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10-Hydroxycamptothecin induces apoptosis in human neuroblastoma SMS-KCNR cells through p53, cytochrome c and caspase 3 pathways

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Neuroblastoma (NB), the most common extracranial solid tumor in childhood, remains one of the most challenging types of cancer to treat. Therefore, the search for novel effective drugs for its treatment is essential. The present study used 10-hydroxycamptothecin (HCPT), which is a naturally occurring alkaloid anticancer agent extracted from the Chinese tree, *Camptotheca acuminata*, and has a strong anticancer activity in vitro and in vivo. HCPT is able to induce apoptosis in cells of various tumor types. However, few studies have been conducted on its efficacy in NB, and its apoptosis-inducing mechanism has not been elucidated. In the present study, the in vitro effects of HCPT on apoptosis in the human NB cell line, SMS-KCNR, and its underlying molecular mechanisms were investigated. Cell proliferation was measured by an MTT assay and apoptosis was measured using DAPI staining and flow cytometric analysis. In addition, western blot analysis was used to evaluate the apoptosis-associated signaling pathways. HCPT was observed to markedly inhibit cell proliferation and induce apoptosis in SMS-KCNR cells at a relatively low concentration (2.5-20 nM). DAPI staining revealed typical apoptotic feature, namely apoptotic body formation. The flow cytometric analysis revealed that the number of apoptotic cells increased from 20.89% (for 2.5 nM) to 97.66% (for 20 nM) following HCPT treatment for 48 h. Western blot analysis revealed that p53, cytoplasmic cytochrome c, cleaved caspase-3 and poly ADP-ribose polymerase (PARP) proteins were significantly upregulated, while the mitochondrial cytochrome c and pro-caspase-3 proteins were downregulated. However, the B-cell lymphoma 2 and Bcl-2-associated X proteins were unaffected. The results indicated that HCPT may inhibit proliferation and induce apoptosis in the SMS-KCNR cells. The possible mechanism of apoptosis induction is the p53-mediated mitochondrial apoptotic signaling pathway, which promotes cytochrome c release and induces apoptosis by activating caspase-3 and PARP. Our study provides experimental evidence for HCPT as a potent therapeutic drug in NB treatment.

Key words: 10-Hydroxycamptothecin, neuroblastoma, apoptosis, p53, cytochrome c, caspase 3

Neuroblastoma (NB) is an embryonal cancer derived from the postganglionic sympathetic nerve system, and is the most common extracranial solid tumor in children [1]. NB is highly malignant and rapidly progressive, and is typically diagnosed at a late stage in the majority of cases [1]. Due to the greater chemoresistance of high-risk NB patients to first-line treatment drugs, the long-term survival rates of these patients remain extremely low [2]. This has become the greatest impediment for the improvement of survival in children with NB, and novel effective drugs are urgently required for high-risk patients.

Camptothecin (CPT) and its derivatives are a class of cell cycle-specific anticancer alkaloids that selectively act on DNA topoisomerase I and have a relatively strong and broad spectrum anticancer activity. The CPT derivatives, irinotecan (CPT-11), topotecan (TPT) and 10-hydroxycamptothecin (HCPT), are widely used in clinical practice. CPT-11 and TPT are most commonly used in the treatment of colon, ovarian and small-cell lung cancer in Europe and America [3-5]. The outcome of recurrent and refractory NB treatment with CPT-11 and TPT has been found to be satisfactory [2,6-8]. However, the high cost of these drugs has limited their clinical use in China. HCPT, which is more active compared with CPT, is a naturally occurring alkaloid anticancer agent extracted from *Camptotheca acuminata*, a native plant in China [9]. Its advantages include strong anticancer activity, low toxicity, no cross-resistance to other commonly used anticancer drugs and low cost. A previous study has identified that the outcome

of treatment with HCPT for non-small cell lung cancer, gastrointestinal tract tumors, liver cancer and nasopharyngeal carcinoma was satisfactory [10]. To improve the survival rates of children with NB (particularly for high-risk patients) and take advantage of the easy access to a domestically manufactured medication, HCPT has been firstly used to treat recurrent and refractory NB in children at the Children's Hospital of Zhejiang University School of Medicine (Hangzhou, China) since 2006, producing satisfactory results [11]. However, its mechanism of action has not been elucidated.

p53 plays a crucial role in mediating DNA damage-induced apoptosis [12]. Although p53 is frequently mutated in many human cancers, it is wild-type in most neuroblastomas [13]. p53 is able to alter the ratio of B-cell lymphoma 2 (Bcl-2) family members in a transcription-dependent manner, alter mitochondrial membrane permeability and promote the release of apoptogenic factors (including cytochrome c) to induce apoptosis [14]. p53 may also induce apoptosis by promoting cytochrome c release in a transcription-independent manner [15]. Cytosolic cytochrome c leads to the activation of caspase-3 and poly ADP-ribose polymerase (PARP), thus subsequently resulting in apoptosis [13].

The aim of the present study was to provide a further theoretical basis to understand the anti-NB mechanisms of HCPT. Therefore, the in vitro killing effect of HCPT on the NB cell line, SMS-KCNR, as well as the impact of different concentrations of HCPT on SMS-KCNR cell proliferation and apoptosis, and its apoptosis-inducing molecular mechanisms were investigated.

Materials and methods

Cell culture and reagents. Human NB cell line, SMS-KCNR (contains wild-type p53), was provided by Professor C Patrick Reynolds (University of Southern California, Los Angeles, CA, USA) and maintained in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. HCPT, purchased from Huangshi Feiyun Pharmaceutical Co., Ltd. (Huangshi, China), was dissolved in dimethyl sulfoxide (DMSO) and stored at 4°C.

Cell proliferation assay. Cell proliferation was measured using an MTT assay. Briefly, SMS-KCNR cells from exponentially growing cultures were seeded into a 96-well plate at a final concentration of 5 $\times 10^4$ cells/well. Next, the cells were treated with different concentrations of HCPT (2.5-200 nM) in triplicate for 24, 48, and 72 h. Cells treated with phosphate-buffered saline (PBS) were used as the control. Following treatment, 20 µL MTT (5 mg/ml, AMRESCO, Solon, OH, USA) was added to each well. Following incubation for 4 h, the MTT medium was discarded, and the purple formazan crystals were solubilized in 150 µL/well warm DMSO. Absorbance in the control and HCPT-treated wells was determined using an Automated Microplate Reader (Bioelisa ELx800™ Absorbance Reader, BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm. The survival rate of the cells was expressed as a percentage of the control wells. Each assay was repeated three times to ensure the reliability.

DAPI staining. DAPI staining was used to visualize the apoptotic nuclear morphology. Following treatment with different concentrations of HCPT (0, 2.5, 5, 10 and 20 nM) for 48 h, the cancer cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.2% Triton-X 100(Bio Basic Inc, Shanghai, China). Subsequently, the cells were stained with 1 mg/ml of DAPI (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37°C. Following two washes with PBS, the samples were observed under a fluorescence microscope (Nikon TE2000, Nikon Corporation, Tokyo, Japan).

Quantitation of apoptosis using flow cytometric analysis. Quantitative assessment of apoptosis was performed using an Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefy, SMS-KCNR cells were exposed to different concentrations of HCPT for 48 h, harvested and washed twice with ice-cold PBS. Subsequently, the cells were resuspended in a binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 2.5 mM CaCl2) and stained with Annexin V-FITC and propidium iodide (PI) at room temperature for 15 min in the dark. The samples were then analyzed immediately using a flow cytometry system (BD FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA). Early apoptotic cells [lower right quadrant (LR)] were designated as Annexin V-positive and PI-negative (Annexin V^+/PI^-) cells, whereas late apoptosis cells [upper right quadrant (UR)] were designated as Annexin V-positive and PI-positive (Annexin V^+/PI^+) cells.

Western blot analysis. Following treatment with different concentrations of HCPT for 48 h, the SMS-KCNR cells were harvested and washed twice with cold PBS. The obtained cells were then lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA and 1 mM phenylmethylsulfonyl fuoride] for 10 min on ice. Subsequent to centrifugation at 12,000 x g for 10 min at 4°C, the supernatant fluid was transferred to a fresh tube and stored at -70°C. For the detection of cytochrome c release, cytoplasmic and mitochondrial proteins were separately extracted by Cytochrome c Releasing Apoptosis Assay Kit (BioVision, Inc., Palo Alto, CA, USA) according to the manufacturer's instructions. Briefy, treated cells ($5x10^7$) were centrifuged (600 x g for 5 min at 4°C) and washed with ice-cold PBS. The cells were resuspended with 1.0 ml of 1X cytosol extraction buffer mix containing dithiothreitol (DTT) and protease inhibitors, and incubated on ice for 10 min. Next, the cells were homogenized in an ice-cold Dounce tissue grinder, and the homogenate was transferred to a microcentrifuge tube and centrifuged at 700 x g for 10 min at 4°C. The supernatant was transferred into a fresh tube, centrifuged at 10,000 x g for 30 min at 4°C and collected as the cytosolic fraction. Subsequently, the pellet was resuspended in 0.1 ml mitochondrial extraction buffer mix containing DTT and protease inhibitors, vortexed for 10 sec and retained as

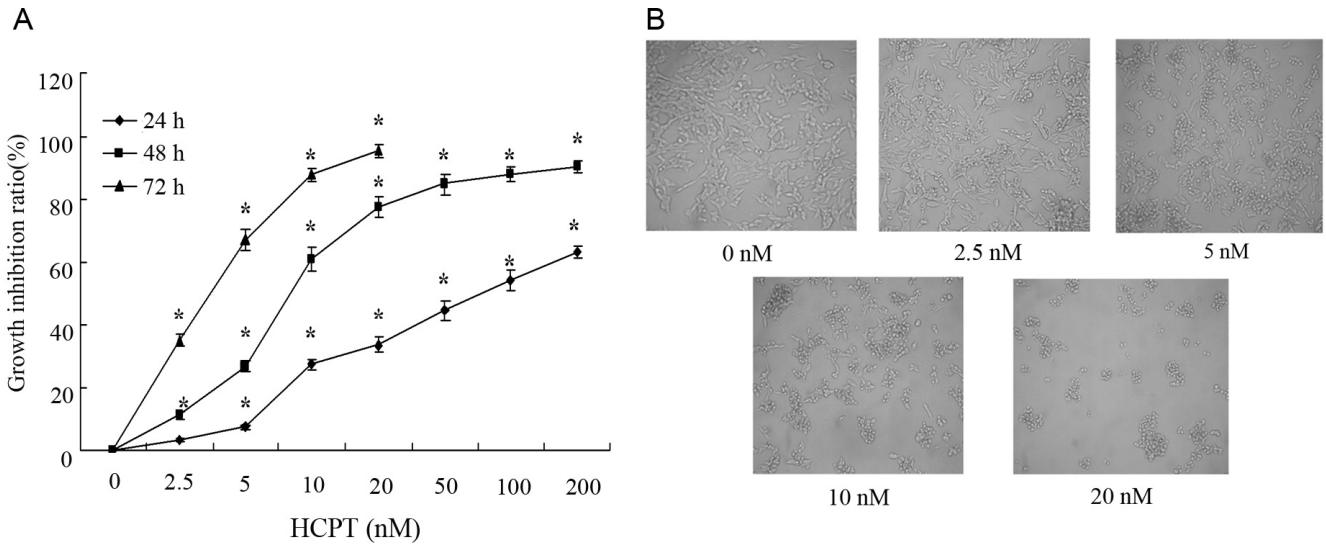

Figure 1. Effect of HCPT on the viability of SMS-KCNR cell lines. (A) Cells were treated with HCPT at concentrations of 0-200nM for 24, 48 and 72 h. The cell viability was measured using an MTT assay. The results are presented as the mean ± standard deviation from three independent experiments at each time point. *P<0.05, vs. the control group (using analysis of variance). (B) Phase-contrast micrographs (×10) of SMS-KCNR cells treated with 0 (control), 2.5, 5, 10 and 20 nM HCPT for 48 h. Following treatment, the number and morphology of cells were significantly altered. HCPT, 10-hydroxycamptothecin.

the mitochondrial fraction. Protein concentrations were determined using a bicinchoninic acid protein assay (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples (50 μg for total cellular and cytoplasmic proteins or 15 μg for mitochondrial proteins) were electrophoresed in a 10% SDS-polyacrylamide gel and subsequently transferred onto polyvinylidene fuoride membranes (Merck Chemicals Co., Ltd, Shanghai, China).The membranes were blocked with 5% non-fat milk in Tris-buffered saline for 2 h at room temperature, followed by incubation with primary antibodies against p53 (1:1000 dilution), Bcl-2 (1:500 dilution), Bcl-2-associated X (Bax) (1:500 dilution), caspase-3 (1:250 dilution), β-actin (1:1000 dilution), cytochrome c (1:200 dilution) (BioVision, Inc.), PARP (1:1000 dilution) and cytochrome c oxidase (COX) IV (1:1000 dilution) (Cell Signaling Technology, Inc., Boston, MA, USA) in Tris-buffered saline at 4°C overnight. Subsequent to washing, the membranes were incubated with a secondary antibody [horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit immunoglobulin IgG antibody at a 1:2000 dilution; Santa Cruz Biotechnology, Inc., Dallas, TX, USA] for 2 h at room temperature. The bands were visualized using an enhanced chemiluminescence (ECL) system (ECL kit; Beyotime Institute of Biotechnology), quantitated by densitometry and normalized to β-actin using Image Pro Plus software (Media Cybernetics, Inc., Rockville, MD, USA).

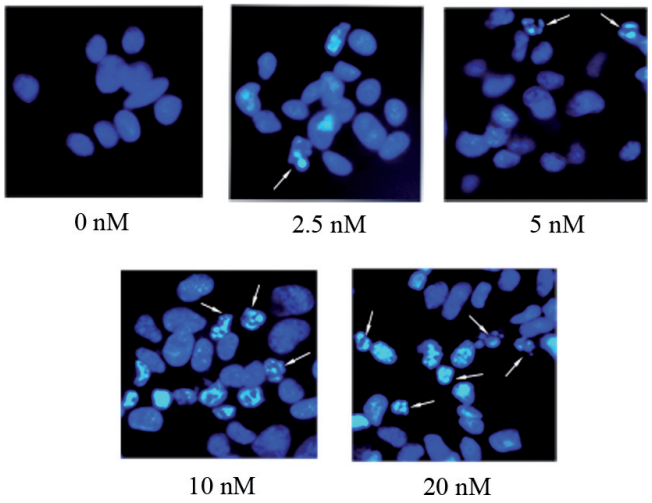

Figure 2. Apoptosis observed by DAPI staining. The SMS-KCNR cells were treated with various concentrations (0, 2.5, 5, 10 and 20nM) of HCPT for 48 h, and then stained with DNA-specific fluorochrome DAPI. Morphological changes were detected by fluorescence microscopy (×40). Apoptotic bodies are indicated using white arrows. HCPT, 10-hydroxycamptothecin.

Statistical analysis. Data are expressed as the mean ± standard deviation. Statistical analysis was performed between various treatment groups and controls using one-way analysis of variance. Data were analyzed using SPSS software (version 18.0; SPSS Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Cytotoxic effect of HCPT on SMS-KCNR cells. The effect of HCPT on the viability of SMS-KCNR cells was measured using an MTT assay. As shown in Fig. 1A, HCPT exhibited

strong cytotoxic effect on SMS-KCNR cells after 24, 48, and 72 h of treatment. HCPT inhibited cell proliferation in a dose- and time-dependent manner. The IC50 values (50% cell growth inhibitory concentration) were 64.67, 9.92 and 3.30 nM for 24, 48 and 72 h of incubation, respectively. In addition, HCPT markedly affected SMS-KCNR cell morphology after a 48-h treatment, as shown in Fig. 1B. Other evident effects included reduced cell numbers, rounded cells and cell detachment, which indicated cell death. Further investigation attempted to determine whether the inhibition effect of cell growth was attributed to the induction of tumor cell apoptosis following treatment.

DAPI staining of SMS-KCNR cells with HCPT. To further confirm the cell apoptosis induced by HCPT in SMS-KCNR cells, a morphological examination of the nuclei was conducted using DAPI staining. As shown in Fig. 2, a number of apoptotic bodies were observed under a fluorescence microscope in cells treated with HCPT for 48 h. The intensities of the apoptotic bodies were increased in an HCPT dose-dependent manner, indicating the induction of apoptosis in the SMS-KCNR cells following HCPT treatment.

Effects of HCPT on cell apoptosis. To further quantify the apoptosis of SMS-KCNR cells induced by HCPT, the cells were stained with Annexin V-FITC/PI and subsequently analyzed using flow cytometry. As shown in Fig. 3, the number of apoptotic cells increased in a dose-dependent manner following treatment with 2.5-20 nM HCPT for 48 h. The proportion of

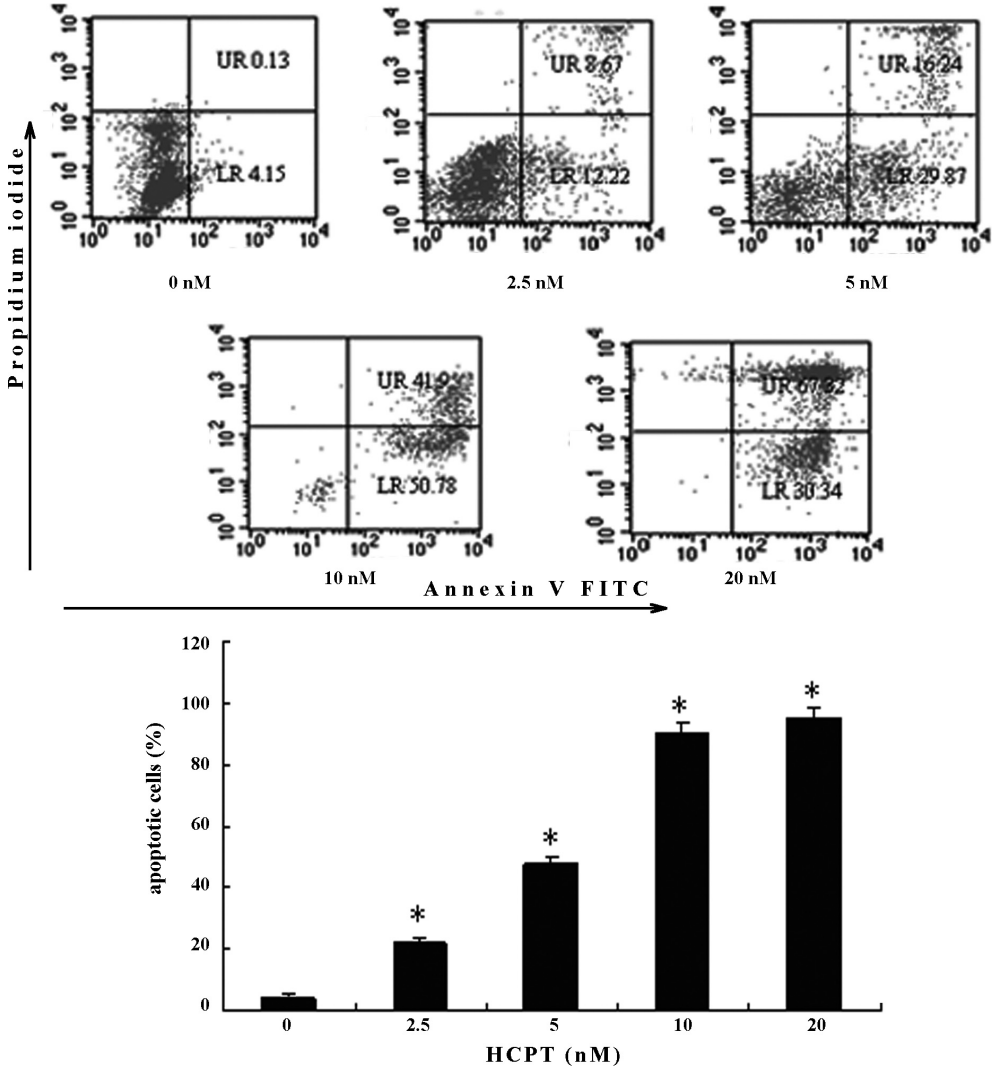

Figure 3. Effects of HCPT on apoptosis in SMS-KCNR cells. Cells were treated with 0 (control), 2.5, 5, 10 and 20 nM of HCPT for 48 h and stained with Annexin V-FITC/PI double staining. The percentage of apoptotic cells was analyzed by flow cytometry. Early apoptotic cells (LR) were designated as Annexin V-positive and PI-negative (Annexin V^+/PI^-) cells and the late apoptosis cells (UR) were designated as Annexin V-positive and PI-positive (Annexin V^+/PI^+) cells. Data were obtained from three independent experiments. *P<0.05, vs. the control group. HCPT, 10-hydroxycamptothecin; FITC, fluorescein isothiocyanate; PI, propidium iodide.

early apoptotic cells [(Annexin V^+/PI^-) cells, LR] significantly increased from 12.22% (2.5 nM) to 29.87% (5 nM), 50.78% (10 nM) and 30.34% (20 nM), whereas that for the untreated control group was only 4.15%. The relatively low rate of early-stage apoptosis in the 20 nM group, as compared with that in the 10 nM group, is due to the transition of cells from early-stage to late-stage apoptosis. In addition, the number of late apoptotic cells [(Annexin V^+/PI^+) cells, UR] increased from 8.67% (2.5 nM) to 67.32% (20 nM), in contrast to 0.13% of cells in the control group. Overall, the total percentage of apoptotic cells (UR + LR) increased from 4.28% in the untreated SMS-KCNR cells to 97.66% following 20 nM HCPT treatment for 48 h.

Apoptosis-associated signaling pathways

Modulation of P53, Bcl-2 and Bax by HCPT treatment. To understand the apoptotic signaling pathways that are activated following HCPT treatment, the levels of p53, Bcl-2 and Bax proteins were evaluated using western blot analysis and calculation of the Bax/Bcl-2 ratio. The findings indicated that treatment of SMS-KCNR cells with HCPT for 48 h resulted in a dose-dependent increase in the p53 protein levels (Fig. 4A); however, no effect was observed on the levels of Bcl-2 and Bax, or the Bax/Bcl-2 ratio following the incubation (Fig. 4B). This suggested that HCPT may have promoted apoptosis through the activation of p53 expression, but independent of Bcl-2 and Bax expression.

HCPT treatment triggers cytochrome c release from mitochondria. To test whether the cytochrome c release was involved in the HCPT-induced apoptosis, cytoplasmic and mitochondrial fractions were separately extracted and analyzed by western blot analysis. The purity of the cytoplasmic fraction was determined using an immunoblot analysis for the presence of marker proteins; COX IV was used as a mitochondrial marker. In the cytoplasmic fraction, low COX IV immunoreactivity was detected, indicating no contamination of mitochondria in cytoplasmic extracts. In addition, western blot analysis revealed that HCPT treatment resulted in a significant dose-dependent increase in the level of cytoplasmic cytochrome c and a concomitant decrease of mitochondrial cytochrome c (Fig. 4C). These results indicated that HCPT induced apoptosis at the mitochondrial level.

Induction of apoptosis by activation of caspase-3 and PARP cleavage. To further investigate the mechanism of HCPT-induced apoptosis, the expression levels of caspase-3, cleaved caspase-3 and PARP were evaluated. Western blot analysis revealed that HCPT treatment resulted in the activation of caspase-3 expression, while the level of pro-caspase-3 protein was decreased and that of the activated 17-kDa subunit was increased in a dose-dependent manner (Fig. 4D). Additionally, while HCPT treatment resulted in the cleavage of PARP, the level of the 89-kDa cleaved subunit was increased in a dose-dependent manner (Fig. 4E). These results suggested that HCPT may induce apoptosis through the activation of the caspase cascade.

Discussion

Apoptosis is a genetically controlled process of programmed cell death, which is important in tumorigenesis and tumor development. The impairment of apoptosis is one of the key characteristics of cancer development, rendering tumors resistant to cytotoxic therapy [16]. Induction of apoptosis in tumor cells is a promising chemopreventive or chemotherapeutic strategy [17,18]. Previous studies have demonstrated that HCPT is able to induce apoptosis in cells of various tumor types, including colon, liver, gastric, prostate and bladder cancer, as well as melanoma [19-24]. However, only one study regarding HCPT-induced apoptosis of NB cells has been conducted [25]. Furthermore, its apoptosis-inducing molecular mechanism has not been elucidated. In the present study, we evaluated the anti-NB effect of HCPT and its molecular mechanisms in vitro.

In our study, HCPT was observed to markedly inhibit the proliferation of SMS-KCNR cells in a dose- and time-dependent manner. Determining whether the decrease in SMS-KCNR cell viability is attributable to apoptosis is crucial, since the induction of apoptosis in cancer cells is a major indicators of anticancer effects [24]. Therefore, fluorescence microscopy was employed to observe the morphological changes in the SMS-KCNR cells(Fig.2), while the extent of apoptosis was quantified by flow cytometric analysis. The prominent characteristics of apoptosis were observed after 48 h of HCPT treatment. A number of apoptotic bodies were observed with a fluorescence microscope using DAPI staining, and the intensities of apoptotic bodies increased in a HCPT dose-dependent manner. Flow cytometric analysis revealed that the number of apoptotic cells increased from 20.89% (following treatment with 2.5 nM HCPT) to 97.66% (following treatment with 20 nM HCPT) in a dose-dependent manner. So consistent with earlier observations in other tumor cells [19-24], we found that HCPT inhibits the proliferation and viability of SMS-KCNR cells by inducing apoptosis.

The two main pathways of chemotherapy-induced tumor cell apoptosis include the mitochondrial and the death receptor pathways [26]. The majority of NB cell lines are resistant to apoptosis induced by death receptor ligands due to the silencing of caspase-8 [26]. Therefore, the mitochondrial pathway may be important in chemotherapy-induced NB cell apoptosis. In the mitochondrial pathway, the tumor suppressor gene p53, Bcl-2 family members and cytochrome c play a vital role. In the present study, western blot analysis of SMS-KCNR cells treated for 48 h with various HCPT concentrations revealed that, as the drug concentration was increased, p53 protein levels increased significantly, Bcl-2/Bax protein levels and ratio remained essentially unchanged, cytoplasmic cytochrome c level increased significantly and mitochondrial cytochrome c level decreased significantly. These results indicated that p53 is activated and released into the cytoplasm with mitochondrial cytochrome c during HCPT-induced SMS-KCNR cell apoptosis, thereby

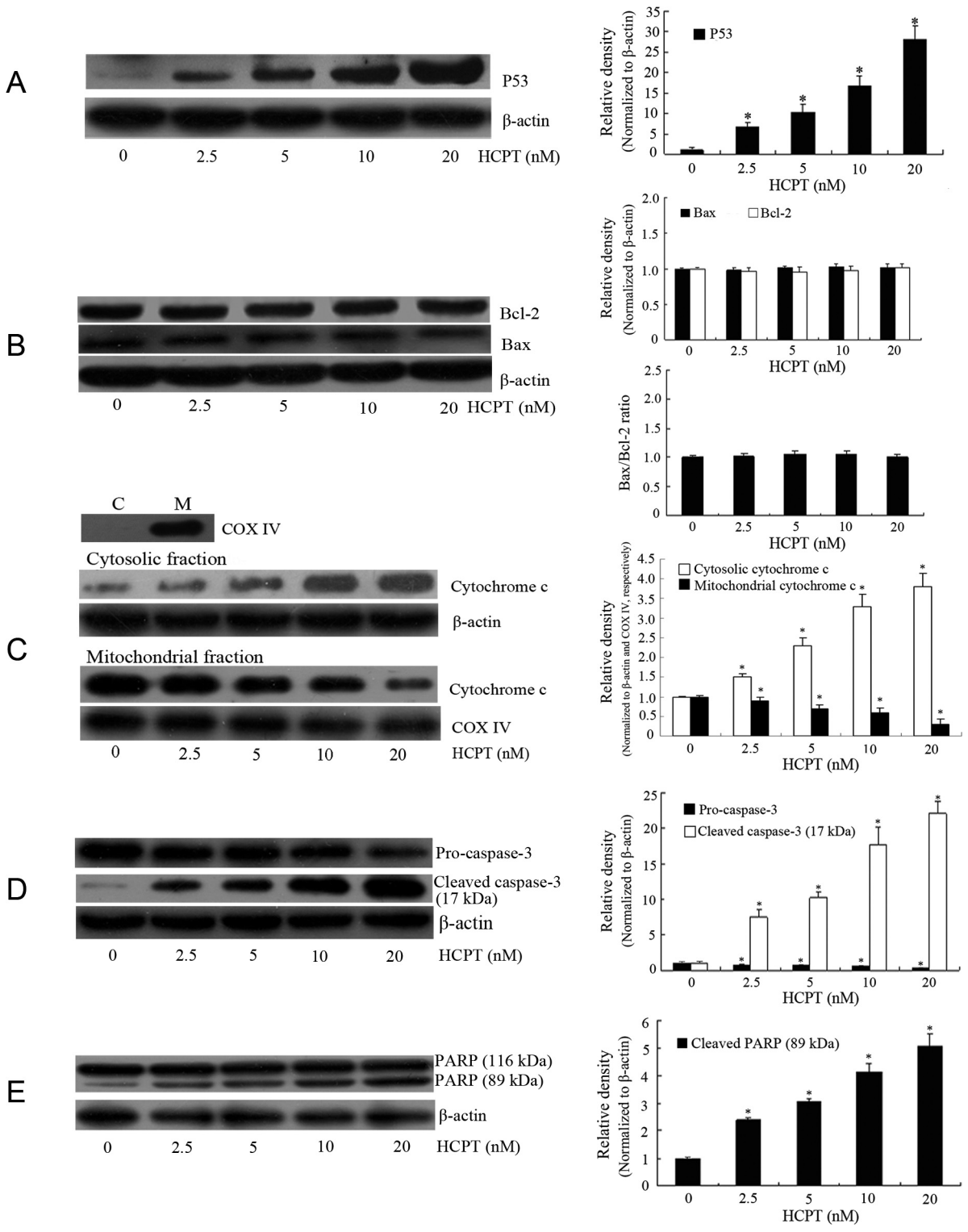

Figure 4. Effect of HCPT on protein expression of p53, Bcl-2, Bax, cytochrome c, caspase-3 and PARP in SMS-KCNR cells. Evaluation of protein expression levels of (A) p53, (B) Bax and Bcl-2, (C) cytochrome c, (D) caspase-3 and (E) PARP by western blot analysis. The SMS-KCNR cells were treated with various concentrations (0, 2.5, 5, 10 and 20 nM) of HCPT for 48 h. Equal amounts of lysates from these cells were immunoblotted with associated individual antibodies. β-actin was used as the loading control, and COX IV was used as the loading control in the mitochondrial fraction. Results were obtained from three independent experiments. The protein expression levels were quantified and normalized against β-actin or COX IV, and expressed as the fold-change relative to the associated controls. *P<0.05, vs. control group. HCPT, 10-hydroxycamptothecin; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X; PARP, poly ADP-ribose polymerase; COX IV, cytochrome c oxidase IV.

demonstrating the involvement of p53 and its mitochondrial signaling pathway. Bcl-2 family members are important to the p53-mediated mitochondrial apoptotic signaling pathway and induce apoptosis by adjusting the permeability of the outer mitochondrial membrane to permit the release of cytochrome c. However, the results of the present study revealed that the expression levels of Bcl-2 and Bax, two important members of the Bcl-2 family, did not change significantly following HCPT treatment. This implied that p53 does not regulate Bcl-2 and Bax during apoptosis, but induces SMS-KCNR cell apoptosis through other members of the Bcl-2 family or alternative mechanisms. Other possible pathways include the following: i) p53-mediated activation of only Bcl-2 homology domain 3 apoptogenic factors, such as Noxa and Puma [27,28], which enables their attachment to anti-apoptotic proteins, including Bcl-2 and Bcl-extra large (Bcl-xL), thereby weakening their anti-Bax and anti-Bcl-2 antagonist/killer activities, and inducing apoptosis; and ii) p53-induced apoptosis through the transcription-independent pathway, in which p53 translocates to the mitochondria and interacts with Bcl-xL to increase the permeability of the mitochondrial outer membrane and induce cytochrome c release, leading to apoptosis [15].

Subsequent to the release into the cytoplasm from the mitochondria, cytochrome c, apoptotic protease activating factor-1 and a caspase-9 precursor form an apoptosome that activates caspase-9 [14]. The activated caspase-9 then cleaves and activates caspase-3 and PARP, finally inducing apoptosis [13, 14]. In the present study, HCPT-induced SMS-KCNR cell apoptosis was found to be accompanied by significant release of cytochrome c from the mitochondria to the cytoplasm. In order to investigate its downstream apoptotic signal transduction process, the expression of caspase-3 and its substrate, PARP, were analyzed. The results revealed that HCPT significantly reduced pro-caspase-3 expression in SMS-KCNR cells, and significantly increased the expression of the activated 17-kDa cleavage fragment and 89-kDa PARP cleavage fragment in a HCPT concentration-dependent manner. This indicates that caspase-3 is activated during SMS-KCNR cell apoptosis and promotes cell apoptosis by acting on its substrate, PARP.

In conclusion, the presented study identified that HCPT may inhibit proliferation and induce apoptosis in SMS-KCNR cells at a relatively low concentration. The possible mechanism of apoptosis induction is the p53-mediated mitochondrial apoptotic signaling pathway, which promotes cytochrome c release and finally induces apoptosis by activating caspase-3 and PARP. HCPT may therefore provide a novel and effective option for the treatment of high-risk patients with NB.

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