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In vitro effects on proliferation, telomerase activity and apoptosis of an eremophilanoid sesquiterpene from *Senecio oldhamianus* Maxim in cultured human tumor cell lines

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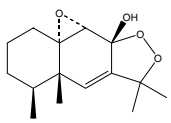
8,11-Dioxol-6-en-9α,10α-epoxy-8β-hydroxyeremophilane (HEM), a new eremophilanoid sesquiterpene, was isolated from *Senecio oldhamianus* Maxim. Its effects of cytotoxicity, telomerase activity, apoptosis and related genes expression in two human tumor cell lines, human hepatoma cells SMMC-7721 and human oophoroma cells HO-8910, were studied. Hydroxycamptothecine (HCPT) was used as a positive control. The IC_{50} of cytotoxicity by HEM were 24.9 ± 2.1 and 19.4 ± 1.6 μM in SMMC-7721 and HO-8910 cells respectively, and 0.35 ± 0.10 and 0.27 ± 0.08 μM for HCPT. HEM inhibited telomerase activity with the IC_{50} 35.9 ± 3.2 μM in SMMC-7721 and 25.6 ± 2.6 μM in HO-8910 cells, while HCPT had no effect on telomerase activity in both tumor cell lines. HEM 20–30 μM induced apoptosis in SMMC-7721 cells from 5.7% to 18.4% and in HO-8910 cells from 7.6% to 67.1%. While HCPT 0.1–0.5 μM induced apoptosis in SMMC-7721 cells from 6.5% to 13.3% and in HO-8910 cells from 9.9% to 30.9%. HEM 30 μM significantly decreased Bcl-2 protein expression to 58.7% in SMMC-7721 and to 57.6% in HO-8910 cells. While HCPT 0.5 μM significantly decreased Bcl-2 protein expression to 64.3% in SMMC-7721 and to 70.0% in HO-8910 cells. HEM 25 μM and 30 μM significantly increased P53 protein expression 2.3–3.6-fold in SMMC-7721 and 3.0–5.7- fold in HO-8910 cells. While HCPT 0.5 μM significantly increased P53 protein expression 3.3-fold in SMMC-7721 and 2.7-fold in HO-8910 cells. Overall, HCPT exhibited a more potent effect on cytotoxicity and apoptosis in the two tumor cell lines than HEM did. However HEM can inhibit telomerase activity in the two tumor cell lines but HCPT cannot.

1. Introduction

Sesquiterpenes have been widely used in indigenous medical practices, including treatment of migraines (Johnson et al. 1985), inflammation (Lyss et al. 1997), and tumors (Lee et al. 1977; Douros et al. 1978; Quintero et al. 1999).
8,11-Dioxol-6-en-9α,10α-epoxy-8β-hydroxyeremophilane (HEM), a new eremophilanoid sesquiterpene, was isolated from *Senecio oldhamianus* Maxim, a Chinese herbal medicine with the effect of promoting circulation of blood, detoxification, detumescence and curing sore, ulcer and incised wounds (Yang et al. 2001).
In recent years, telomerase, apoptosis and their relations to cancer have been widely discussed. Telomerase, a reverse transcriptase that synthesizes telomeric repeats, is found in germ line cells, stem cells, and mitogen-stimulated leukocytes, but is rare in most of the somatic tissues. Because more than 80% of human tumors exhibit telomerase activity (Kim 1997; Nakamura et al. 1997), it is suggested that activation of telomerase in tumor cells has a major impact on their continuous growth, conversely, inhibition of telomerase may induce their growth arrest. The correlation between telomerase activity and human tumors has led to the hypothesis that tumor growth requires reactivation of telomerase and that telomerase inhibitors represent a class of chemotherapeutic agents. So telomerase has become a potential target for antitumor therapy (Hoos et al. 1998; Meyerson et al. 1997). To induce apoptosis is another mechanism of antitumor drugs. Many reports indicated that exposure of tumor cells to antitumor drugs (Hickman 1992) or to telomerase activity inhibitors (Herbert et al. 1999) can induce apoptosis directly. Here we describe the *in vitro* biological activities of HEM in this respect.



2. Investigations and results

2.1. HEM exhibited cytotoxicity in two tumor cell lines

The cytotoxicities of HEM and hydroxycamptothecine (HCPT, a positive control) toward SMMC-7721 and HO-8910 cells were determined in 96-well microtiter plates

Table: IC_{50} (μM) of cytotoxicity and telomerase activity for HEM and HCPT (n = 3)

	Cytotoxicity		Telomerase activity	
	SMMC-7721 cells	HO-8910 cells	SMMC-7721 cells	HO-8910 cells
HEM	24.9 ± 2.1	19.4 ± 1.6	35.9 ± 3.2	25.6 ± 2.6
HCPT	0.35 ± 0.10	0.27 ± 0.08	NE	NE

Means ± SE, NE: no effect

after 48 h incubation using the sulforhodamine B (dyeing) method. The IC_{50} of HEM and HCPT were 24.9 ± 2.1 and 0.35 ± 0.10 μM in SMMC-7721 cells, and were 19.4 ± 1.6 and 0.27 ± 0.08 μM in HO-8910 cells respectively (Table). The results indicate that HCPT exhibited more potent cytotoxicity.

2.2. HEM inhibited telomerase activity in two tumor cell lines

After 48 h incubation with different concentrations of HEM or HCPT, 2×10^5 cells were harvested and the telomerase activities were detected according to the telomeric repeat amplification protocol (TRAP) described in the experimental section. The absorbance ($A_{450\,nm} - A_{690\,nm}$) of SMMC-7721 and HO-8910 untreated cell samples were 0.97 and 2.73 (Fig. 1). This means that the telomerase activity in SMMC-7721 cells is about 35.5% of that in HO-8910 cells. But HEM exhibited the same effect on telomerase activity in the two tumor cell lines. The absorbance declined while the concentration of HEM increased from 10 to 42 μM in both tumor cell lines (Fig. 1A) and the IC_{50} of telomerase activity inhibited by HEM were 35.9 ± 3.2 and 25.6 ± 2.6 μM in SMMC-7721 and HO-8910 cells respectively (Table). However, HCPT (0.05–0.5 μM) showed no effect on telomerase activity (Fig. 1B).

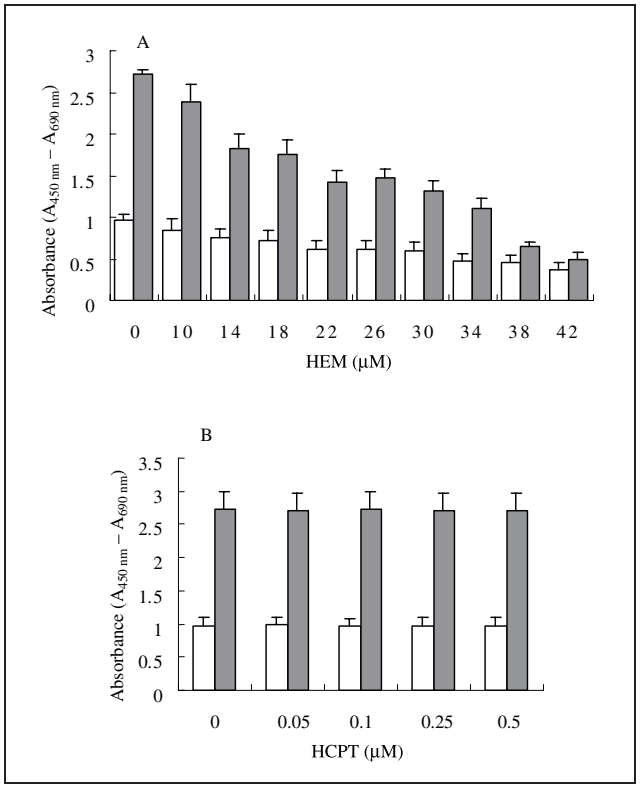


Fig. 1: HEM inhibited telomerase activity (A) while HCPT did not (B) in the two tumor cell lines. □ SMMC-7721 cells; ■ HO-8910 cells

2.3. HEM induced apoptosis in two tumor cell lines

Apoptosis was detected by the flow cytometry method and a fluorescence microscope as described. After treatment with HEM or HCPT for 48 h, the percentages of apoptosis in SMMC-7721 and HO-8910 cells were increased with increasing concentrations of the compounds. HEM (20–30 μM) induced apoptosis in SMMC-7721 cells from 5.7% to 18.4% and in HO-8910 cells from 7.6% to 67.1% (Fig. 2A). While HCPT (0.1–0.5 μM) induced apoptosis in SMMC-7721 cells from 6.5% to 13.3% and in HO-8910 cells from 9.9% to 30.9% (Fig. 2B). The fluorescence microscope showed that after treatment with HEM or HCPT, micronuclei and nuclear fragments were observed (Fig. 3).

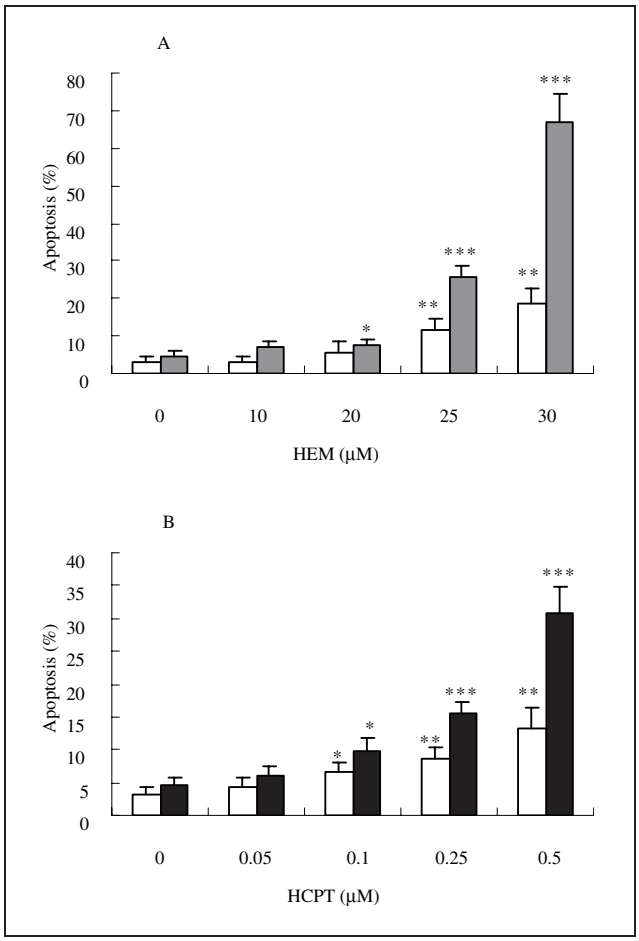


Fig. 2: The percentages of apoptosis in SMMC-7721 and HO-8910 cells after treated with HEM (A) or HCPT (B) for 48 h (* $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ vs control). □ SMMC-7721 cells; ■ HO-8910 cells

2.4. Down-regulation of Bcl-2 and up-regulation of P53 proteins expression in two tumor cell lines

The cell plasma was labeled with anti-Bcl-2 and anti-P53 monoclonal antibodies to measure the expression of Bcl-2 and P53 proteins analyzed by flow cytometry. HEM (30 μM) significantly decreased Bcl-2 protein expression to 58.7% in SMMC-7721 and to 57.6% in HO-8910 cells

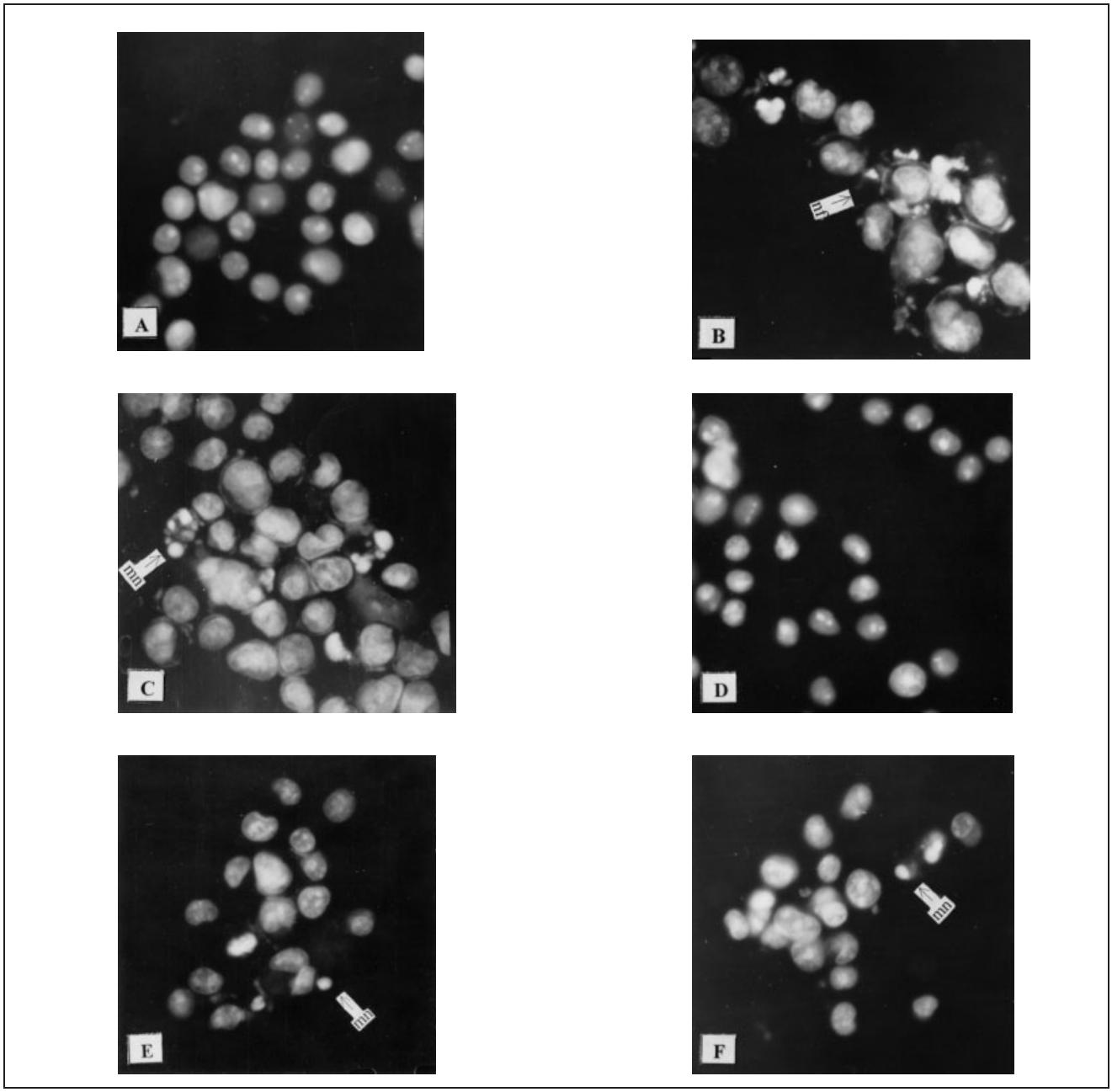


Fig. 3: Fluorescence photomicrographs (300 ×) of apoptosis in SMMC-7721 (A–C) and HO-8910 cells (D–F) treated with HEM or HCPT: control (A, D); cells treated with HEM at 25 μM for 48 h (B, E); cells treated with HCPT at 0.25 μM for 48 h (C, F). Micronuclei (mn) and nuclear fragment (nf)

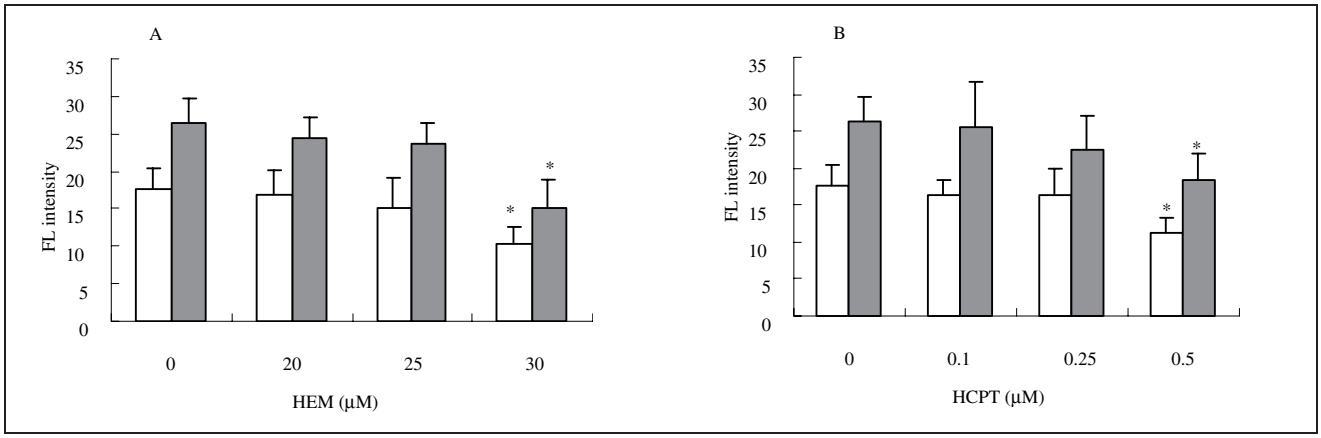


Fig. 4: Down-regulation of Bcl-2 protein expression induced by HEM (A) or HCPT (B) in SMMC-7721 and HO-8910 cells (* $p < 0.05$ vs control); □ SMMC-7721 cells; ■ HO-8910 cells

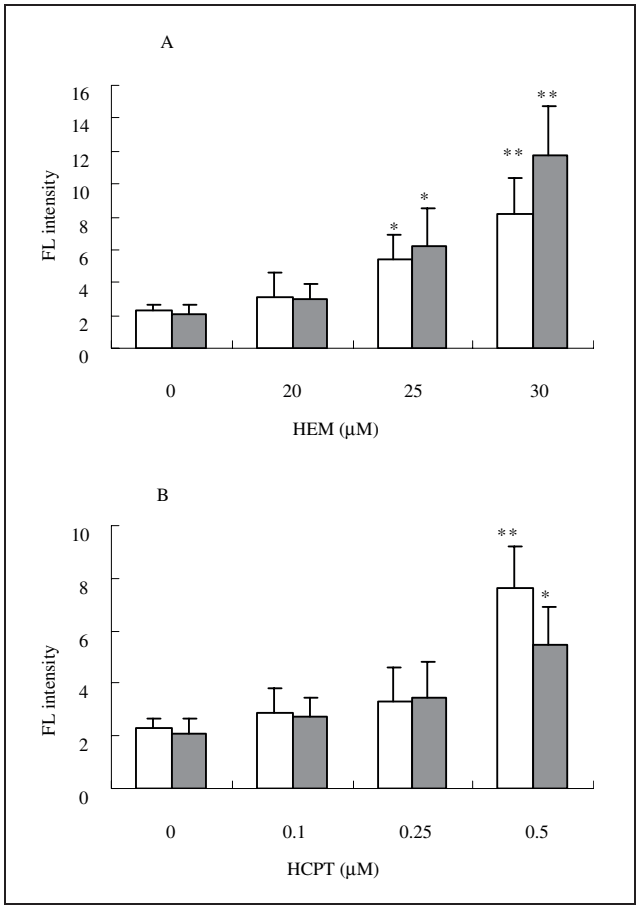


Fig. 5: Up-regulation of P53 protein expression induced by HEM (A) or HCPT (B) in SMMC-7721 and HO-8910 cells (* $p < 0.05$; ** $p < 0.01$ vs control). □ SMMC-7721 cells; ■ HO-8910 cells

(Fig. 4A) while HCPT 0.5 μM significantly decreased Bcl-2 protein expression to 64.3% in SMMC-7721 and to 70.0% in HO-8910 cells (Fig. 4B). HEM 25 and 30 μM significantly increased P53 protein expression 2.3- and 3.6-fold in SMMC-7721 and 3.0- and 5.7-fold in HO-8910 cells (Fig. 5A) while HCPT 0.5 μM significantly increased P53 protein expression 3.3-fold in SMMC-7721 and 2.7-fold in HO-8910 cells (Fig. 5B).

3. Discussion

Anticancer drugs have been shown to trigger apoptosis in various cancer cells, and the induction of apoptosis in cancer cells is an appropriate aim in the therapy of malignant tumors (Fisher 1994; Hickman 1992). The concentrations 10–30 μM for HEM and 0.05–0.5 μM for HCPT were selected to examine their apoptotic induction and related gene expression according their IC_{50} of cytotoxicity. In other words, the concentration range was selected around their IC_{50} to compare the antitumor potential of HEM with that of HCPT. We finally found that in this concentration range both tested compounds can induce apoptosis and regulated two genes relating to apoptosis. Bcl-2 and P53 are two of the most prominent proteins involved in apoptosis (Blagosklonny 2001). Bcl-2 seems to be at the convergence of many apoptotic pathways. P53, a tumor suppressor protein regulates Bcl-2 gene expression *in vitro* and *in vivo* (Miyashita et al. 1994). Therefore, the changes in Bcl-2 and P53 found in our study may be responsible for the apoptosis induction by HEM and HCPT. Thus HEM and HCPT exhibit the same mechanism of apoptosis but HCPT was more potent than HEM.

Most interestingly, we found that HEM and HCPT exhibited different effects on telomerase activity in this study. HEM is able to inhibit telomerase activity in the two tumor cell lines, but HCPT cannot. Whereas telomerase inhibition ability may be an important effect of HEM, HCPT has no effect on telomerase. Therefore, HEM might be a candidate of an anticancer drug.

4. Experimental

4.1. Chemicals and reagents

HEM (purity > 98%) was isolated from Chinese herb *Senecio oldhamianus* Maxim. RPMI 1640 medium was purchased from Gibco. Fetal calf serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). Sulforhodamine B, diethyl pyrocarbomate, dimethylsulphoxide (DMSO) and propidium iodium (PI) were purchased from Sigma-Aldrich Co. (USA). Hydroxycamptothecine (HCPT) was purchased form Hainan Weikang Pharmaceutical Co., Ltd. (Hainan, China). RNase A was purchased from Shanghai Sangon Biotech Co (Shanghai, China). Telomerase PCR ELISA kit was purchased from Roche Diagnostics Corporation (Germany). Monoclonal antibodies (Pharmingen), Bcl-2 (clone124, FITC-conjugated), P53 (clone G59–12, FITC-conjugated) and their control mouse IgG1 were purchased from Jingmei Biotech. Ltd. (Shenzhen, China).

4.2. Cell culture and drug treatment

Human hepatoma cells SMMC-7721 or human oophoroma cells HO-8910 were cultured in RPMI 1640 medium with 10% heat-inactivated fetal calf serum, 100 μg/ml streptomycin and 100 units/ml penicillin at 37 °C in a humidified atmosphere containing 5% CO_2. For telomerase activity and flow cytometry assays, exponentially growing cells were harvested and seeded in 6-well tissues culture plate at 1×10^5 cells/ml. After incubation for 24 h, cells were exposed to different concentrations of HEM or HCPT for 48 h (the stock solution contains HEM 200 μg, DMSO 2 μl and cell culture medium 1 ml).

4.3. Cytotoxicity assay

The cytotoxicities of HEM or HCPT toward SMMC-7721 and HO-8910 cell lines were determined in 96-well microtiter plates by the sulforhodamine B method described by Skehan et al. (1990) with some modifications. Briefly, exponentially growing cells were harvested and seeded in 96-well plates with the final volume 100 μl containing 4×10^3 cells per well. After 24 h incubation, cells were treated with various concentrations of HEM or HCPT for 48 h. The cultures were fixed at 4 °C for 1 h by addition of ice-cold 50% trichloroacetic acid (TCA) to give a final concentration of 10%. Fixed cells were rinsed 5 times with deionized water and stained for 10 min with 0.4% sulforhodamine B dissolved in 0.1% acetic acid. The wells were washed 5 times with 0.1% acetic acid and left to dry overnight. The absorbed sulforhodamine B was dissolved in 150 μl unbuffered 1% Tris base [tris(hydroxymethyl) aminomethane] solution in water (pH 10.5). The absorbency of extracted sulforhodamine B at 570 nm was measured by a microplate reader (Bio-Rad). The experiments were carried out in triplicate. Each run entailed 5–6 concentrations of the compounds being tested. The survival rates of cells exposed to the compounds were calculated by assuming the survival rate of untreated cells to be 100%.

4.4. Telomerase activity assay

Telomerase activity was detected using a PCR-based telomeric repeat amplification protocol (TRAP) enzyme-linked immunosorbent assay (ELISA) kit (Telomerase PCR ELISA kit), following the manufacturer's description with some modifications. Briefly, cells were harvested and counted using a hemocytometer. 2×10^5 cells were lysed in 200 μl lysis reagent and incubated on ice for 30 min. After centrifugation at 16000 g for 20 min at 4 °C, 2 μl supernatant was added to 25 μl reaction mixture and sterile water was added to a final volume of 50 μl. Then PCR was performed in a GeneAmp PCR System 9600 Thermal Cycler followed this protocol: primer elongation (20 min, 25 °C), telomerase inactivation (5 min, 94 °C), product amplification through 30 cycles reaction (denaturated for 30 s at 94 °C, annealed for 30 s at 50 °C and polymerized for 90 s at 72 °C), then after another 10 min incubated at 72 °C, the products can be kept at 4 °C until the next step. The PCR products were denatured and hybridized to the digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. The resulting products were immobilized via the biotin labeled primer to a streptavidin-coated microtiter plate. The immobilized PCR products were then detected with the antibody against digoxigenin (anti-DIG-POD) that was conjugated to peroxidase. Finally, the probe was visualized by virtue of peroxidase metabolizing TMB to form a colored reaction product. The absorbances at 450 nm (with a reference wavelength of 690 nm) were

measured with a microplate reader (Bio-Rad). Untreated cells were used as positive control and the same cell extract heated 10 min under 65 °C was used as negative control.

4.5. Nuclei apoptosis assessed by flow cytometry and fluorescence microscope

After exposure to HEM or HCPT for 48 h, the apoptosis was assessed by flow cytometry according to the method of Telford et al. (1992) with some modifications. In brief, about 10^6 cells were harvested, washed with ice cold PBS and fixed with ice cold 90% ethanol, then kept overnight at 4 °C. Cells were rinsed with PBS and stained with 10 μg/ml PI working solution (PBS containing 0.2% Triton X-100, 0.1 mM EDTA and 100 μg/ml RNase A) for 30 min at room temperature in the dark. Then they were washed with PBS to remove PI, and determined on a flow cytometer (Facscalibure, Becton-Dickinson, San Jose, CA) within 1 h. The data were analyzed by Cellquest version 1.2.2 software (B-D). Fluorescence microscope observations were carried out with the same samples.

4.6. Bcl-2 and P53 expression assay

Cells (about 10^7 cells/ml) were trypsinized, harvested and suspended in ice-cold PBS, then fixed and permeabilized in 70% methanol at 4 °C for 1 h. 10^6 cells were incubated with anti-Bcl-2 or P53 monoclonal antibodies conjugated with FITC in the dark for 30 min at 4 °C. Their irrelevant mouse IgG1 was used as a negative control to determine background fluorescence. After washing twice with ice-cold PBS, the samples were determined by a flow cytometer (Facscalibure, Becton-Dickinson, San Jose, CA). The Bcl-2 and P53 levels were expressed by the mean fluorescence (FL) intensity.

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