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Preclinical report

Apoptosis induction and cell cycle perturbation in human hepatoma Hep G2 cells by 10-hydroxycamptothecin

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10-Hydroxycamptothecin (HCPT), a DNA topoisomerase I inhibitor, is an antitumor alkaloid isolated from a Chinese tree, *Camptotheca acuminata*, and exhibits a remarkable anti-hepatoma effect. We studied HCPT to determine whether or not its anti-hepatoma activity occurs through apoptosis induction and cell cycle disturbance using the MTT method, DAPI staining, agarose gel electrophoresis and flow cytometric analysis. The results showed that HCPT inhibited proliferation of human hepatoma Hep G2, Bel-7402 and Bel-7404 cells at an optimal concentration of 0.1 μg/ml. This growth inhibition was dose and time dependent, and was accompanied by evidence of apoptotic changes and cell cycle perturbation in Hep G2 cells. Chromatin condensation and nuclear fragmentation were observed in Hep G2 cells by fluorescence microscopy. Agarose gel electrophoresis showed internucleosomal DNA fragmentation ('ladder pattern') of Hep G2 cells following treatment with HCPT, in a concentration- and time-dependent manner. Flow cytometry showed that HCPT induced a massive hypodiploid cell population and arrested cells in G_2/M phase (at low dose) or in S phase (at high dose) in Hep G2 cells. The results of this study suggest that the anti-hepatoma effect of HCPT may result from apoptosis induction and cell cycle disturbance.

Key words: 10-Hydroxycamptothecin, apoptosis, cell cycle, flow cytometry, Hep G2 cells.

Introduction

Primary liver cancers remain one of the most common malignancies worldwide. Treatment options are limited and prognosis is poor. Surgical resection remains the mainstay of therapy; however, metastases are often present when the cancer is discovered, precluding a curative resection. Chemotherapy adds little to overall survival in patients in whom a curative resection is impossible.[1] Therefore, novel strategies and agents that target specific molecular pathways, as well as triggering a process of cell death (i.e. apoptosis), are being evaluated in the treatment of several neoplasms.

Apoptosis is an active process of cell death. It is a normal physiological event during morphogenesis, maturation and tissue renewal. Apoptosis also plays an important role in cancers. Since most treatment modalities exert their cytotoxic effects through the induction of apoptosis, treatment efficacy may well be related to the inherent ability of the target cells to respond by apoptosis while the resistance of certain tumors to treatment may well reflect failure to activate the cell's apoptotic cascade. Therefore, the ability to assess the presence and timing of apoptosis occurring during and as the result of treatment may have prognostic value in the clinic.[2] In recent years, strategies aimed at modulating the apoptotic pathway have been described as possible treatments for different cancers.

DNA topoisomerase I (Topo I) inhibitors represent an exciting and promising new class of anticancer therapeutic agents currently undergoing clinical evaluation. 10-Hydroxycamptothecin (HCPT), an analog of camptothecin (CPT, a well-known Topo I inhibitor[3,4]), is an alkaloid originally isolated from *Camptotheca acuminata*, a tree native to China. HCPT has a wide spectrum of anticancer activity *in vitro* and *in vivo* mainly through inhibitory effects on Topo I.[5] Among natural CPTs, HCPT has been shown to be more active and less toxic.[6–8] It has

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been well accepted as an effective anticancer drug in China and exhibits a remarkable effect in the treatment of human hepatic cancer in the clinic.[9] However, the capability of HCPT to induce apoptosis in human hepatoma cells has not been tested before. The purpose of this study was to evaluate the anti-hepatoma effect of HCPT, and whether or not it induces apoptosis and disturbs the cell cycle *in vitro*.

Materials and methods

Cell culture and treatment

The human hepatoma Hep G2 cell line was obtained from the ATCC (Rockville, MD). Human hepatoma Bel-7402 and Bel-7404 cell lines were from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 nutrient mixture (Gibco/BRL Life Technologies, Grand Island, NY) containing 15% calf serum, HEPES 15 mmol/l, sodium bicarbonate 2.0 g/l, penicillin 1.0×10^5 U/l, streptomycin 100.0 mg/l and L-glutamine 2.0 mmol/l, in an incubator with a humidified 5% CO_2+95% air atmosphere at 37°C.[10] For all experiments, cells were treated with various concentrations of HCPT (HCPT injection, 5 mg HCPT in 2 ml sterilized water, was produced by Huanshi Feiyun Pharmaceutical, Hubei, China) at 24 h after plating. Analysis was performed at different times during culture of the cells, as described below.

Cell viability and growth kinetics

To evaluate the effect of HCPT on human hepatoma cells cultured *in vitro*, kinetics of cell growth and survival were measured by the 3-[4,5-dimethylthial-2-yl]-3,5-diphenyltetrazolinium bromide (MTT)-based colorimetric assay.[11,12] Triplicate wells were used for each concentration of HCPT.

Detection of apoptotic nuclei

Morphological changes characteristic of apoptosis were determined by staining cell nuclei with DAPI. Briefly, cells were plated onto poly-L-lysine-coated 24-well cell culture plates (Costar, Cambridge, MA, USA) and incubated overnight. The cells were exposed to various concentrations of HCPT for different times and then washed twice with PBS. The cells were then fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) solution (pH 7.4) for 30 min at room temperature, washed twice with PBS and stained with a 0.5 mg/ml solution of DAPI (Sigma, St Louis, MO) in PBS for 10 min at room temperature. The plates were washed twice with PBS and photographed using an Olympus UV light fluorescence microscope.

DNA extraction and gel electrophoresis

The fragmentation of Hep G2 cells DNA was assayed with a modification of the method of Balachandran *et al.*[13] DNA was extracted from control and treated cells including attached and floating cells. Briefly, 1.0×10^6 Hep G2 cells were washed twice with PBS and lysed overnight at 37°C in lysis buffer containing 10 mmol/l Tris-HCl, pH 8.0, 10 mmol/l EDTA, 0.4% sodium dodecylsulfate, 150 mmol/l NaCl and 1 mg/l proteinase K (Sigma). After complete digestion, saturated phenol was added to the cell lysates and mixed fully. Samples were then centrifuged at 6000 r.p.m. for 5 min. An equal volume of chloroform was added to the supernatant isolated from the previous step, mixed fully and centrifuged as above. Supernatant was added to 2.5-fold volumes of absolute cold ethanol and NaCl at 0.2 mol/l final concentration for DNA precipitation. The DNA pellets were obtained by centrifugation at 12000 r.p.m. for 10 min and washed twice with 70% ethanol. The DNA pellets were then air dried, dissolved in TE buffer (10 mmol/l Tris-HCl, pH 7.8, 1 mmol/l EDTA) containing 1 mg/ml RNase A for 30 min at 37°C. Electrophoresis was performed on 1.5% agarose gels (Sigma) in TBE buffer (90 mmol/l Tris, pH 8.0, 90 mmol/l boric acid and 2 mmol/l EDTA) at 60 V constant current for 4 h. The DNA fragments were visualized by UV fluorescence after staining with 0.5 μg/ml ethidium bromide (Sigma).

Flow cytometry experiment

Cellular DNA content was detected by flow cytometry via determination of propidium iodide (PI).[14] After trypsinization, cells were washed with PBS and cell pellets were fixed in 70% ethanol at 4°C overnight. After being washed twice with PBS, the cells were stained with 1.0 ml of PI solution containing 50 μg/ml PI (Sigma), 10 μg/ml RNase A (Sigma), 0.5% (v/v) Triton X-100 and 0.1% (w/v) trisodium citrate for 30 min at room temperature in the dark before cytofluorometry.

Results

Cytotoxic effect

HCPT inhibited the proliferation of Hep G2, Bel-7402 and Bel-7404 cells (Figure 1). The decrease in viable cells was proportional to the time and dosage. Cell growth was slightly inhibited by HCPT at lower concentrations (0.001 and 0.01 μg/ml), but completely inhibited by HCPT at higher concentrations (0.1 and 1.0 μg/ml). From the growth curves in Figure 1, 0.1 μg/ml of HCPT was chosen as an optimal concentration for further observations. After 6 days of continuous exposure to HCPT, the IC_{50} values for Hep G2, Bel-7402 and Bel-7404 cells were found to be 0.012, 0.907 and 0.045 μg/ml, respectively.

Morphological changes

As shown in Figure 2, characteristic nuclear morphological changes of apoptosis, including chromatin condensation and nuclear fragmentation, were observed in Hep G2 cells after 48 h of treatment with 1.0 μg/ml HCPT.

DNA fragmentation

To determine whether HCPT-treated Hep G2 cells demonstrated the characteristic DNA laddering of apoptosis, we examined genomic DNA extracted from Hep G2 cells treated with various concentrations of HCPT for different times by agarose gel electrophoresis. As shown in Figure 3, internucleosomal fragmentation of the DNA was observed in the Hep G2 cells. The extent of the laddering was drug dose and time dependent. DNA laddering was obviously observed at concentrations of 0.1 and 1.0 μg/ml but not at the concentration of 0.01 μg/ml after exposure to HCPT for 48 h (Figure 3A). After treatment with 0.1 μg/ml HCPT, DNA laddering was detected at 24 h of incubation and became remarkable after 36 h of treatment (Figure 3B).

Apoptotic population

The nuclear DNA of apoptotic cells was cleaved into regularly sized fragments by activated nucleases and some of these fragments may escape the cell before it loses membrane integrity. The hypodiploid population formed by cells having a reduced DNA content in DNA content frequency histograms obtained by flow

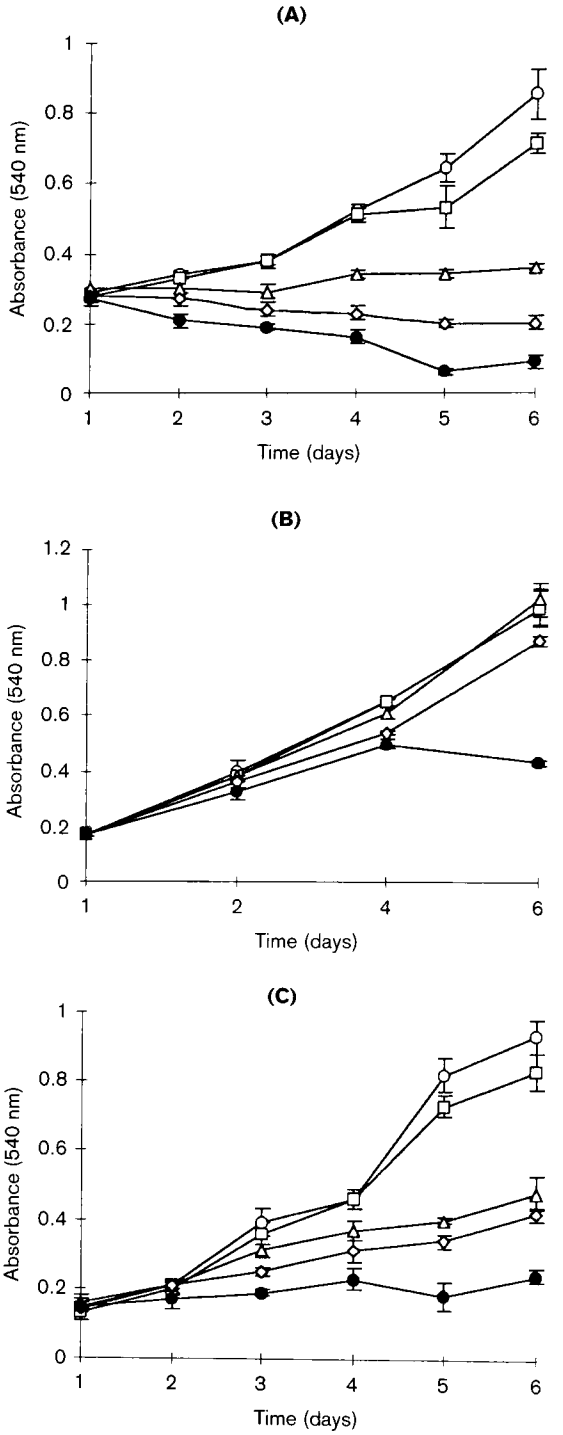

Figure 1. Effect of HCPT on the growth of human hepatoma HepG2 (A), Bel-7402 (B) and Bel-7404 (C) cells. ○, Control; □, HCPT 0.001 μg/ml; △, HCPT 0.01 μg/ml; ◇, HCPT 0.1 μg/ml; ●, HCPT 1.0 μg/ml. (Mean ± SD.) The cytotoxic effects were assessed by the MTT method, as described in Materials and methods.

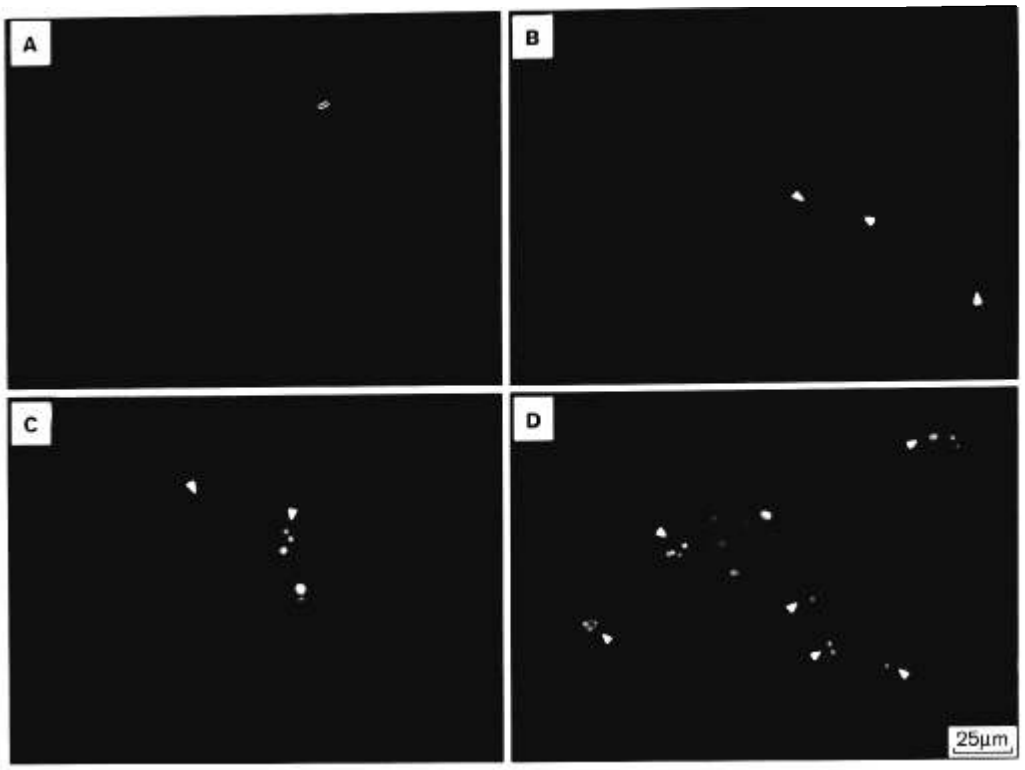

Figure 2. Nuclear morphological appearance of Hep G2 cells cultured without (A) or with 0.01 (B), 0.1 (C) and 1.0 (D) μg/ml HCPT for 48 h. The morphological changes in cells were detected by DAPI staining and observed under a UV light fluorescence microscope, as described in Materials and methods. Arrowheads denote condensed and fragmented nuclei. Magnification ×320.

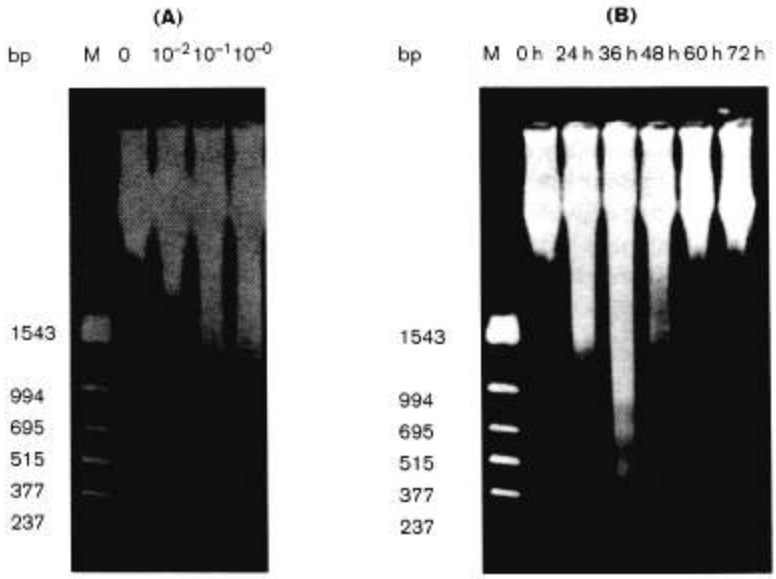

Figure 3. Agarose gel (1.5%) electrophoresis of DNA extracted from Hep G2 cells. (A) DNA fragmentation in Hep G2 cells treated for 48 h with the indicated concentration (μg/ml) of HCPT. (B) DNA fragmentation in Hep G2 cells treated with 0.1 μg/ml HCPT for the indicated times. M: molecular weight markers.

cytometric analysis represents the presence of apoptotic cells, which allowed quantitative comparison of the degree of apoptosis induced by different treatments of HCPT. As shown in Figure 4, HCPT induced the apoptosis of Hep G2 cells both dose and time dependently. In control cells, the apoptotic population was 2.1%. It increased to 13.1, 33.9 and 43.1% after treatment of cells with 0.01, 0.1 and 1.0 μg/ml HCPT for 48 h, respectively (Figure 4A). Figure 4(B) shows that apoptotic population of Hep G2 cells increased with increasing lengths of exposure to 0.1 μg/ml HCPT. The apoptotic population increased to 15.3, 22.4, 29.7, 34.5, 43.2 and 48.9% after 12, 24, 36, 48, 60 and 72 h of cultivation with 0.1 μg/ml HCPT, respectively.

Cell cycle distribution

The proportion of non-apoptotic Hep G2 cells in the different phases of the cell cycle was determined by flow cytometry. Untreated Hep G2 cells demonstrated a relatively normal distribution pattern, with most cells in the G_1 phase (55.0%), a lower S phase (21.9%) and G_2 (23.1%) peak of the cycle. The change of cell cycle distribution of Hep G2 cells treated for 48 h with

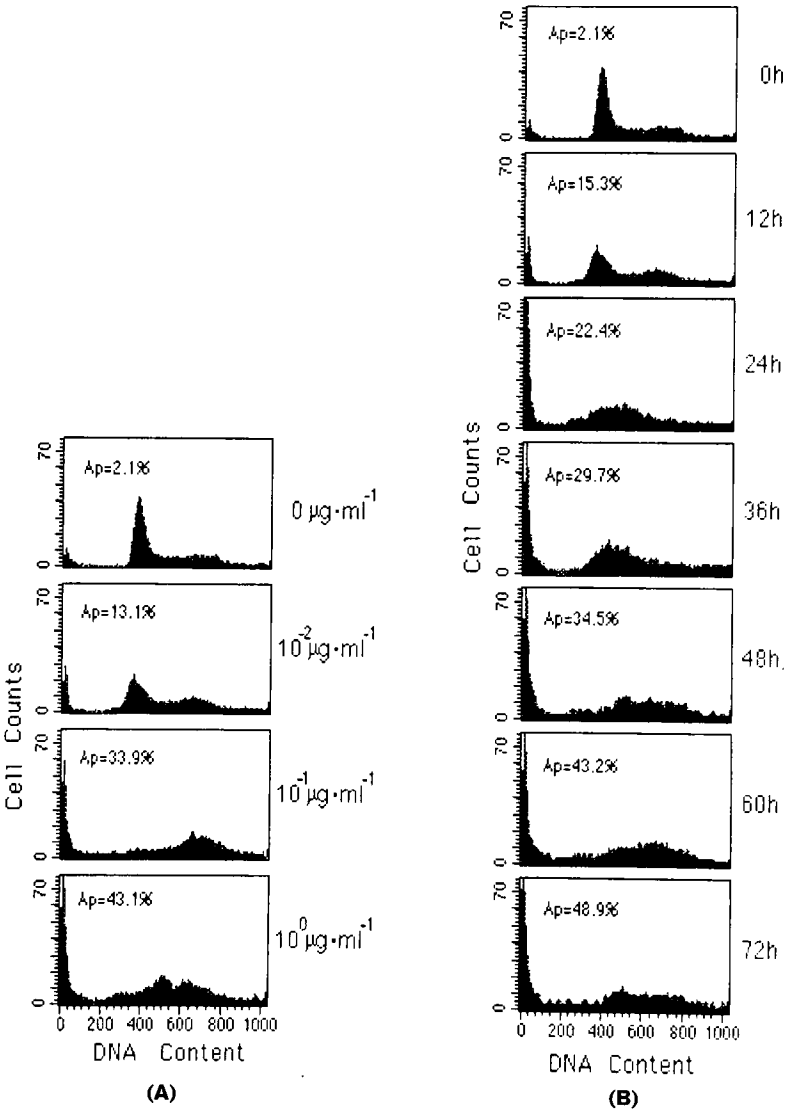

Figure 4. Effect of HCPT on the apoptotic population of Hep G2 cells. (A) Treatment for 48 h with the indicated concentration of HCPT. (B) Treatment with 0.1 μg/ml HCPT for the indicated times. The results were obtained by flow cytometry assay, as described in Materials and methods.

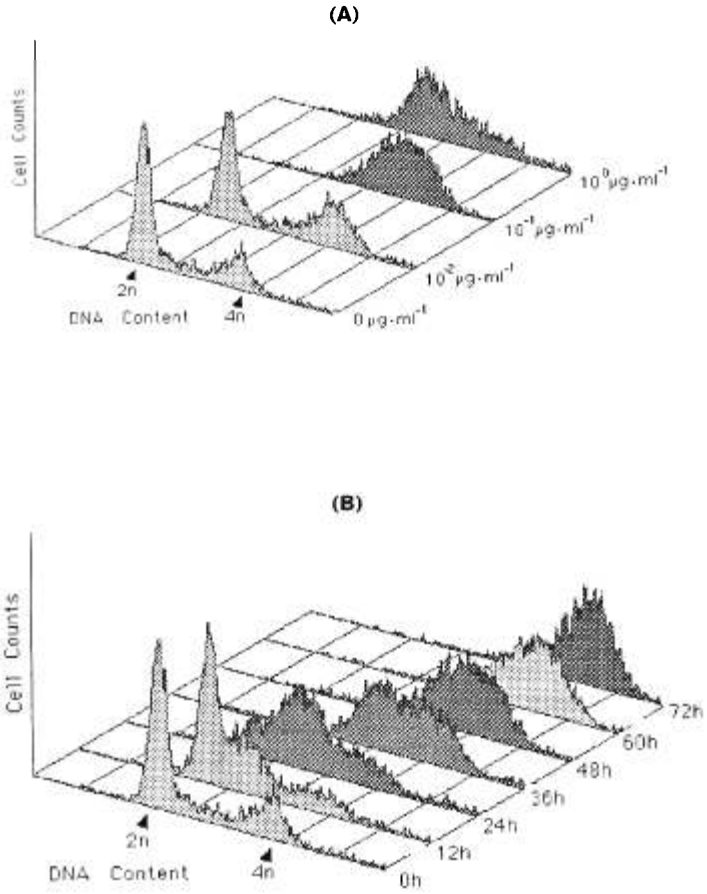

Figure 5. Effect of HCPT on cell cycle distribution of the non-apoptotic Hep G2 cells. (A) Treatment for 48 h with the indicated concentration of HCPT. (B) Treatment with 0.1 μg/ml HCPT for the indicated times. The results were obtained by flow cytometric analysis, as described in Materials and methods.

different doses of HCPT is shown in Figure 5(A) and Table 1. HCPT at low doses (0.01 and 0.1 μg/ml) mainly produced Hep G2 cells arrested in G_2/M, but almost all of the cells ($94 \pm 4\%$) were blocked in S phase after treatment with a high dose (1.0 μg/ml) of HCPT. The alteration of cell cycle distribution of Hep G2 cells treated with 0.1 μg/ml HCPT for different times is shown in Figure 5(B) and Table 2. An S phase accumulation can be observed at the early stage of treatment (12 and 24 h). After that, the proportion of S phase cells gradually decreased while the proportion of G_2/M phase cells increased. The proportion of G_2/M phase cells was $92 \pm 4\%$ after 72 h treatment of HCPT.

Table 1. Cell cycle distribution of the non-apoptotic Hep G2 cells treated with HCPT for 48 h

HCPT (μg/ml)	Cell cycle distribution (%)		
	G_0/G_1	S	G_2/M
0	55 ± 4	22 ± 3	23 ± 3
0.01	46 ± 5^a	18 ± 3^a	36 ± 4^b
0.1	0	33 ± 4^b	67 ± 5^c
1.0	0	94 ± 4^c	5.8 ± 1.9^c

$n = 3$ samples (10 000 cells were measured in each sample). Mean $\pm$ SD.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ versus control.

Discussion

This study demonstrates that HCPT is capable of inhibiting the proliferation of three kinds of human hepatoma cell lines *in vitro* in a dose- and time-dependent manner. This proliferation-inhibiting effect of HCPT on human hepatoma cells conforms to its strong anti-hepatoma effect in the clinic.

In Hep G2 cells, the inhibition of proliferation was accompanied with characteristics of apoptosis includ-

Table 2. Cell cycle distribution of the non-apoptotic Hep G2 cells treated with 0.1 μg/ml HCPT

Time (h)	Cell cycle distribution (%)		
	G_0/G_1	S	G_2/M
0	55±4	22±3	23±3
12	49±4[a]	43±4[c]	7.6±1.1[c]
24	10±3[c]	90±6[c]	0
36	0	67±5[c]	33±3[b]
48	0	33±4[b]	67±5[c]
60	0	11.2±2.0[b]	89±5[c]
72	2.1±0.9[c]	5.8±1.6[c]	92±4[c]

$n=3$ samples (10000 cells were measured in each sample). Mean±SD.
[a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ versus control.

ing nuclear morphological changes, DNA fragmentation and massive hypodiploid DNA content. The apoptosis-inducing effect of HCPT may be related to its Topo I inhibitory activity. Topo I is an important nuclear enzyme for various DNA functions including transcription and replication. Topo I inhibitors can lead to the formation of reversible Topo I-DNA cleavable complexes in cells. It is likely, therefore, that collison of the replication fork with the cleavable complex is the event which directly triggers the apoptotic pathway (see Darzynkiewicz *et al.*[15] and references therein). HCPT has been shown to be more active than either CPT or topotecan.[6] In the present study, HCPT was found to induce the apoptosis in Hep G2 cells dose- and time-dependently.

We found that 48 h of treatment with HCPT at low doses (0.01 and 0.1 μg/ml) mainly produced non-apoptotic Hep G2 cells arrested in G_2/M phase, but almost all of the cells (94.2±2.4%) were blocked in S phase by a high dose (1.0 μg/ml) of HCPT. Early studies indicated that the DNA Topo I inhibitor CPT allowed initiation of DNA synthesis, but it effectively prevented cells from progressing to mitosis[16] and arrested cells in S phase.[15] CPT-11, a new CPT analog and Topo I inhibitor, induced an increase in the S phase cell population in esophageal and colon tumor lines.[17] It has been subsequently shown that cell death induced by CPT, selective to S phase cells, occurs by apoptosis.[15,18] As a Topo I inhibitor like CPT, HCPT may also lead to a S phase arrest and induce S-phase-specific apoptosis in the HepG2 cells. In this study, a high dose (1.0 μg/ml) of HCPT led to full S phase accumulation and a higher apoptotic population in Hep G2 cells. However, we propose that a low dose (0.1 μg/ml) of HCPT could not fully block the cells in S phase, so some of the cells still can enter the G_2/M phase. Because of the DNA injury caused by HCPT, the cells arrested in S phase gradually died due to apoptosis. So, the low S phase population in the low-dose HCPT-treated group may result from the S-phase-specific apoptosis. In the high-dose HCPT-treated group, because of a full arrest, the remaining non-apoptotic cells were still S phase cells though the apoptotic population was higher than that of the low-dose HCPT-treated group. The S phase arrest effect of HCPT can be further demonstrated by the results of the temporal change of cell cycle distribution in the 0.1 μg/ml HCPT-treated group. Treatment with 0.1 μg/ml HCPT led to a high proportion of S phase cells and a low proportion of G_2/M phase cells with a lower apoptotic population at the early stage of treatment (12 and 24 h). Prolongation of incubation time caused an increase in apoptotic cells and a decrease in the fraction of cells in S phase. Almost all of the remaining non-apoptotic cells (91.9±5.1%) were in G_2/M phase at 72 h. We proposed that, at the early stage, most cells have not been induced to undergo apoptosis; therefore, S phase cells and G_2/M cells co-exist in the culture. With the increase of time of treatment, apoptosis was induced in these S phase cells, and resulted in a decrease of the S phase population and an accumulation of G_2/M cells at the late stage (36–72 h). The decrease in the proportion of S phase cells in non-apoptotic cells after HCPT treatment for 36–72 h demonstrated the specificity of HCPT's effect in terms of cell cycle phase in inducing apoptosis. In summary, HCPT inhibited the proliferation of hepatoma cells and the inhibition may be related to its S-phase-specific apoptosis induction.

Conclusion

Our findings suggest the following. (i) HCPT is capable of inhibiting the proliferation of three kinds of human hepatoma cell lines at an optimal concentration of 0.1 μg/ml *in vitro*. (ii) This growth inhibition was accompanied with evidence of apoptotic changes and cell cycle perturbation in Hep G2 cells. (iii) The anti-hepatoma effect of HCPT may result from apoptosis induction and cell cycle perturbation.

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