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Purification and Characterization of CDMHK, a Growth Inhibitory Molecule Against Cancer Cell Lines, from *Myxobacterium* sp. HK1 Isolated from Korean Soil

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Abstract *Myxobacterium* sp. HK1, isolated from Korean soil, degrades cellulose, differentiates to fruiting body, and its 16s rDNA has 95% similarity to *Polyangium* sp. An anticancer molecule, CDMHK, was identified from culture broth of *Myxobacterium* sp. HK1, and purified by Diaion HP20, Silica gel, Sephadex LH-20 chromatography, and preparative HPLC using an YMC OSD-A C18 column. The molecular structure and formula were determined to be $C_{12}H_{19}N_3O_2$ (M.W 237) by MS spectrometry, 300 MHz ^1H and ^{13}C NMR. The CDMHK was not active against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. However, this molecule inhibited the growth of various cancer cell lines. The ED_{50} values of CDMHK were determined to be 0.147, 0.086, 0.18, 0.166, and 0.142 μg/ml against A549, SK-OV-3, SK-MEL-2, VF498, and HCT15 cancer cell lines, respectively. In addition, the CDMHK was able to induce apoptosis of the CCRF-CEM cancer cell line, evidenced by DNA fragmentation assay and DAPI staining.

Key words: *Myxobacterium* sp. HK1, growth inhibitor, cancer cell lines, apoptosis

Myxobacteria are Gram-negative soil bacteria that are assigned to the suborders Cystobacterineae and Sorangineae, both of which belong to the δ-group of the Proteobacteria [25]. They are distinguished from most other bacteria by their ability to glide in swarms, to feed cooperatively, and to form fruiting bodies upon starvation [4, 22, 28]. In addition, they have been shown to produce a wide variety of secondary metabolites with unique structures and biological activities [24].

The myxobacteria produce a large number of bioactive molecules with antifungal, antibiotic, and anticancer activities. More than 80 basic structures with nearly 350 structural variants have been identified, some of which may have chances for medical application [26]. When 2,150 bacteriolytic myxobacteria isolates were tested, 55% of them turned out to be producers of bioactive substances, and this ratio increased to 95% among 720 cellulose decomposing isolates [2]. These biologically active compounds were diverse in structures, belonging to different molecular classes such as polyketides from acetate and propionate, linear and cyclic peptides, and heterocyclic compounds [2].

Bioactive compounds from myxobacteria show various inhibition mechanisms against target cells. For example, myxopyronin isolated from *Myxococcus fulvus* [11], corallopyronin isolated from strains of *Corallococcus coralloides* [13], and sorangicin isolated from *Sorangium cellulosum* strains [14] block bacterial RNA polymerases; myxovalargin produced from *Myxococcus fulvus* [12] inhibits protein synthesis in eukaryotes; myxothiazol isolated from a strain of *Myxococcus fulvus* [5] blocks the cytochrome complex in the respiration chain; myxalamide from *Myxococcus xanthus* [8] and aurachin from *Stigmetella aurantiaca* [18] inhibit the NADH ubiquinone oxidoreductase; soraphen isolated from *Stigmatella aurantiaca* [6] blocks the fungal acetyl-CoA carboxylase; and epothilone [10] isolated from *Sorangium cellulosum* acts upon the microtubule of eukaryotic cells, blocks cell division, and leads to cell death (apoptosis). Its action spectrum is narrow. Bacteria are not affected and fungi are rarely affected. Epothilone acts upon cancer cell lines, such as breast, intestinal, and ovarian

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cancers. There is a good chance that epothilone will become important for clinical cancer treatment [10].

Based on the above considerations, we isolated myxobacteria from Korean soil, which utilizes cellulose, and determined cell cytotoxicity against various cancer cell lines. An anticancer molecule, CDMHK, was purified from one isolate, *Myxobacterium* sp. HK1, and characterized.

MATERIALS AND METHODS

Sampling and Treatment of Soils

Soils for isolating cellulolytic myxobacteria were sampled from top-soil, containing decayed leaves and trees, in South Korea. One g of soil was treated with 1 ml of an antifungal reagent, Fungizone (Invitrogen), for 12 h and then dried overnight at 50°C.

Isolation of Cellulose-Utilizing Myxobacteria

One g of treated soil was inoculated on mineral salt agar [$MgSO_4 \cdot 7H_2O$, 0.02% (w/v); KNO_3, 0.075%; K_2HPO_4, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.002%; $CaCl_2 \cdot 2H_2O$, 0.004%; Bacto-agar, 1.5%; pH 7.2] overlaid with a Whatman filter paper No. 42 [20], and incubated at 30°C for up to 20 days. Under dissection microscope, fruiting body isolated on mineral salt agar was inoculated on casitone agar [Bacto-casitone, 1% (w/v); $MgSO_4 \cdot 7H_2O$, 0.01%; KH_2PO_4, 0.27%; K_2HPO_4, 0.35%; Bacto-agar, 1.5%; pH 7.2] and cultivated at 30°C for 2 days.

Identification of Isolated Myxobacteria

For identification of isolated myxobacteria, 16s rDNA sequencing was done. The degenerated primer based on a conserved sequence of 16s rDNA was used to amplify 16s rDNA from the isolated bacterium using PCR. Nucleotide sequence of forward primer, called N16sF, was 5'-AGAGTTTGATC(A/C)TGGCTCAG-3' (posed at 27 on 16s rDNA) and the sequence of reverse primer, called N16sR, was 5'-GG(C/T)TACCTTGTTACGACTT-3' (posed at 1492 on 16s rDNA). A 1.4 kb PCR fragment was ligated into pCR2.1-TOPO (Invitrogen), and the pCR-16DNA generated was sequenced at BIONEX (Korea). Analysis of phylogenic similarity of isolated myxobacteria was performed by ClustalX V1.83 and Treeview software.

Purification of an Anticancer Molecule, CDMHK

Myxobacterium sp. HK1 was cultivated in 15 l of casitone medium at 30°C for 10 days. Cell-free supernatant was harvested by centrifugation (6,000 ×*g*, 4°C, 10 min). The supernatant was subjected to Diaion HP20 resin (Mitsubishi chemical) column chromatography (80×200 mm) using stepwise distilled water and methanol gradient [distilled water→60% methanol (v/v)→absolute methanol]. Fractions (2 l) were collected, and the fractions showing anticancer activity were concentrated using a rotary vacuum evaporator (EIYLA). After extracting concentrated fractions with ethyl acetate, the ethyl acetate phase was recovered, and loaded on a Silica gel (Merck) chromatography column (45×100 mm). Elution was performed by stepwise gradient of hexane and ethyl acetate [1:4 (v/v)→1:1→absolute ethyl acetate]. Active fractions were collected, concentrated, and redissolved in absolute methanol. Concentrated sample was loaded on a Sephadex LH20 chromatography column (16×500 mm), and the column was eluted with absolute methanol. Each fraction showing anticancer activity was concentrated and purified by preparative HPLC on a YMC ODS-A C18 column (10×150 mm) using an 85%–100% methanol gradient.

Physicochemical Property and Structure of CDMHK

The CDMHK was subjected to instrumental analyses for its physicochemical property and structure. The UV-visible absorption spectrum was measured with the Waters UV6000 system. FAB-MS and HRFAB-MS were done on a JMS-700 Mstation mass spectrometry, and ^{1}H-NMR and ^{13}C-NMR spectra were measured with a Bruker NMR spectrometer in chloroform-*d*.

Determination of Cell Cytotoxicity

For cytotoxicity assay, cells were seeded onto 96-well microculture plates at 1×10^4 cells/well and allowed to adhere for 24 h. The medium was removed and replaced with fresh medium with or without increasing concentrations of cytotoxic agents. The cells were then incubated for an additional 48 h. The medium was again removed and replaced with fresh medium without additional cytotoxic agents, and the cells were further incubated for 24 h. Cell survival was then quantified using the tetrazolium dye MTT as described previously [3, 15, 17, 21]. Each experimental data point represents the average value obtained from six replicates, and each experiment was repeated at least three times.

DNA Fragmentation Assay and DAPI Staining

In order to determine apoptotic DNA fragmentation induced in CCRF-CEM cancer cells following the treatment by CDMHK, the isolation of apoptotic DNA fragments was performed as described previously [1, 23]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer [1% Tween 20, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5]. After centrifugation for 5 min at 1,500 ×*g*, the supernatant was collected, brought to 1% SDS, and treated with RNase A for 2 h at 50°C, and subsequently with Proteinase K for 2 h at 37°C. The DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 5 M ammonium acetate. The DNA fragmentation was visualized by electrophoresis on a 2% agarose gel.

To identify the induction of apoptosis, the cells were subjected to DAPI staining. DAPI is a fluorescent stain to examine nuclei for morphologic assessment of changes

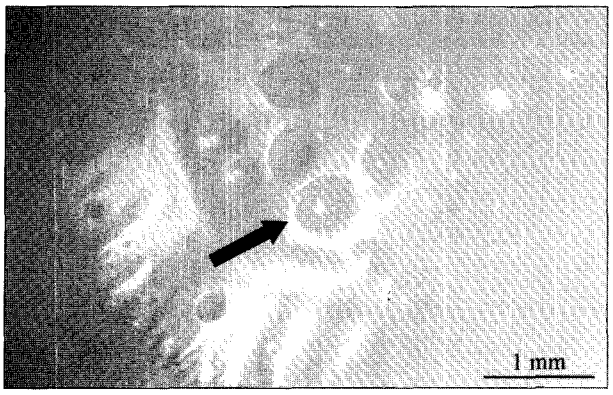

Fig. 1. Fruiting body of *Myxobacterium* sp. HK1.
Black arrow indicates a fruiting body of *Myxobacterium* sp. HK1 on mineral salt agar.

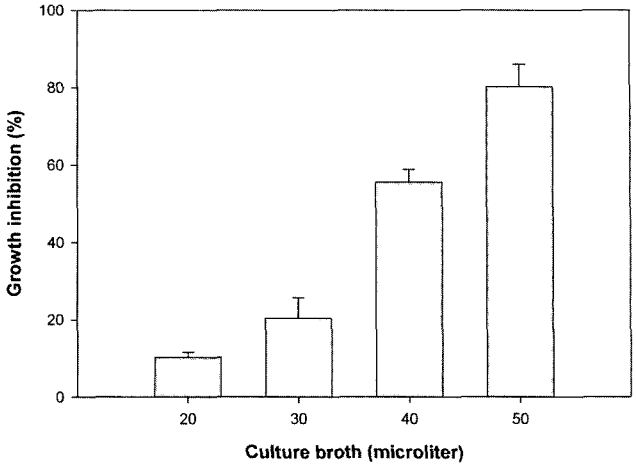

Fig. 3. Cell cytotoxicity of the ethyl acetate extract of culture broth from *Myxobacterium* sp. HK1.
Human acute leukemia CCRF-CEM was used. Growth inhibition was determined by MTT assay and 20, 30, 40, and 50 μl of culture broth equivalent were added independently.

during apoptosis under a fluorescence microscope [16]. In brief, following 24 h of treatment with CDMHK, the cells were harvested by trypsinization and washed with PBS. The pellet was then fixed in 4% formaldehyde. The cells were resuspended in 500 μl of DAPI solution [0.2 μg/ml DAPI (Roche), 0.1% Triton X-100, 2% paraformaldehyde, 1× PBS] for 30 min. Cells were then spotted onto a microscope slide and allowed to air-dry. Images were obtained using an Olympus BX61 fluorescent microscope with digital camera.

Results and Disscusion

Isolation of *Myxobacterium* sp. HK1 from Korean Soil

After incubating sample soils on mineral salt agar overlaid by Whatman filter paper No. 42 for 10 days at 30°C, 32 microorganisms that decayed filter paper appeared. After incubating for another 5 days, the fruiting body of cellulolytic myxobacterium was observed on mineral salt agar overlaid with the filter paper. The fruiting body of selected myxobacterium was then isolated using a sterilized needle and inoculated on casitone agar. Under a dissection microscope, the fruiting body of the isolated *Myxobacterium* sp. HK1 was observed as an ovoid structure with approximately 500 μm diameter (Fig. 1).

Identification of *Myxobacterium* sp. HK1

To identify the isolated *Myxobacterium* sp. HK1, the 16S rDNA sequence of *Myxobacterium* sp. HK1 (1,506 bp) was analyzed. After extraction of genomic DNA from *Myxobacterium* sp. HK1, PCR for 16S rDNA was performed. The result of 16S rDNA sequencing and comparative analysis of phylogenic similarity with other myxobacteria revealed 95% similarity to *Polyangium* sp (Fig. 2). *Polyangium* sp. can utilize cellulose and is very close to *Sorangium* sp. [9].

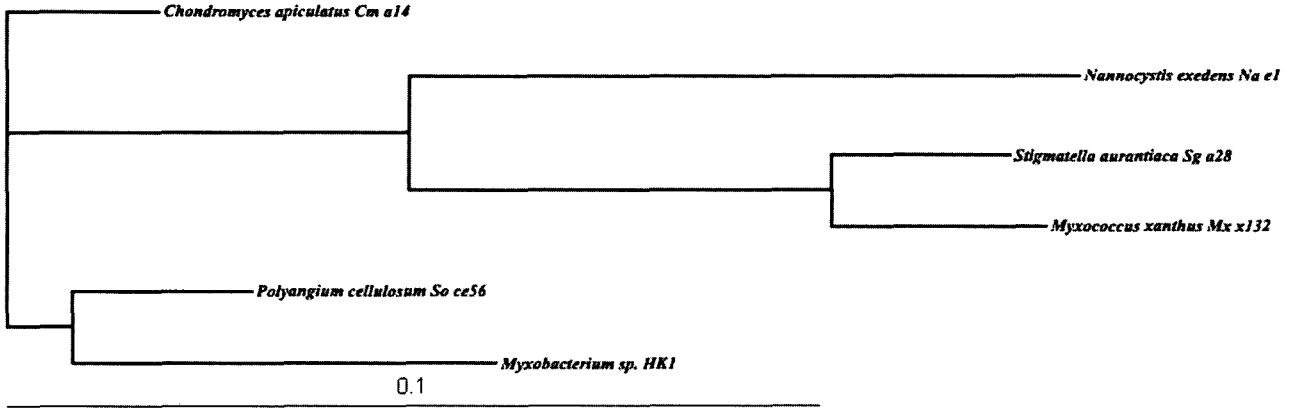

Fig. 2. Analysis of the phylogenic similarity of *Myxobacterium* sp. HK1.
This analysis was performed by ClustalX V1.83 and Treeview software.

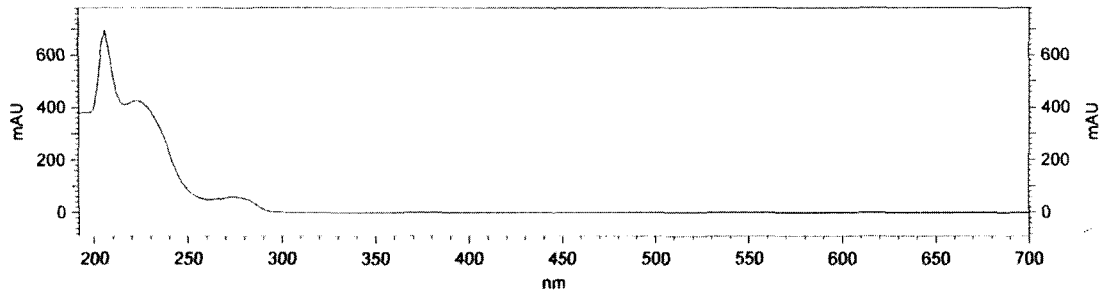

Fig. 4. UV spectrum of the purified CDMHK.
CDMHK (1 μg) was dissolved in 1 ml of methanol and analyzed by scanning from 200 to 700 nm.

Polyangium sp. and *Sorangium* sp. can produce various bioactive molecules as secondary metabolites [9], and their fruiting bodies are ovoid shaped.

Purification and Characterization of CDMHK

Myxobacterium sp. HK1 was cultivated in 5 ml of casitone medium for 7 days in a shaking incubator (30°C, 200 rpm). After centrifugation, cell-free supernatant was extracted with an equal volume of ethyl acetate. The ethyl acetate extract was concentrated by vacuum evaporator and tested for growth inhibitory activity against CCRF-CEM (human acute leukemia cell line). As shown in Fig. 3, the culture broth of *Myxobacterium* sp. HK1 had growth inhibitory activity against CCRF-CEM.

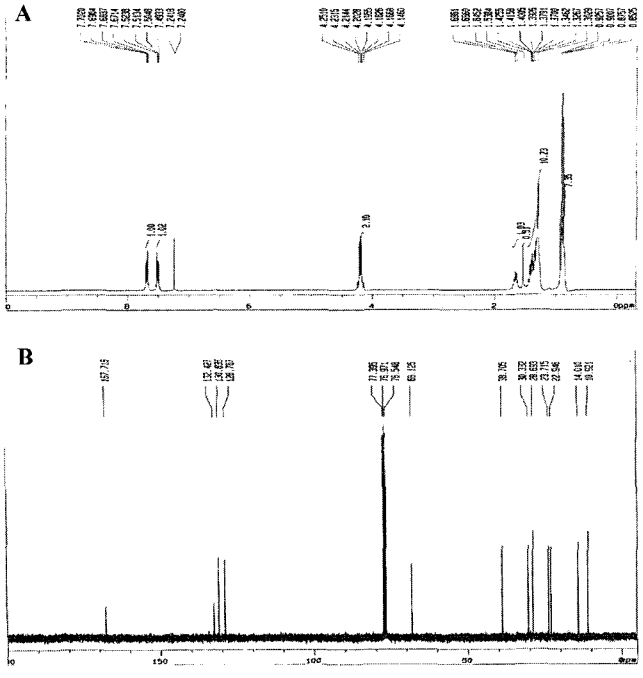

Fig. 5. ^{1}H-NMR spectrum (A) and ^{13}C-NMR spectrum (B) of the purified CDMHK.
After 3 mg of the CDMHK was dissolved in chloroform-*d*, the NMR spectrum was measured by 300 MHz NMR spectroscopy.

The purification procedure of an anticancer molecule from *Myxobacterium* sp. HK1 is described in Materials and Methods. The final yield of purified substance from 15 l of culture broth of *Myxobacterium* sp. HK1 was 10.05 mg. The purified substance was a white powder, soluble in methanol but insoluble in water. It showed UV absorbance at 208 nm, slightly similar to that of melithiazol B [27] and jerangolids [7], but the UV spectrum of the purified substance from *Myxobacterium* sp. HK1 (Fig. 4) was not exactly matched to any known bioactive molecules isolated from myxobacteria. Based on FAB-MS spectrometry, its molecular weight was determined to be 237. As revealed by MS, NMR ^{1}H (Fig. 5A), and ^{13}C NMR (Fig. 5B), the molecular formula of the purified substance was assigned to be $C_{12}H_{19}N_3O_2$ (Fig. 6). This structure of the purified substance from *Myxobacterium* sp. HK1 suggested the identity of a new compound, compared with any bioactive molecules from myxobacteria. The purified substance was designated as CDMHK.

Cell Cytotoxicity of CDMHK

The cytotoxic effect of the CDMHK from *Myxobacterium* sp. HK1 on various cancer cell lines of human origins was

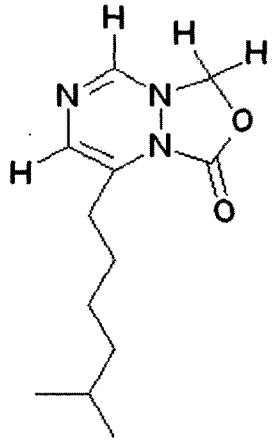

Fig. 6. Proposed model of the purified CDMHK.
This model was constructed by MS, ^{1}H, and ^{13}C-NMR spectra.

Table 1. Cell cytotoxicity of CDMHK against human cancer cell lines.

Cell line[a]	ED_{50} (μg/ml)[b]
	CDMHK
A549	0.620±0.01
SK-OV-3	0.363±0.01
SK-MEL-2	0.759±0.012
XF498	0.70±0.02
HCT15	0.599±0.01

[a]A549, lung carcinoma; SK-OV-3, adenocarcinoma; SK-MEL-2, malignant melanoma; XF498, central nervous system carcinoma; HCT15: colon cancer cell line.
[b]ED_{50}: calculated dose when cell growth inhibition was 50%.

determined by MTT assay. Most human cell lines tested were found to be sensitive, and cytotoxicity of CDMHK was observed at concentrations ranging from 0.363 to 0.759 μg/ml. The CDMHK was cytotoxic to various cancer cell lines, and ED_{50} values appeared to be 0.147 μg/ml for A549, 0.142 μg/ml for HCT15, 0.18 μg/ml for SK-MEL-2, 0.086 μg/ml for SK-OV-3, and 0.166 μg/ml for XF498 (Table 1). Human malignant melanoma SK-OV-3 was more sensitive than other cell lines. In particular, CDMHK was more superior against SK-OV-3 (human ovarian cancer cell line) than apicularens B from *Chondromyces* sp [19]. These results suggest that the CDMHK from *Myxobacterium* sp. HK1 is effective on various carcinomas.

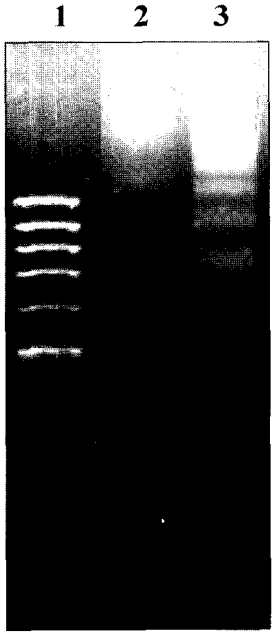

Fig. 7. CDMHK-induced apoptotic DNA fragmentation against human acute leukemia CCRF-CEM.
Lane 1 indicates 1 kb DNA ladder, and Lanes 2 and 3 indicate nontreated genomic DNA and apoptotic DNA fragments after treatment of 0.1 μg/ml of CDMHK, respectively.

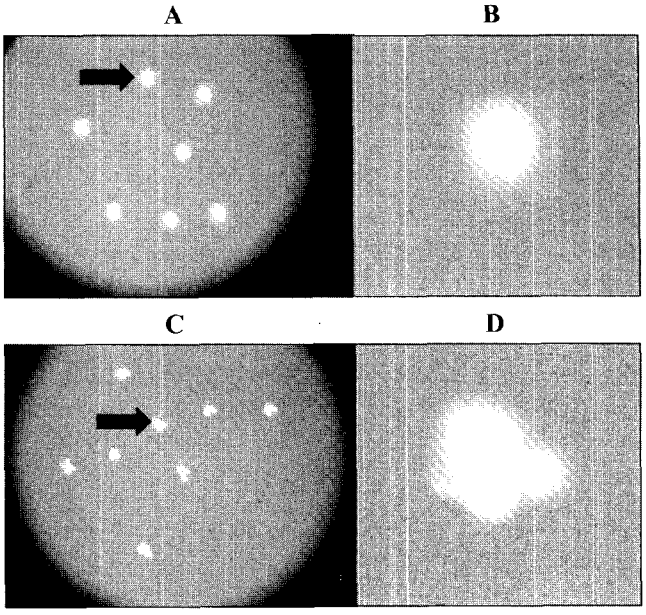

Fig. 8. CDMHK-induced apoptosis against human acute leukemia CCRF-CEM.
Upper panels (A and B) indicate nontreated CCRF-CEM cell and lower panels (C and D) indicate 0.1 μg/ml of CDMHK-treated cell. Panels A and C were observed under fluorescence microscopy at 600×. Panels B and D are magnified panels A and D, repectively.

Pattern of Cell Cytotoxicity by CDMHK

To determine cellular mechanisms underlying the cytotoxicity, the effect of CDMHK on human acute leukemia CCRF-CEM was investigated by focusing on the induction of apoptosis, using DNA fragmentation assay and DAPI staining. When the cells were treated with 0.1 μg/ml CDMHK concentration for 24 h, DNA fragmentation and bodies of chromatin condensation were detected (Figs. 7 and 8). These results demonstrate that the cytotoxic effect of CDMHK from *Myxobacterium* sp. HK1 is attributable to a mechanism that is very similar to apoptosis. In conclusion, CDMHK is a promising anticancer agent that is applicable to various cancer cell lines.

Acknowledgments

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