ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Apr；21 (4)：364 – 368
http：//www.chinaphar.com

Differentiation-inducing action of 10-hydroxycamptothecin on human hepatoma Hep G2 cells[1]

ZHANG Xiong-Wen，JIANG Jian-Fei，XU Bin[2] （*Department of Pharmacology，Shanghai Institute of Materia Medica，Chinese Academy of Sciences，Shanghai 200031，China*）

KEY WORDS 10-hydroxycamptothecin；experimental liver neoplasm；cell differentiation；cell cycle；protein p53；immunohistochemistry；proliferating cell nuclear antigens；telomerase；flow cytometry

ABSTRACT

AIM：To study the mechanism of differentiation-inducing action of 10-hydroxycamptothecin (HCPT) on human hepatoma Hep G2 cells. **METHODS**：The proliferating cell nuclear antigen (PCNA) expression was studied by immunocytochemical staining method. The cell cycle distribution and the wild-type protein p53 expression were measured by flow cytometry. Telomerase activity was assayed with telomeric repeat amplification protocol (TRAP). **RESULTS**：After treatment with HCPT at differentiation-inducing concentrations 5 – 20 $\mu g \cdot L^{-1}$ for 6 d，Hep G2 cells were mainly arrested at G2/M phase and the PCNA expression rate was lower than that of control cells. When Hep G2 cells grew in a medium containing HCPT 5 $\mu g \cdot L^{-1}$ for 6 d，the p53 expression level markedly increased in comparison with the control cells. The telomerase activity did not change in Hep G2 cells treated with HCPT 5 – 20 $\mu g \cdot L^{-1}$ for 8 d. **CONCLUSION**：The differentiation-inducing effect of HCPT on Hep G2 cells is related with the cell cycle arrest at G_2/M phase，down-regulation of PCNA and up-regulation of wild-type protein p53.

INTRODUCTION

10-Hydroxycamptothecin (HCPT)，an alkaloid isolated from the Chinese plant *Camptotheca acuminata*，is an anticancer agent in experimental research and clinical study[1]. HCPT could inhibit the activity of DNA topoisomerase I (Topo I)[2]. Our previous study showed that HCPT induced differentiation of murine erythroleukemia cells[3] and HL-60 cells[4]. We have observed the differentiation-induction of HCPT 5 $\mu g \cdot L^{-1}$ on human hepatoma Hep G2 cells. In this work the mechanism of differentiation-induction action of HCPT was investigated.

MATERIALS AND METHODS

Cell culture and drug treatment The human hepatoma Hep G2 cell line was obtained from the American Type Culture Collection. 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，USA) containing 15 % calf serum[5]. For all experiments，cells were treated with various concentrations of HCPT (HCPT injection，5 mg HCPT in 2 mL sterilized water，Huangshi Feiyun Pharmaceutical Co，Hubei，China) at 24 h after plating. Analysis was performed at different time intervals during culture of the cells.

Immunocytochemical assay of proliferating cell nuclear antigen (PCNA) expression After a 6-d treatment with HCPT，cells (1.0×10^5 per well) were collected and smeared on the slides precoated with 0.01 % poly-*L*-lysine (Sigma，St Louis，MO，USA). The PCNA immunoreactivity was detected by the ABC (avidin-biotin-peroxidase complex) method. The primary antibody used was a mouse anti-human PC 10 antibody (DAKO，Denmark) (1∶100) and the secondary antibody used was a biotinylated-antibody rabbit anti-mouse IgG (Shanghai Chinese-American Company，China) (1∶50). Photos were taken and quantitative analysis was conducted with an image analysis system (Leica，German). The experiment was performed at least three times.

Flow cytometric analysis of cell cycle After a 6-d treatment with HCPT，cellular DNA content was detected by flow cytometry via determination of propidium

[1] Project supported by the National Natural Science Foundation of China，№ 39570824.
[2] Correspondence to Prof XU Bin. Phn 86-21-6431-1833，ext 312. Fax 86-21-6437-0269. E-mail bxu@server.shcnc.ac.cn
Received 1999-05-25 Accepted 1999-09-27

iodide (PI)[6]. After trypsinization, cells (1.0×10^6 per sample) were washed with PBS and cell pellets were fixed in 70 % ethanol at 4 ℃ overnight. After being washed twice with PBS, the cells were stained with 1.0 mL of PI solution containing PI (Sigma, St Louis, MO, USA) 50 mg·L^{-1}, RNase A (Sigma, St Louis, MO, USA) 10 mg·L^{-1}, Triton X-100 0.5 % (v/v), and trisodium citrate 0.1 % (w/v) at room temperature in the dark for 30 min before cytofluorometry. The experiment was performed at least three times.

Indirect immunofluorescence assay of p53 expression The protein p53 level in cells was measured by flow cytometry[7]. Each sample contained 1.0×10^6 cells. The primary antibody used was a mouse primary antibody against p53 (DAKO, Denmark) (1∶50) and the secondary antibody used was a FITC-conjugated-antibody rabbit anti-mouse IgG (DAKO, Denmark) (1∶50). The antigen density was measured by Becton Dickson FACStar plus flow cytometer and the percentage of p53 positive cells were recorded. The experiment was performed at least three times.

TRAP assay of telomerase activity The telomerase activity of Hep G2 cells were measured by TRAP[8,9]. Briefly, after exposure to HCPT 5 – 20 μg·L^{-1} for 8 d, cell pellets were resuspended in 3-[(3-cholamidopropyl) dimethyl ammonio]-1-propanesulphonate (CHAPS) based telomerase lysis buffer 200 mL. The suspension was centrifuged at 15 000 × *g* at 4 ℃ for 30 min and the supernatant was used for telomerase assay. The protein concentration was measured by BCA assay (Pierce, USA). Telomerase reaction products were amplified at 29 PCR cycles at 94 ℃ for 30 s and 60 ℃ for 30 s. The PCR products were examined by 10 % nondenature polyacrylamide gel electrophoresis. The experiment was performed at least three times.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed by *t*-test.

RESULTS

Cell cycle distribution of Hep G2 cells The proportions of Hep G2 cells in the different phases of the cell cycle were determined by flow cytometry. Untreated Hep G2 cells demonstrated a relatively normal distribution pattern, with most cells in the G_0/G_1 phase (62.3 %), less cells in S phase (19.8 %) and G_2/M phase (17.8 %). The change of cell cycle distribution of Hep G2 cells treated with different doses of HCPT was shown in Fig 1. HCPT obviously blocked the progression of Hep G2 cells and arrested in G_2/M. After a 6-d treatment of HCPT 5, 10, and 20 μg·L^{-1}, the proportion of G_2/M phase cells was found to be 29.2 %, 50.6 %, and 80.1 %, respectively (Fig 1).

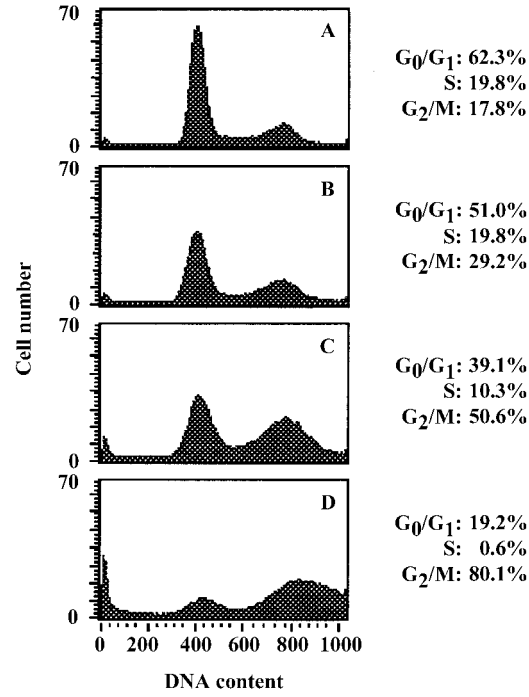

Fig 1. Flow cytometric analysis of the cell cycle distribution of the human hepatoma Hep G2 cells untreated (A) or treated with HCPT 5 μg·L^{-1}(B), 10 μg·L^{-1}(C) and 20 μg·L^{-1}(D) for 6 d.

PCNA expression Using immunocytochemical staining method, positive reaction of PCNA (yellow gains) can be seen in the nuclei of Hep G2 cells. After treatment with HCPT 5 – 20 μg·L^{-1} for 6 d, the positive rate of expression and the mean of grey colour of PCNA in Hep G2 cells were significantly less than those of control cells (Fig 2, Tab 1).

Tab 1. Influence of HCPT on PCNA expression in human hepatoma Hep G2 cells. $^cP < 0.01$ *vs* control. $\bar{x} \pm s$.

Group	*n*	Positive rate/%	Mean grey
Control	292	96	0.32 ± 0.06
HCPT 5 μg·L^{-1}	274	59	0.22 ± 0.08^c
HCPT 20 μg·L^{-1}	287	32	0.23 ± 0.08^c

Protein p53 expression HCPT 5 μg·L^{-1} enhanced the p53 expression in Hep G2 cells. The p53

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Apr；21 (4)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

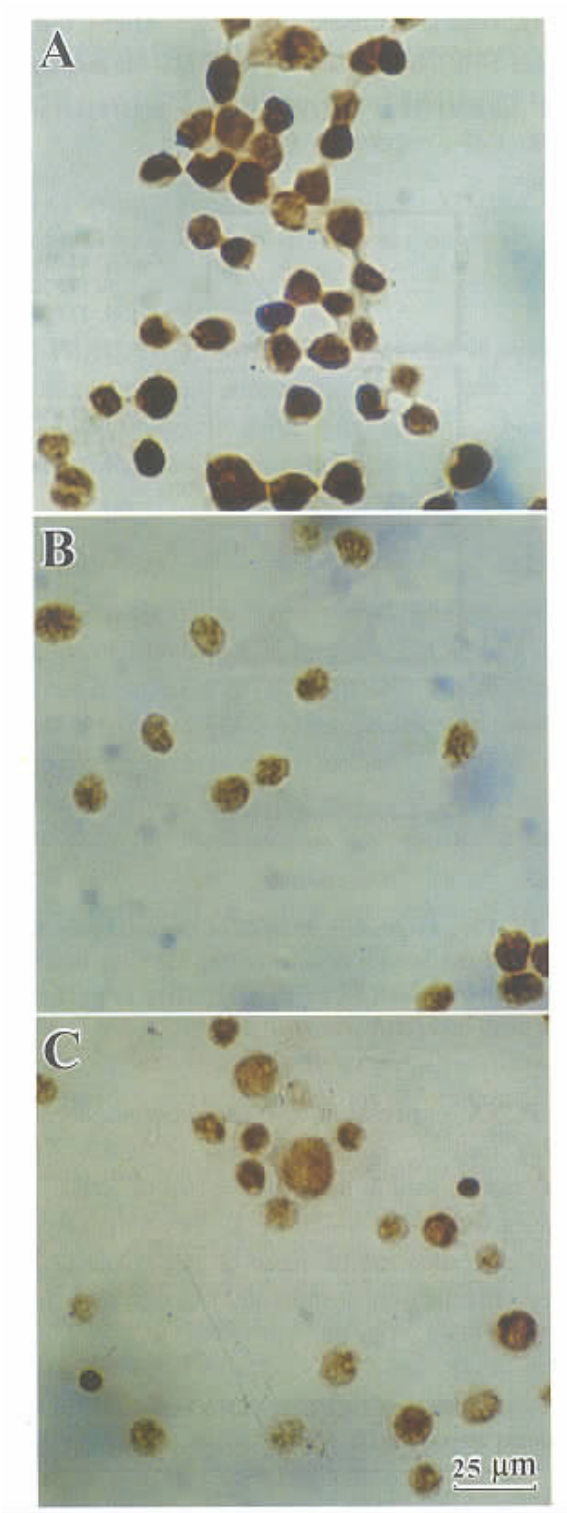

Fig 2. Immunocytochemical detection of proliferating cell nuclear antigen (PCNA) expression in human hepatoma Hep G2 cells untreated (A) or treated with HCPT 5 μg·L^{-1}(B) and HCPT 20 μg·L^{-1}(C) for 6 d. ×400.

positive rate of control cells was 6.8 %. After 2 d, 4 d, and 6 d treatment of HCPT, the p53 positive rate was increased to 30 %, 34 %, and 26 %, respectively (Fig 3).

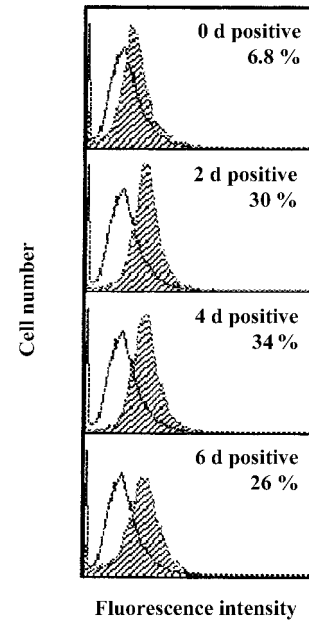

Fig 3. Flow cytometric analysis of p53 expression in human hepatoma Hep G2 cells treated with HCPT 5 μg·L^{-1} for 0 – 6 d. Shaded profiles, cells incubated with anti-p53 antibody. Open profiles, cells incubated with secondary antibody alone.

Effect of HCPT on telomerase activity of Hep G2 cells After incubation with HCPT 5 – 20 μg·L^{-1} for 8 d, the telomerase activity of Hep G2 cells had no significant change (Fig 4).

DISCUSSION

The relationship between the differentiation induction and the cell cycle distribution is not yet clear. It has been reported that different phases of the tumor cells possess different sensitivity to induction of differentiation. Cytarabine was most effective to induce differentiation at the G_1/S phase in K562 cells[10], but Me_2SO at S phase in mouse erythroleukaemia cells[11] and camptothecin at S and M phase in K562 cells[12]. In the present study, we found that HCPT 5 μg·L^{-1} could produce marked differentiation-inducing effect and arrest Hep G2 cells mainly at G_2/M phase.

PCNA is considered as an auxiliary protein of DNA polymerase δ and participates in the DNA replication process of cell proliferation. The expression level of PCNA is related to the proliferation activity of cells and the PCNA positive cells have been reported in G_1 late phase or S early phase cells[13]. In our experiments, HCPT

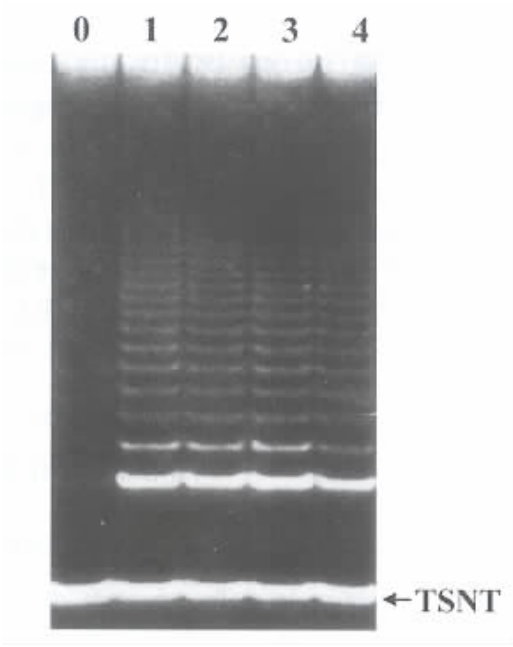

Fig 4. Telomerase activity in human hepatoma Hep G2 cells untreated (lane 1) or treated with HCPT 5, 10, and 20 μg·L^{-1} (lane 2 – 4) for 8 d. Lane 0: Negative control. Five separate experiments were made in duplicate.

decreased the PCNA positive rate of Hep G2 cells suggesting that the proliferation activity of cells was inhibited. Such findings were in agreement with the results in cell cycle assay that HCPT mainly arrested cell cycle at G_2/M phase.

Two kinds of p53, ie, wild-type p53 and mutant p53, have been found. In Hep G2 cells only wild-type p53 was expressed. Wild-type p53 has been shown to be an important transcription factor in inducing differentiation and regulation of cell proliferation. In our study, HCPT enhanced the expression of wild-type p53 in Hep G2 cells. Such enhancement may be related to the proliferation-inhibiting and differentiation-inducing effects of HCPT on hepatoma cells.

In our study, the malignant phenotypes of Hep G2 cells, such as biochemical characteristics and proliferating ability, could be reversed by HCPT 5 μg·L^{-1}, but the morphological malignant phenotype of Hep G2 cells was not reversed. The present work showed that the telomerase activity of Hep G2 cells under the influence of HCPT at differentiation-inducing concentrations was not altered. The possible reason for explanation may be the fact that the Hep G2 cells have not been completely differentiated into mature hepatocytes. Thus, HCPT could not activate the telomerase repressing mechanism which is based on the complete differentiation of Hep G2 cells.

REFERENCES

1 Xu B, Yang JL. Hydroxycamptothecin as an antitumor agent. In: Chang HM, Yeung HW, Tso WW, Koo A, editors. Advances in Chinese medicinal materials research. Singapore: World Scientific Publishing Company; 1985. p 377 – 89.

2 Wall ME, Wani MC. Camptothecin and analogs: from discovery to clinic. In: Potmesol M, Pinedo H, editors. Camptothecins: new anticancer agents. Boca Raton: CRC Press; 1995. p 21 – 9.

3 Xu B. The influence of several anticancer agents on cell proliferation, differentiation and the cell cycle of murine erythroleukemia cells. Am J Chin Med 1982; 9: 268 – 76.

4 Ling YH, Tseng MT, Nelson JA. Differentiation induction of human promyelocytic leukemia cells by 10-hydroxycamptothecin, a DNA topoisomerase I inhibitor. Differentiation 1991; 46: 135 – 41.

5 Zhang XW, Xu B. Study on the growth of tumor cells cultured in serum-free medium. Chin J Oncol 1999; 21: in press.

6 Lou LG, Xu B. α-Anordrin-induced apoptosis of leukemia K562 cells is not prevented by cicloheximide. Acta Pharmacol Sin 1997; 18: 169 – 72.

7 Aiello A, Delia D, Borrello MG, Biassoni D, Giardini R, Fontanella E, *et al*. Flow cytometric detection of the mitochondrial Bcl-2 protein in normal and neoplastic human lymphoid cells. Cytometry 1992; 13: 502 – 9.

8 Broccoli D, Young J. Telomerase activity in normal and malignant hematopoietic cells. Proc Natl Acad Sci USA 1995; 92: 9082 – 6.

9 Kim NW, Piatyszek MA. Specific association of human telomerase activity with immortal cells and cancer. Science 1994; 266: 2011 – 5.

10 Wanda PE, Walker MM. Hemoglobin induction by Ara-C in human erythroleukaemia cells (K562) is cell-cycle dependent. Leukemia Res 1989; 13: 683 – 8.

11 Levy J, Terada M, Rifkind R, Marks P. Induction of erythroid differentiation by dimethylsulphoxide in cells infected with Friendly virus: relationship to cell cycle. Proc Natl Acad Sci USA 1984; 81: 7461 – 5.

12 McSheehy PMJ, Gervasoni M, Lampasona V, Erba E, D'Incalci M. Studies of the differentiation properties of camptothecin in the human leukaemic cells K562. Eur J Cancer 1991; 27: 1406 – 11.

13 Bravo R, Frank R, Blundell PA, Macdonald-Bravo H. Cyclin/PCNA is the auxiliary protein of DNA polymerase δ. Nature 1987; 326: 515 – 7.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Apr；21 (4)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

10-羟基喜树碱对人肝癌 Hep G2 细胞的分化诱导作用[1]

章雄文，蒋建飞，胥　彬[2]（中国科学院上海药物研究所药理室，上海 200031，中国）

关键词　10-羟基喜树碱；实验性肝肿瘤；细胞分化；细胞周期；p53 蛋白；免疫组织化学；增殖细胞核抗原；端粒酶；流式细胞术

目的：研究羟基喜树碱 HCPT 对人肝癌 Hep G2 细胞分化诱导作用的机制．方法：用免疫细胞化学染色检测增殖细胞核抗原表达；流式细胞仪检测细胞周期和野生型 p53 蛋白表达；端粒重复扩增法检测端粒酶活性．结果：以诱导分化剂量（5 – 20 $\mu g \cdot L^{-1}$）的 HCPT 处理 6 d，人肝癌 Hep G2 细胞明显受阻于 G_2/M 期，PCNA 阳性表达率降低．经 HCPT 5 $\mu g \cdot L^{-1}$ 处理 6 d，Hep G2 细胞的野生型 p53 蛋白表达明显增强，但端粒酶活性没有改变．结论：HCPT 诱导人肝癌 Hep G2 细胞分化的作用与细胞阻滞于 G_2/M 期、PCNA 表达降低及野生型 p53 蛋白表达增强有关．

（责任编辑　刘俊娥）