The microtubule-dependent motor protein Eg5 is essential for the development and function of the mitotic spindle. Now it has become an anti-mitotic drug target in high throughput screening for anticancer drugs in vitro. Here is a protocol for cloning, expression and purification of a human Eg5 that codes for motor and linker domain in Escherichia coli BL21 (DE3) cells. The effects of temperature, pH, metal ions and DMSO on ATPase activity were investigated. A new compound CPUYL064 showed good inhibitory effect against Eg5 (IC_{50} value, 100 nM). It inhibited the proliferation of human hepatocellular liver carcinoma cell line HepG2 in a dose- and time-dependent manner. CPUYL064 induced a clear G_2/M phase arrest and caused the monastral spindle in HepG2 cells. Induction of apoptosis was further confirmed by changes in membrane phospholipids, changes in mitochondrial membrane potential and by detection of DNA fragmentation. These results indicate that CPUYL064 could be developed as a new, potent mitotic arrest compound.

Key words  Eg5; molecular cloning; characterization; inhibitor; G_2/M arrest; apoptosis
Cells were harvested after 20 h of growth at 20 °C and then disrupted by sonication. The soluble lysate was clarified by centrifugation and applied to a SP-Sepharose column (Amersham Pharmacia Biotech) in a buffer A (20 mM Na-PIPES, pH 6.3; 20 mM NaCl; 1 mM MgCl2; 1 mM Na-EGTA). Protein was eluted with a linear gradient of 20—1000 mM NaCl. Eg5 was identified by SDS-PAGE, and then applied to Mono-Q columns (Amersham Pharmacia Biotech) in a buffer B (20 mM Tris—HCl, pH 8.8; 1 mM MgCl2; 1 mM Na-EGTA). A gradient from 0—1000 mM NaCl was used to elute Eg5.15) Fractions were analyzed by SDS-PAGE. The most concentrated fraction was dialyzed against ATPase buffer (20 mM Na-PIPES, pH 7.5; 1 mM MgCl2; 1 mM Na-EGTA) and then aliquoted, frozen in liquid nitrogen, stored at −80 °C.

ATPase Activity Assay All experiments were done at room temperature. The reagents were added to wells of a 96-well clear plate and the final reaction of the assay contained 20 mM PIPES, pH 7.5, 5.0 mM MgCl2, 1 mM EGTA, 10 μM paclitaxel, 0.6 μM tubulin (MT), 0.5 mM ATP, 2% DMSO containing inhibitors (This DMSO concentration had no effect on the ATPase activity as showed later) in a reaction volume of 100 μL. Reactions were started by adding ATP. The plates were incubated at 37 °C for 30 min. Following incubation, the malachite-green based reagents were added to detect the release of inorganic phosphate.16) The plates were incubated for an additional 5 min at the room temperature, and then 10 μL 34% sodium citrate was added. The absorbance at 610 nm was determined using Multiskan Spectrum Microplate Spectrophotometer (Thermo Electron Corporation). The controls without Eg5 or MTs are the background and should be subtracted from all values. The controls with MTs but without Eg5 give the nucleotide hydrolysis by MTs and should be subtracted from corresponding values with Eg5 and the same concentration of MTs. The data were analyzed using Microsoft Excel to obtain the IC50 of the test compounds.

Effects of Temperature, pH, Metal Ions and DMSO on ATPase Activity The optimum temperature for the ATPase activity was measured at pH 7.5 and at various temperatures from 25 to 70 °C. The optimum pH for the enzyme activity was measured between pH 4.0 and pH 10.0 at 37 °C. The DNA was then dissolved and subjected to 1.0% agarose gel electrophoresis. According to the manufacturer’s instructions, the cells were treated with the drug (8 to 16 μM) for 12 h, and then collected and washed with PBS. The washed cell pellet was resuspended in binding buffer containing FITC-conjugated annexin V and PI. The sample was incubated for 15 min in the dark at 4—8 °C before analysis by flow cytometer.

JC-1 Mitochondrial Membrane Potential Detection Assay The fluorescent cationic dye JC-1 (Biovec) was used for detection of mitochondrial membrane transition events in live cells, to provide an early indication of the initiation of cellular apoptosis. For this assay, cells were treated with the drug (8 to 16 μM) for 12 h, and then collected. The pellet was resuspended in incubation buffer containing JC-1 at 37 °C in a 5% CO2 incubator for 15—20 min. Cells were washed and analyzed for apoptosis using a flow cytometer.

DMSO Electrophoresis Analysis Cells (1×106) were treated with the drug (8 to 16 μM) for 30 h, and then collected by centrifugation. The genomic DNA was extracted using Quick Apoptotic DNA Ladder Detection Kit (Biovision). The DNA was then dissolved and subjected to 1.0% agarose gel electrophoresis.

Statistical Analysis All experiments were performed at least three times. Statistical analysis was performed using Student’s t-test and statistical significance was expressed as *p<0.05, **p<0.001.

RESULTS

Expression and Purification of Eg5 A DNA fragment encoding N-terminal Eg5 protein (Swiss-Prot no. P52732) was amplified by PCR and sub-cloned into the pET28a vector using standard cloning methods. This ORF encoded a putative ATPase with the deduced molecular weight of 44 kDa, and the deduced isoelectric point of pH 8.08 using Genelrunner software, based on the deduced amino acid sequence. In the presence of 0.5 mM IPTG, Eg5 was expressed in E. coli
BL21 (DE3). A shift to 20 °C for 20 h helped in the solubility of the protein. Recombinant Eg5 was purified in a two-step process (Figs. 2A, B). The protein was first eluted from SP-Sepharose column by a gradient of 20 to 1000 mM NaCl (Fig. 2A). The chromatogram contained a large flowthrough peak. Many impurities were removed at this washing step. Then a second peak followed by the Eg5 peak (P1) appeared. The fraction containing Eg5 was dialyzed against buffer B, and applied to Mono-Q column (Fig. 2B). Eg5 Fraction (P2) was analyzed by SDS–PAGE. The purity of the Eg5 is higher than 90% as determined by SDS–PAGE (Fig. 2C). Approximately 0.4 mg of Eg5 has been purified consistently from 1 g of cell paste.

**Enzyme Characterization and the Inhibition of ATPase Activity**

The optimum temperature of the enzyme activity was investigated at PIPES buffer (pH 7.5), and the reaction mixtures were incubated at different temperatures for 30 min (Fig. 3A). The MT-activated ATPase activity increased from 58% at 25 °C to 100% at 37 °C, and decreased quickly to 55% at 42 °C, and gradually to 20% at 70 °C. The optimum temperature was 37 °C. The optimum pH of the enzyme activity was investigated at 37 °C for 30 min with a pH range from 4.0 to 10.0 (Fig. 3B). The relative activity increased from 57% at pH 4.0 to 100% at pH 7.5, then decreased to 44% at pH 10.0. The effect of 10 mM various metal ions was determined by incubating the enzyme and the metal ions for 30 min in PIPES buffer (pH 7.5) at 37 °C (Fig. 3C). Most of the assayed metal ions showed significant effect on ATPase activity. Mn²⁺ and Mg²⁺ increased the ATPase activity by up to 131% and 158%, respectively. Other metal ions decreased the ATPase activity greatly according to the results. The effect of DMSO on the enzyme activity was investigated by incubating the reaction mixtures for 30 min at 37 °C in PIPES buffer (pH 7.5) containing 0, 1, 2, 5, 10, 20 and 50% (v/v) DMSO.

**Fig. 2. Purification of Eg5**

(A) SP-Sepharose column elution profile at 280 nm. After sample injection and the washing step using buffer A (20 mM Na-PIPES, pH 6.3; 20 mM NaCl; 1 mM MgCl₂; 1 mM Na-EGTA), the protein was eluted with a linear gradient of 20—1000 mM NaCl. Eg5 was in the P1 peak. (B) Mono-Q column elution profile at 280 nm. After sample injection a step gradient using 0—1000 mM NaCl in buffer B (20 mM Tris–HCl, pH 8.8; 1 mM MgCl₂; 1 mM Na-EGTA) was employed. Eg5 was in the P2 peak. (C) SDS–PAGE of the purified Eg5: lane 1, size standards (molecular mass indicated in kDa); lane 2, purified Eg5.

**Fig. 3. Enzyme Characterization of Eg5 in the MT-Activated ATPase Activity Assay**

(A) Effect of temperature. The activity of recombinant Eg5 was measured at pH 7.5 and at various temperatures (25—70 °C). (B) Effect of pH. The activity was measured at 37 °C and at various pH (4.0—10.0). (C) Effect of metal ions. The enzyme was incubated in PIPES buffer (pH 7.5) for 30 min at 37 °C with 10 mM of various metal ions. The activities were measured then. (D) Effect of DMSO. The enzyme was incubated in PIPES buffer (pH 7.5) for 30 min at 37 °C with various concentrations of DMSO in the reaction mixtures.
DMSO (Fig. 3D). In these tests it showed that 0—2% (v/v) DMSO had no significant effect on the ATPase activity. However, more than 5% (v/v) DMSO in the reaction mixture inhibited the enzyme activity very strongly.

With a fixed concentration of MTs (0.6 $\mu$M), the IC$_{50}$ values were determined by measuring the MT-activated ATPase activity. Monastrol showed the inhibitory effect with a determined IC$_{50}$ value 7.9 $\mu$m, comparable to a reported value 34 $\mu$m.10) CK0106023 had a good potency against the recombinant Eg5 with the determined IC$_{50}$ value 25 $\mu$m, comparable to a reported value 12 $\mu$m.13) CPUYL064 had a determined IC$_{50}$ value of 100 $\mu$m (data not shown). It appeared more potent than monastrol by a 79-fold factor, a little less potent than CK0106023 (three times less active).

**Effects of CPUYL064 on Cell Proliferation** The WST assay was used to examine the effects of CPUYL064 on HepG2 cells. Cells were treated with increasing doses of drug up to 36 h. As shown in Fig. 4, after 24 h incubation, CPUYL064 caused dose-dependent growth inhibition in HepG2 cells with an IC$_{50}$ of 5 $\mu$m. A trend of increasing cytotoxicity with increasing time was then observed. The growth of HepG2 cells was significantly inhibited by CPUYL064.

**Cell Cycle Analysis** HepG2 cells were incubated with the drug (8, 12 or 16 $\mu$m) for 12 or 20 h, and the cell cycle was analyzed on a flow cytometer. As shown in Fig. 5, a dose-dependent accumulation of cells at the G$_2$/M phase was seen as early as after 12 h treatment (from 11.72 to 47.21%). This G$_2$/M accumulation was strengthened following 20 h treatment and induced a profound sub-G$_0$ peak at higher doses (12, 16 $\mu$m). Thus, the anti proliferative effect of CPUYL064 was attributable to the induction of cell cycle arrest at the G$_2$/M phase of the cell cycle.

**Morphological Observation of Nuclei** The mitotic stage of the arrested cells by immunofluorescent staining of their DNA, as showed in Fig. 6, was examined. Untreated control cells showed the normal morphology of nuclei (Fig. 6A), CPUYL064-treated cells displayed monastral spindle phenotype (Figs. 6B, C). This result was consistent with those above, and suggested CPUYL064 could be treated as a new, potent Eg5 inhibitor.

**Apoptosis Detection** Because a profound sub-G$_0$ peak was seen in Fig. 5, we measured the induction of apoptosis using a double staining method employing FITC-annexin V and PI on a flow cytometry. Flow cytometry analysis of HepG2 cells revealed that an early apoptotic population in CPUYL064-treated cells (Fig. 7A) was significantly greater than that in control cells (from 1.1 to 7.5%). The amount of apoptotic cells in treated cells (12 h, 16 $\mu$m) was 6.8-times greater compared with control cells.

Another apoptotic characteristic is the changes of mitochondrial membrane potential. As seen in Fig. 7B, treatment of HepG2 cells with CPUYL064 for 12 h decreased the mitochondrial membrane potential in a dose-dependent manner. The green signal increased from 13.5% in control cells to...
Internucleosomal DNA fragmentation is a hallmark of apoptosis in mammalian cells. As seen in Fig. 7C, treatment with high doses of CPUYL064 (12 or 16 μM) for 30 h resulted in the formation of definite fragments that could be seen as a characteristic ladder pattern. The ladder-like DNA is clearer with an increase in concentration of the drug from 8 to 16 μM.

**DISCUSSION**

Cancer is the second leading cause of death in industrialized nations. Effective therapeutics is greatly needed. Traditional drugs aimed at blocking DNA replication or microtubule dynamics have several undesired side effects including neurotoxicity and cells may develop resistance during prolonged treatment. Mitosis-specific kinesins contribute to the proper execution of mitosis. They are essential for the proper chromosome alignment, segregation and centrosome separation and now have become antimitotic drug targets in high throughput screening *in vitro*. In this study a method of producing a human mitotic kinesin protein Eg5 motor domain through bacterial system was provided. It provides the most economical means of obtaining substantial quantities of Eg5 to screen a small molecular library and to screen traditional Chinese medicines in our school.

This protein codes for N-terminal Eg5 protein including motor and link domain. The deduced molecular weight was in good agreement with what the SDS-PAGE analysis showed (Fig. 2C). The Eg5 was purified on a SP-sepharose column at pH 6.3. It was eluted when a 230 mM NaCl concentration appeared. Then it was further purified on a Mono Q column at pH 8.8. The protein was eluted at a 280 mM NaCl concentration.

To understand the nature of Eg5 as an enzyme, some of the biochemical properties were studied. The optimum temperature of the enzyme in MT-activated ATPase assay was 37 °C. Low temperature could affect the tubulin polymerization although there was taxol in the assay buffer. When temperature got higher, the ATP was not stable. Eg5 showed maximum activity at pH 7.5. The substrate ATP could autohydrolyse when it was in an alkaline or acidic condition. The malachite green based reagents that were used to analyze the inorganic phosphate produced in the assay needed an appropriate acidic condition. When pH exceeded 9, the color developed in the malachite green phosphate assay might not be...
appropriate. In both assays we used sodium citrate to slow down the autohydrolysis of ATP. Eg5 responded differently to divalent cations. The Mn$^{2+}$ and Mg$^{2+}$ stimulated the ATPase activity while others decreased the ATPase activity greatly. The study of the effect of DMSO on the enzyme activity showed that Eg5 had a resistance against DMSO when the concentration was less than 2%. When IC$_{50}$ values of the small molecule test compounds are being measured, the DMSO concentration shall not exceed 2%.

A variety of structures have now been reported as inhibitors of Eg5 since the first inhibitor monastrol was identified. A series of monastrol derivatives have been designed and synthesized. The structure–activity relationship of these monastrol derivatives reveals that: enhancement of inhibition cannot be achieved by variation of the aromatic substitution pattern of the 4-aryl moiety in 4-aryl-3,4-dihydropyrimidine-2(1H)-ones or -thiones (Fig. 1 PART 1); conformational rigidization through cyclisation of the side chains, which results in a cyclic ketone, leads to a significantly better inhibition (Fig. 1 PART 3); the two additional methyl groups at 7-position in dimethylenastron lead to a distinct increase of Eg5 inhibition (Fig. 1 PART 3).\textsuperscript{22,23}

CPUYL064 is from a new series of dihydropyrimans, which were synthesized according to the structures of monastrol and dimethylenastron. As seen in Fig. 1, the PART 1* and PART 2* in CPUYL064 are designed based on the PART 1 and PART 2 in monastrol. The PART 3* in CPUYL064 is designed based on the PART 3 in dimethylenastron. CPUYL064 was identified and characterized as a potent inhibitor of Eg5. It inhibited Eg5 ATPase activity with an IC$_{50}$ of 100 nM.

CPUYL064 had a significant inhibitory effect on the growth rate of HepG2 cells. The cytotoxic effect was dose- and time-dependent. As the concentration of the drug increased, the relative viability decreased, and a significant inhibition was observed after 24 h over the concentration ranging from 2 to 16 $\mu$M ($p<0.05$). At the same concentration of the drug with the time lapsing, the relative viability also decreased. When the concentration of CPUYL064 was 4 $\mu$M, the relative viability of HepG2 cells was 51.07% (24 h), and 25.06% (36 h).

Cell-cycle analysis revealed that inhibition of cell viability by CPUYL064 resulted from cell cycle arrest at the G$_{2}$/M phase, accompanied by an increasing in sub-G$_{0}$ fraction, indicating apoptotic cell death. G$_{2}$/M arrest was shown early after 12 h treatment, while apoptotic cell death was a later event. High doses of the drug for 12 h or low doses for a long time (20 h) could result a sub-G$_{0}$ peak. Other apoptotic features further confirmed the induction of apoptosis by CPUYL064.

Our microscopic studies showed that CPUYL064 caused mitotic arrest in HepG2 cells by inducing the formation of mononasters (a rosette of condensed mitotic chromosomes), the characteristic effect of the Eg5 kinesin inhibition.\textsuperscript{10} This effect was observed after 20 h of drug exposure (12 $\mu$M).

Apoptosis, which is identified as one of the most fundamental biological processes in eukaryotes in which individual cells die by activating intrinsic ‘suicide’ mechanisms, has been thought to have a key role in damaging cancer cells, by causing a variety of insults.\textsuperscript{24,25} Other apoptotic features were detected as early as 12 h after treatment. The assays include cell-surface annexin V binding, which measured the appearance of phosphatidylserine on the external plasma membrane, changes in mitochondrial membrane potential, which is measured by JC-1, and DNA fragments. Taken together, these results suggest that cells begin apoptosis after 12 h treatment in a dose-dependent manner.

In summary, our results suggest that the new compound CPUYL064 is a promising anticancer drug targeting Eg5 ATPase activity. It may have clinical utility in the treatment of cancer.

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