitor. TerE did not affect microtubule integrity in interphase, but induced formation of a monoastral spindle in M phase. TerE inhibited both motor and microtubule-stimulated ATPase activities of human Eg5, but did not affect conventional kinesin from either *Drosophila* or bovine brain. Although terpendoles have been reported as inhibitors of acyl-CoA:cholesterol *O*-acyltransferase (ACAT), the Eg5 inhibitory activity of TerE was independent of ACAT inhibition. Taken together, we demonstrate that TerE is a novel Eg5 inhibitor isolated from a fungal strain.

Introduction

Mitosis is the process by which eukaryotic cells ensure equal distribution of their chromosomes at cell division. Progression through mitosis is inhibited by the spindle checkpoint until chromosomes are properly aligned on the mitotic spindle. Since most cells in the human body are not actively dividing in comparison to cancer cells, a drug that activates the spindle checkpoint might be useful for the treatment of malignant tumors that show rapid and abnormal cell proliferation. Indeed, some of the most useful cancer therapeutic agents, such as vinblastine and taxol, are known to activate the spindle checkpoint control through the inhibition of microtubule dynamics [1, 2]. However, these drugs directly target tubulins and inhibit several dynamic processes dependent upon microtubule activity, such as maintenance of organelles, cell shape, cell motility, and transport of vesicle and organelles [3–5]. Moreover, microtubule inhibitors sometimes cause severe side effects in nonproliferating neuronal cells [6]. In order to avoid such side effects, specific inhibitors that affect the function of the target molecule required for the M phase progression would be useful. Recently, mitotic kinesin has been recognized as an attractive target for antitumor compounds. Monastrol was isolated as a chemical inhibitor of the mitotic kinesin, Eg5 [7], from a chemical library of synthetic compounds. Since natural products are also an important source of specific inhibitors so-called bioprobes [8], we have exploited microbial products to obtain M phase-specific inhibitors that do not interact directly with tubulins [9]. We have rediscovered TerE, which was originally reported as a minor derivative of ACAT inhibitor [10, 11]. In this report, we show that TerE acts as a novel inhibitor of Eg5.

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Results

Identification of Terpendole E as a Specific Inhibitor of M Phase Progression

We screened more than 4000 fungal extracts to find cell cycle inhibitors using the mouse temperature-sensitive cell line tsFT210 and found such an activity in a culture broth of a soil-isolated fungus. Next, we purified the active principles from the fermentation broth and identified them as terpendoles C, E, H, and I (TerC, TerE, TerH, and TerI), which were originally isolated as ACAT inhibitors. Since tsFT210 is a temperature-sensitive mutant of the cdc2 kinase, tsFT210 cells are arrested at the boundary of G2/M phase when cultured at 39°C (Figure 1B). When the culture temperature of the arrested cells was decreased to 32°C, most cells passed through M phase and entered G1 phase after 4 hr (Figure 1C). In the presence of colchicine, a tubulin polymerization inhibitor, the cells released from the G2 arrest were synchronously accumulated in M phase (Figure 1D), which was confirmed by the observation of nuclear morphology. TerE at a concentration of 50 μM caused the same accumulation of cells in M phase (Figure 1E) as colchicine did; however, TerC, TerH and TerI isolated from the same strain had no inhibitory activity on the cell cycle progression in M phase (data not shown). TerE also inhibited the cell cycle at M phase in asynchronously cultured cells (Figure 1F). These observations suggest that the molecular target of TerE is a factor indispensable for M phase progression.

Terpendole E Did Not Affect Tubulin Polymerization In Vitro but Induced Monoastral Phenotype in Metaphase Cells

Many M phase inhibitors directly interact with tubulin and affect microtubule polymerization. Therefore, we examined whether TerE had stimulatory or inhibitory

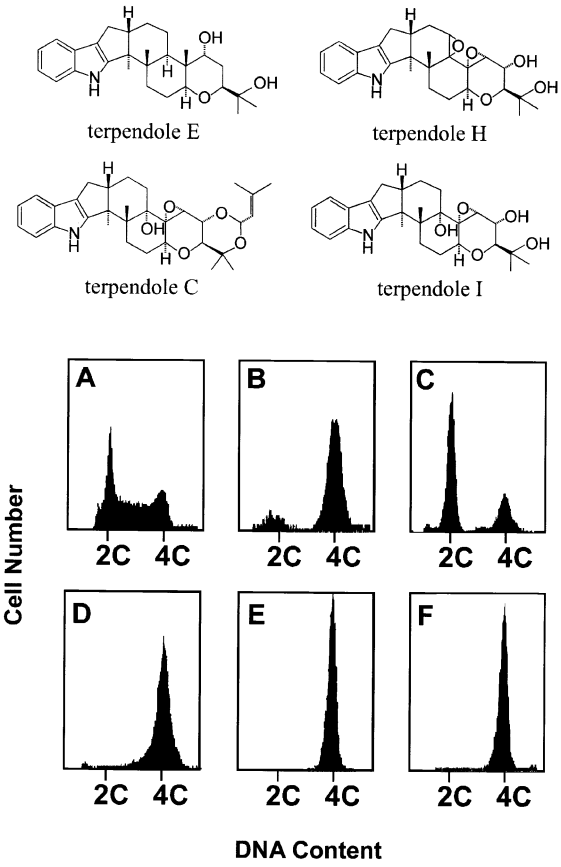

Figure 1. Structures of Terpendoles and M Phase Arrest by Terpendole E

DNA distribution of tsFT210 cells was analyzed by flow cytometry after propidium iodide staining. The cells were cultured at 32°C (A) and synchronized at the G2/M boundary by incubation at 39°C for 17 hr (B). When the cells were transferred at 32°C to release them from G2 arrest, the cells pass through M phase and entered G1 phase after 4 hr (C). The cells were released from the temperature arrest in the presence of 10 μM colchicine (D) or 50 μM TerE (E). At 32°C, the cell cycle progression was also monitored in the presence of 50 μM TerE (F).

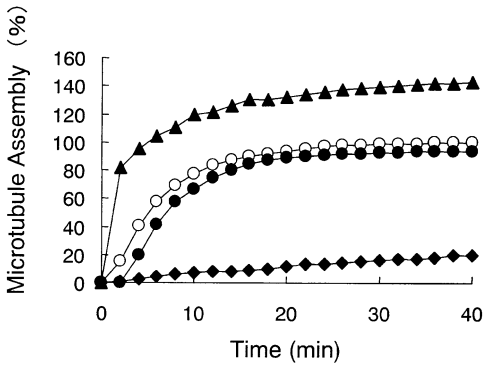

Figure 2. In Vitro Microtubule Polymerization Assay

Microtubule assembly in vitro was monitored under the presence or absence of chemicals. Microtubule proteins (2 mg/ml) were incubated with 100 μM TerE (closed circle), 11 μM vinblastine (closed diamond), 20 μM taxol (closed triangle), or without chemical (open circle).

effects on microtubule polymerization in vitro. As shown in Figure 2, taxol enhanced microtubule polymerization; on the contrary, vinblastine inhibited microtubule polymerization. Under the same conditions, TerE had no effect on microtubule polymerization in vitro.

Next, we investigated the distribution of microtubules in several cell lines (3Y1, A549, and HeLa cells) by indirect immunofluorescent microscopy (Figure 3). Treatment of cells with colchicine, a potent inhibitor of microtubule assembly, disrupted the microtubule network (data not shown). In contrast, TerE treatment of interphase cells (Figures 3IB, 3IIB, and 3IIIB) did not affect the microtubule network in comparison with the control (Figures 3IA, 3IIA, and 3IIIA) even at a concentration of 100 μM. This result suggests that TerE does not target microtubules directly but inhibits some other molecule(s) functioning in M phase.

To reveal the molecular target of TerE, cells were treated with TerE and arrested in M phase. Microtubule morphology and DNA segregation were observed by double staining of microtubules and DNA (Figures 3IC, 3ID, 3IIC, 3IID, 3IIIC, and 3IIID). Untreated control cells showed a typical bipolar mitotic spindle (Figures 3IC, 3IIC, and 3IIIC). In contrast, TerE-treated cells showed a monoastral microtubule array that was surrounded by a ring of chromosomes (Figures 3ID, 3IID, and 3IIID). This phenotype resembles that of a mutant of the mitotic kinesin Eg5, a member of the BimC family, and also resembles that of cells treated with monastrol, an Eg5 inhibitor. Inhibition of Eg5 results in unseparated centrosomes. To test if TerE treatment induces the appearance of unseparated centrosomes like Eg5 inhibition, we stained cells with Hoechst33258 and anti-γ-tubulin antibody to observe chromatin and centrosomes, respectively. As expected, γ-tubulin signals were associated with the poles of the spindle in control cells (Figures 3IE, 3IIE, and 3IIIE), while two well-separated γ-tubulin signals were not seen in TerE-treated cells (Figures 3IF, 3IIF, and 3IIIF). These observations indicate that TerE inhibits the segregation of centrosomes but not the duplication of centrosomes. In fact, the average distance between γ-tubulin spots in TerE (50 μM) -treated 3Y1 cells was decreased to nearly 12.5% (0.62 ± 0.37 μm, n = 113) of the control (4.95 ± 1.04 μm, n = 84). These results suggest that Eg5 activity is inhibited by treatment with TerE in situ.

Terpendole E Did Not Inhibit Golgi Trafficking

Several motor proteins, including conventional kinesin and dynein, play a major role in intracellular retrograde and anterograde trafficking, respectively. To test whether TerE affect such motor proteins, we investigated the effect of TerE on brefeldin A (BFA)-induced retrograde trafficking from Golgi to endoplasmic reticurum (ER) and also anterograde ER-to-Golgi trafficking after BFA removal in NRK cells. BFA disintegrates both Golgi stack and *trans* Golgi network, and induces a merge of them with the ER and lysosome/endosome, respectively [12]. As shown in Figure 4A, the Golgi was dispersed by BFA after treatment for 8 min. TerE did not inhibit BFA-induced Golgi dispersion (Figure 4B). When BFA was removed from cells, Golgi stacks were reconstructed in control cell as a result of anterograde

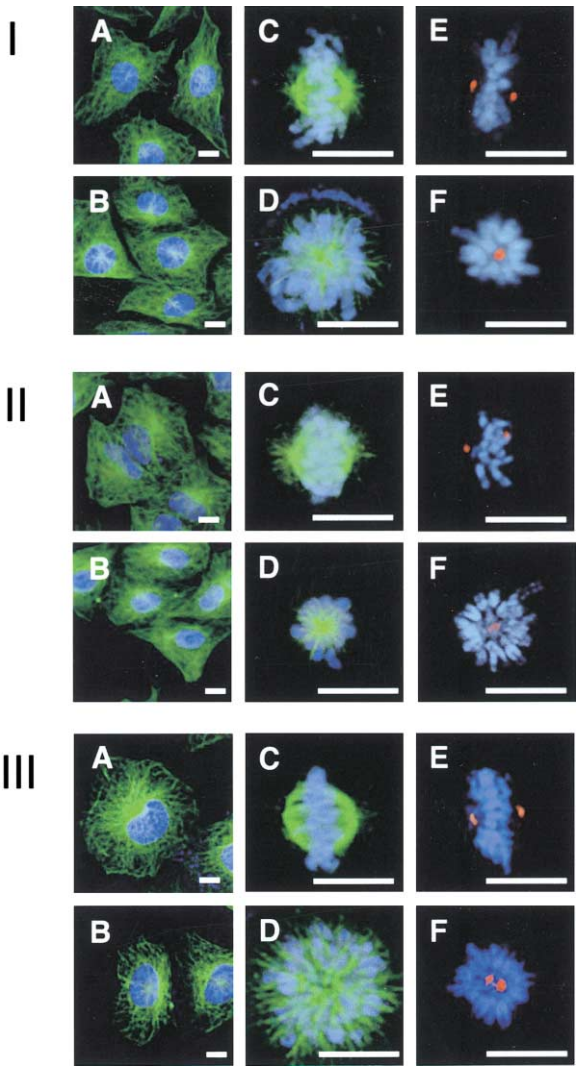

Figure 3. Effects of Terpendole E on Cell Morphology

Three cell lines, 3Y1(I), A549 (II), and HeLa (III), were used for the same experiments. Immunofluorescence staining of β-tubulin (green in [A]–[D]), γ-tubulin (red in [E] and [F]), and DNA (blue) of cells treated for 12 hr with 1% MeOH (control) (A, C, and E) or 50 μM TerE (B, D, and F). Cells in interphase were observed: without treatment (A) and TerE-treated cells (B). Cells in mitosis were observed (C–F). Normal bipolar spindles and DNA alignment at the metaphase plate of control cells (C and E) were replaced with a monoastral spindle surrounded by chromosomes in TerE-treated cells (D and F). Unseparated spindle poles in TerE-treated cells were observed by γ-tubulin staining (F). Scale bars indicate 10 μm.

ER-to-Golgi trafficking of Golgi proteins (Figure 4C). Golgi stacks were reconstructed in TerE-treated cells, too (Figure 4D). These observations indicate that TerE affects neither retrograde nor anterograde trafficking of Golgi proteins and that TerE does not inhibit motor proteins at least required for the trafficking.

Terpendole E Inhibited Eg5 but Not a Conventional Kinesin

To investigate the direct effect of TerE on Eg5, we measured motor activities of Eg5 and conventional kinesins on microtubules in vitro. A fragment of human Eg5 (1–439 aa) was cloned by RT-PCR from HL60 cells and fused to glutathione S-transferase (GST) as described in Experimental Procedures. E439GST was expressed in *E. coli*, and purified via its affinity with glutathione (GSH)-agarose beads and taxol-stabilized microtubules. The gliding movement of microtubules was observed by dark-field microscopy (Figure 5A). Purified E439GST moved microtubules at a speed of 0.042 ± 0.008 μm/s (Figures 5B and 5C). The motor activity of E439GST was drastically reduced to 0.0079 ± 0.0028 μm/s by TerE (Figures 5D and 5E). In the same experimental conditions, TerE did not inhibit the motor activity of K430GST and purified bovine conventional kinesin (data not shown). A dose-dependent curve of E439GST against Eg5 is shown in Figure 6A (closed circle, IC_{50}, 14.6 μM). This inhibition was reversible, and microtubule gliding was restored by washing out TerE (Figure 6B).

As the motility of motor proteins is coupled to ATP hydrolysis, we investigated the effects of TerE on the ATPase activity of Eg5 (Figure 6C). ATPase activity was measured as the release of phosphate from the γ position of ATP using malachite green. This assay was performed in the absence or presence of microtubules. In the absence of microtubules, the ATPase activity of Eg5 was quite low (0.021/s ± 0.0045). However, ATPase activity was drastically stimulated in the presence of microtubules (2.07 ± 0.26 ATPase/s). TerE inhibited the ATPase activity of Eg5 in both cases as follows. In the case of unstimulated ATPase activity, it was weakly inhibited to 28.9% by 100 μM of TerE. In the case of microtubule-stimulated ATPase activity, it was strongly inhibited in a dose-dependent manner (IC_{50}, 23.0 μM; Figure 6C and Table 1), suggesting that TerE largely affects the microtubule-stimulated process of ATP hydrolysis. These results clearly indicate that TerE inhibits both the motility on microtubules and the ATPase activity of Eg5 in vitro.

Terpendole E Is a Selective Inhibitor of Eg5 Rather Than an ACAT Inhibitor

Since terpendole derivatives were isolated as ACAT inhibitors, we next compared the inhibitory activity of TerE with that of other ACAT inhibitors such as terpendole derivatives and FR179254. The results are summarized in Table 1. It has been reported that TerC and FR179254 are potent ACAT inhibitors, whereas TerE, TerH, and TerI hardly inhibit ACAT activity. TerC, TerH, TerI, and FR179254 did not cause M phase arrest or inhibition of microtubule motility activity of either E439GST or K430GST even at 100 μM (Table 1). Furthermore, the in vitro motility assay system was reconstituted without ACAT. These results clearly indicate that the inhibition of Eg5 activity by TerE is not caused through ACAT inhibition.

Discussion

We have identified TerE as an M phase inhibitor from fungal metabolites (Figure 1). To understand the molecular mechanism of the TerE-induced M phase arrest, we examined the machinery required for M phase progression. TerE did not affect tubulin polymerization in vitro and in situ (Figures 2, 3IB, 3IIB, and 3IIIB), TerE inhibited the formation of bipolar spindles during mitosis (Figures

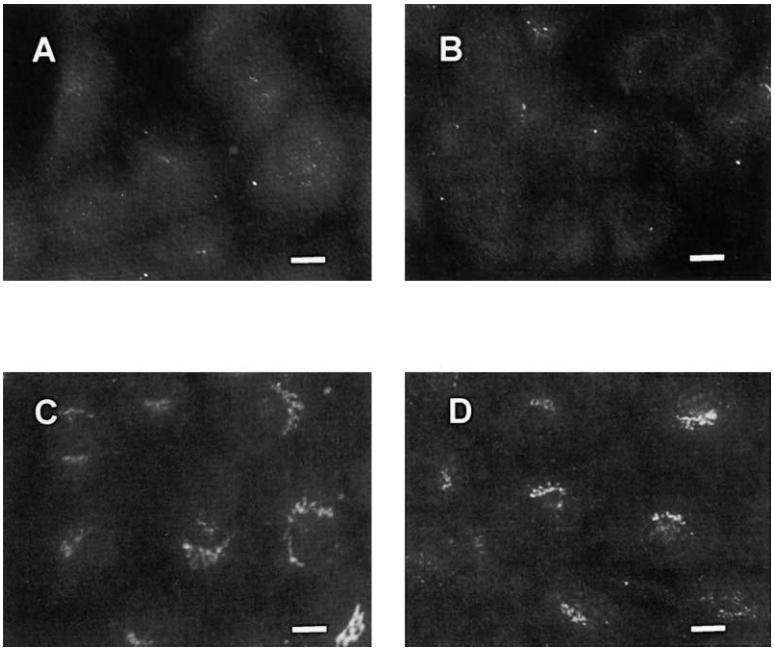

Figure 4. Terpendole E Affected Neither Retrograde nor Anterograde Trafficking of Mannosidase II, a Golgi-Resident Protein

Retrograde (A and B) and anterograde (C and D) trafficking of mannosidase II were followed with NRK cells treated with the vehicle, 0.5% MeOH (A and C) or 100 μM TerE (B and D). Results at 8 and 60 min after the induction of retrograde and anterograde trafficking are shown, respectively. Scales bars indicate 10 μm.

3ID, 3IF, 3IID, 3IIF, 3IIID, and 3IIIF). This phenotype resembles that of a mutant of the mitotic kinesin Eg5, a member of the BimC family [13–18]. The phenotype also resembles that of cells treated with monastrol, an Eg5 inhibitor [7]. Eg5 functions in the formation and maintenance of bipolar spindles by creating pushing forces that keep the spindle pole apart [19, 20]. To investigate the inhibitory activity of TerE against Eg5, we have

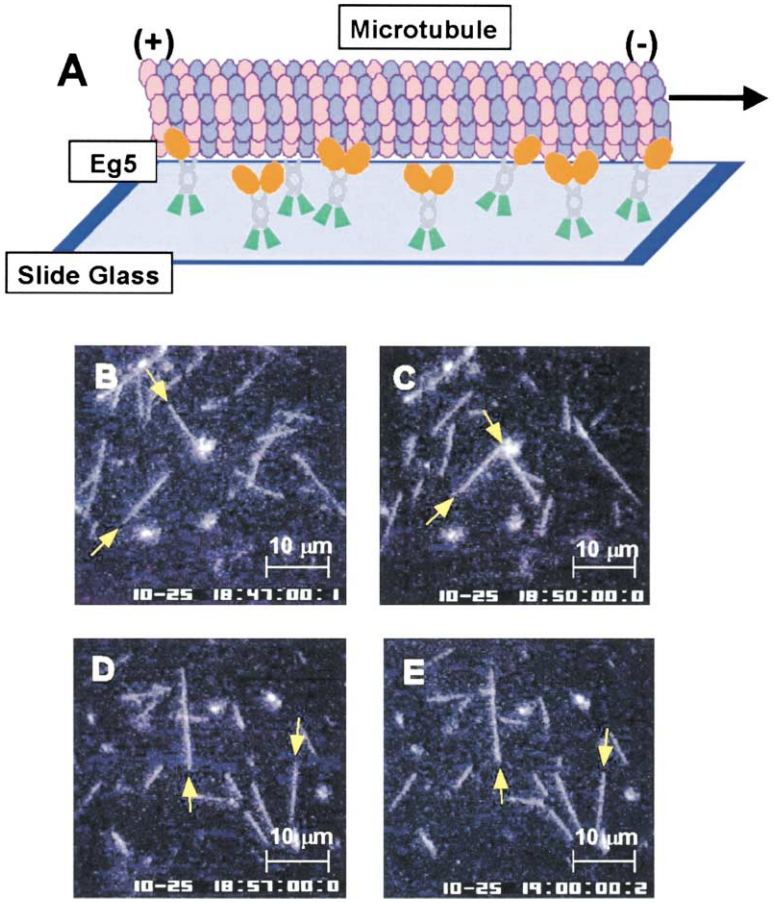

Figure 5. In Vitro Motility Assay with a Dark-Field Microscope

E439GST or K430GST molecules were attached on the glass surface. Polymerized microtubules placed on the glass moved with the minus end leading (A). The movement of microtubules was recorded onto VTR, and the speed was measured. The movement of the microtubules driven by E439GST was about 7.6 μm during 3 min (B and C). In the presence of TerE (50 μM), the movement was drastically reduced, and the movement was about 1.4 μm during 3 min (D and E).

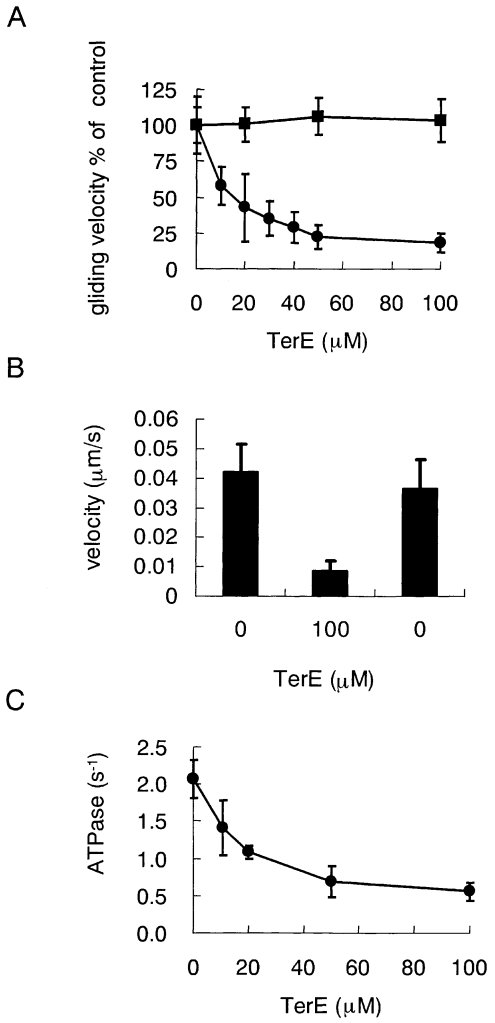

Figure 6. Terpendole E Inhibits Microtubule Motility Driven by Eg5 In Vitro

(A) TerE inhibits the microtubule gliding driven by Eg5, E439GST (closed circle) with a dose-dependent manner, but not by conventional kinesin, K430GST (closed square).

(B) TerE reversibly inhibits the microtubule gliding driven by Eg5 (E439GST). For the washout experiment, we measured Eg5-driven microtubule gliding in the absence of TerE (left column) and then added 100 μM TerE into the chamber (middle column). After incubation for 15 min, the drug was depleted from the assay chamber, and gliding velocity was immediately measured again (right column).

(C) TerE inhibits the microtubule-stimulated ATPase activity of Eg5 (E439GST) in a dose-dependent manner.

cloned Eg5 and a conventional kinesin. The kinesin activity was monitored by movement of microtubules on a glass slide coated with recombinant kinesins. The motor domain of the kinesin superfamily is highly conserved [21]; however, TerE selectively inhibited the microtubule motility driven by Eg5 but not by conventional kinesin (Figure 6A). To eliminate the possibility that TerE is a nonselective inhibitor of motor proteins including kinesin and dynein [20], inhibitory effects of TerE on retrograde and anterograde trafficking of Golgi proteins were examined. TerE did not inhibit retrograde and anterograde trafficking of mannosidase II, a Golgi-resident protein (Figure 4), suggesting that TerE does not inhibit the activity of motor proteins involved in intracellular protein transport. Moreover, other terpendole derivatives, TerC, TerH, and TerI, did not cause M phase arrest or inhibit the motor activities of kinesins. It is interesting that subtle structural differences between TerE and other terpendoles cause different effects upon cellular activity.

We assume that TerE must bind to Eg5 directly, because TerE affected the ATPase activity of Eg5 in the absence of microtubules. However, since the motility of motor proteins is coupled with ATP hydrolysis, there remains the possibility that TerE inhibits directly the motility of Eg5, and inhibition of ATPase results from inhibition of motility.

Significance

Bioprobes are chemical compounds that perturb specific cellular functions and are valuable tools for dissecting complex processes in mammalian cells. We have been developing bioprobes that inhibit the progression of M phase, for example, tryprostatin A. In this paper, we have rediscovered terpendole E (TerE), which was originally isolated as an acyl-CoA:cholesterol acyltransferase inhibitor, as a specific inhibitor of mitotic kinesin, Eg5 from microbial metabolites. TerE inhibited chromosome segregation and resulted in monopolar spindles through the inhibition of Eg5 activity, but TerE did not inhibit conventional kinesins. Monastrol, a synthetic compound, is known to be an inhibitor of Eg5; however, this is the first report to find the Eg5 inhibitor from natural products. TerE should be a useful tool to investigate the mitotic process.

Experimental Procedures

Isolation and Identification of Terpendoles

A soil-isolated fungus strain RK99-F33 was found to produce several cell cycle inhibitors in the culture broth. The taxonomic studies revealed that the strain RK99-F33 was very similar to *Albophoma yamanashiensis*, which was previously reported as a terpendole producer. The strain RK99-F33 was cultured for 4 days at 28°C in cylindrical flasks containing 70 ml of the producing medium (containing 1.5% glucose, 1.0% soluble starch, 0.3% beef extract, 0.2% malt extract, 0.2% yeast extract, 1.5% dried yeast, 0.3% corn steep liquor, 0.05% K_2HPO_4, 0.05% NaCl, 0.05% $CaCO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% agar, adjusted at pH 7.0). Ten liters of the culture broth was extracted with the same volume of ethyl acetate twice, and then the organic layer was collected and concentrated in vacuo. The concentrated extract was applied on a silica gel column (Silica gel 60, Merck) equilibrated with chloroform. Active principals were eluted by the 5:1 mixture of chloroform-methanol. The active fractions were combined and concentrated in vacuo. Subsequently, the concentrated material was applied on a preparative HPLC (Pegasil ODS, Senshu Science Co.). After elution with 70% acetonitrile, we obtained four active compounds, **1** (7 mg), **2** (42 mg), **3** (20 mg), and **4** (15 mg), by activity- and UV spectrum-guided fractionation. These compounds, **1**, **2**, **3**, and **4**, were identical to terpendoles C, E, H, and I, respectively, (Figure 1) by extensive analyses of ^1H and ^{13}C NMR as well as FAB-MS spectrometry. Their molecular ion peaks were found in FAB-MS spectra as follows: TerC, *m/z* 519.3 (M^+, $C_{32}H_{41}NO_5$); TerE, *m/z* 437.2 (M^+, $C_{28}H_{39}NO_3$); TerH, *m/z* 451.2 (M^+, $C_{27}H_{33}NO_5$); and TerI: *m/z* 453.2 (M^+, $C_{27}H_{35}NO_5$).

Chemicals and Antibodies

Colchicine and FR179254 [22] were purchased from Sigma and Calbiochem, respectively. Brefeldin A was purified by our hands.

Table 1. The Effects of Terpendoles in Several Assay Systems

	In Situ		In Vitro			
			Residual Activity Percent of Velocity		Residual Activity Percent of ATPase	
	M Phase Arrest	MT Network in Interphase	E439GST	K430GST	E439GST	K430GST
Terpendole E	+++	–	20.5 ± 6.6 (14.6 μM[a])	103.5 ± 14.8	27.0 ± 5.8	107.1 ± 15.1
Terpendole C	–	–	103.5 ± 23.9	99.2 ± 24.9	88.4 ± 2.0	NT
Terpendole H	–	–	92.6 ± 11.9	89.6 ± 15.8	87.9 ± 2.0	NT
Terpendole I	–	–	91.3 ± 9.2	95.6 ± 8.0	78.3 ± 12.0	NT
FR179254	–	NT	113.4 ± 20.1	115.6 ± 24.7	NT	NT

100 μM drugs were tested in several in situ and in vivo assay systems. For in situ assay, exponentially growing 3Y1 cells were treated with each drug for 24 hr (cell cycle) or 12 hr (MT network). M phase arrest was monitored by a flow cytometer, and microtubule (MT) network in inerphase cells was observed under fluorescent microscope. NT, not tested.
[a] IC_{50} values of TerE against gliding velocity and ATPase activity of Eg5 were determined from the data of Figures 6A and 6C, respectively.

Monoclonal antibodies against β and γ-tubulins are products of Sigma T4026 and T6557, respectively. Anti-mannosidase II monoclonal antibody was purchased from Berkeley Antibody Co. (Richmond, CA). Alexa 488 goat anti-mouse IgG was purchased from Molecular Probe Inc. (Eugene, OR).

Bioassay to Detect Cell Cycle Inhibitors by Flow Cytometry

The bioassay to detect cell cycle inhibitors was carried out as described previously [9]. Temperature-sensitive mutant tsFT210 cells have a defect in cdc2 kinase. The cells were maintained in suspension culture in RPMI1640 medium supplemented with 5% calf serum at 32°C and synchronized in G2 phase at 39°C (restrictive temperature) for 17 hr. In the random-cultured assay, cells cultured at 32°C were seeded into a 12-well plate at a density of 2×10^5 cells/ml in 1 ml of fresh medium. Then, cells were continuously cultured in the presence of inhibitors at 32°C for 17 hr. In the synchronous-cultured assay, cells were seeded at a density of 2×10^5 cells/ml into a 12-well plate and were preincubated at 39°C for 17 hr to synchronized in G2 phase. Then, 10 μl of inhibitor solution was added, and the cells were incubated at 32°C for 4 hr.

The DNA distribution of tsFT210 cells was analyzed by flow cytometry (Profile II; Coulter Co., Hialeah, FL) after treatment with propidium iodide solution for 30 min.

Preparation of Tubulin and Polymerization Assay In Vitro

Microtubule proteins were prepared from bovine brain by the polymerization-depolymerization method as described previously [23]. Turbidity assays of microtubules were performed by incubating microtubule protein in BRB80 buffer (80 mM PIPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 6.8) instead of MES buffer. Increase of absorbance at 350 nm was monitored in cuvettes at 37°C using a thermostatic spectrophotometer (Ultrospec 2000, Pharmacia).

Cell Culture and Immunofluorescence Procedure

Rat normal fibroblast 3Y1 cells, human lung carcinoma A549 cells, human cervix epidermoid carcinoma HeLa cells, and normal rat kidney NRK cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum and cultured in a humidified atmosphere containing 5% CO_2.

DNA/chromatin was stained with Hoechst 33258. Tubulins were stained by anti-β-tubulin antibody and anti-γ-tubulin antibody. The microtubules and DNA were observed under a microscope, Olympus BX60 (Tokyo, Japan) with CCD camera, Orca-ER (Hamamatsu Photonics Inc., Shizuoka, Japan).

Retrograde and Anterograde Trafficking of Golgi Proteins

Brefeldin A (BFA)-induced retrograde trafficking of Golgi proteins in NRK cells treated with 100 μM TerE were assayed as previously [24]. To investigate the effects of TerE on anterograde trafficking of Golgi proteins after BFA removal, NRK cells were incubated in the presence of 10 μg/ml of BFA for 30 min and then MeOH or 100 μM TerE was added. After incubation for 60 min more, the cells were rinsed to remove BFA and incubated with MeOH or 100 μM TerE for further 60 min. Golgi structures were stained using anti-mannosidase II and incubated with Alexa 488 goat anti-mouse antibodies.

Construction of E439GST and K430GST

The C terminus truncated fragments of human Eg5 and *Drosophila* conventional kinesin were fused to GST. First, E439GST (1–439 amino acids of human Eg5 [human kinesin like spindle protein, accession no. U37426]) was cloned from HL60 human leukemia cells with reverse-transcription-PCR using primers 5′-GGCATATGGCGT CGCAGCCAAA-TTCGTCTG-3′ and 5′-ATCCATGGACAACTCTGTAA CCCTATTCAG-3′ containing NdeI and NcoI sites (underlined), respectively. Second, K430GST (1–430 amino acids of *Drosophila* conventional kinesin [kinesin heavy chain, accession no. M24441]) was cloned from cDNA by PCR using primers 5′-CCTGTAAGCATAT GTCCGCGGAACGAG-3′ and 5′-CCTGCTCG-TCCATGGCAACCGA TGC-3′ containing NdeI and NcoI sites (underlined), respectively. The GST gene was amplified from pGEX-6P-2 (Amersham Pharmacia Biotech) by PCR using 5′-GGCCATGGCCCCTATACTAGGT TATTG-3′ and 5′-GGAAGCTTTCAGTCACGAT-GCGGCCGCTCG-3′ as the forward and reverse primers, respectively, the forward primer introducing NcoI site (underlined) onto the 5′ end of the DNA, and the reverse primer containing pGEX-6P-2 HindIII site (underlined) followed behind the stop codon. The DNA was subcloned into pGEM-T easy vector (Promega) and sequenced. The Eg5 and kinesin fragments and GST were digested with NdeI/NcoI and NcoI/HindIII, respectively, and ligated into the NdeI/HindIII sites of the *E. coli* expression vector pRSET-B (Invitrogen).

Expression and Purification of E439GST and K430GST

E439GST and K430GST were expressed in *E. coli* BL21(DE3) harboring the pRSET-E439GST and pRSET-K430GST plasmids, respectively. Overnight cultures were diluted to 20-fold into LB medium supplemented with 50 μg/ml ampicillin and grown at 37°C until the OD_{600} was 1.0. The cells were shaken for a further 30 min at 30°C before induction with isopropyl β-D-thiogalacto-pyranoside (0.4 mM), and after a further 4 hr shaking at 30°C, the cells were harvested by centrifugation. The cell pellet was washed twice with buffer A (20 mM PIPES, 5 mM $MgCl_2$, pH 6.8) and resuspended in buffer A supplemented with protease inhibitors (1 mM PMSF, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg /ml antipain). Cells were disrupted by sonication on ice, followed by centrifugation (80,000 rpm, 2°C, 15 min) to remove debris. GSH-agarose beads (Sigma) were added to the clarified supernatant and incubated for 30 min. The agarose beads were washed three times with buffer A supplemented with 150 mM NaCl and 10 μM ATP. GST-fusion proteins were eluted with 500 μl of buffer A supplemented with 30 mM reduced glutathione (Sigma), 150 mM NaCl, and 10 μM ATP.

Eluted E439GST and K430GST were further purified using their affinity toward polymerized tubulin. Tubulin (2 mg/ml in BRB80 supplemented with 1 mM GTP, 2.5 mM $MgCl_2$, 10% dimethyl sulfoxide) was polymerized for 30 min at 37°C and stabilized by adding 10 μM taxol. Polymerized microtubules were mixed with the purified E439GST or K430GST and incubated for 15 min with 500 μM AMP-PNP (Sigma). After removing the unbound proteins by centrifugation

(75,000 rpm, 27°C, 15 min), GST-fusion proteins bound to microtubules were eluted with ATP-containing buffer (40 mM NaCl, 7 mM ATP, 7 mM $MgCl_2$, 200 mM potassium acetate, 10 μM taxol in BRB80) by incubation for 15 min at 25°C. Microtubules were finally removed by centrifugation (85,000 rpm, 27°C, 15 min).

In Vitro Motility Assay

In vitro motility assay was performed as previously reported [25] with slight modifications. In detail, a coverslip (18 × 18 mm; Matsunami Co., Kishiwada, Japan) was placed on the center of a glass slide (76 × 26 mm) between two strips of grease to make a flow chamber (18 × 12 × 0.063 mm). E439GST (100 μg/ml), K430GST (100 μg/ml), and bovine brain kinesin (15 μg/ml) were prepared in motility buffer (MB; 20 mM PIPES, 4 mM $MgSO_4$, 10 mM potassium acetate, 1 mM EGTA, 0.2 mM EDTA, 10 μM ATP, 0.5% β-mercaptoethanol, pH 6.8). 15 μl of diluted motor protein was introduced into the flow chamber to adhere the motor proteins directly to the glass. After 5 min incubation, 25 μl of casein solution (0.5 mg /ml) was introduced into the chamber and incubated for a further 5 min [26]. After washing the chamber with 25 μl of MB two times, 15 μl of 0.2 μM taxol-stabilized microtubules in MB containing 10 μM taxol (MBP) was introduced into the chamber and incubated for 5 min. After washing the chamber with 25 μl of MBP twice, microtubule movement was initiated by adding MBP with 1 mM ATP (MBPA). For inhibition of microtubule movement, MBPA containing 50 μM TerE was introduced into the chamber. Microtubule movement was observed by dark-field microscopy and recorded with a video tape recorder using a silicon-intensified target camera (Ikegami CTC-9000).

Measurement of ATPase Activity

ATPase activity of E439GST and K430GST was measured in buffer A. For microtubule-stimulated ATPase activity, 4 μM polymerized microtubule, 10 μM taxol, 1 mM EGTA, and 1 mM dithiothreitol were added to the buffer. The concentration of E439GST and K430GST was 0.3 μM in the presence of polymerized tubulin, but 4 μM in the absence of tubulin. The reaction was initiated by the addition of 1 mM ATP at 25°C and terminated by the addition of 0.3 μM perchloric acid. Inorganic phosphate was measured by the modified malachite green method [27].

Acknowledgments

The authors are grateful to Drs. S. Omura and H. Tomoda (Kitasato University) for the gift of terpendole C as an authentic sample. We also acknowledged Dr. G. Okada (RIKEN Institute) for taxonomic study of the producer strain, and Drs E. Schiebel and K. Labib (CRC Paterson Institute) for helpful comments on the manuscripts. This study was supported by a Grant of Bioarchitect Basic Research in RIKEN, and a grant from the Ministry of Education, Culture, Sports, Science, and Technology.

Received: September 18, 2002
Revised: January 2, 2003
Accepted: January 6, 2003

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