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Anti-hepatoma activity of taxol *in vitro*

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KEY WORDS taxol；hepatocellular carcinoma；cultured tumor cells；cell division；cell cycle；apoptosis

ABSTRACT

AIM：To investigate the *in vitro* anti-hepatoma activity of taxol against SMMC-7721 human hepatoma cells. **METHODS**：The hepatoma cell growth was measured by MTT-microculture tetrazolium assay. Cell-cycle kinetics and apoptosis were analyzed by flow cytometry and microscopic examination. **RESULTS**：Taxol inhibited the hepatoma cell growth in concentration- and time-dependent manners with IC_{50} of 18.96 nmol·L^{-1}. Marked cell accumulation in G_2/M phase and multinucleated cells were also observed after treatment with taxol 10 nmol·L^{-1}. In addition，taxol at 10 nmol·L^{-1} could induce the apoptosis of hepatoma cells. **CONCLUSION**：Taxol suppresses the growth of SMMC-7721 human hepatoma cells *in vitro* by causing cell-cycle arrest，aberrant mitosis，and apoptosis of the human hepatoma cells.

INTRODUCTION

Taxol，a complex diterpene with a taxane ring system，was discovered in 1971 while screening for anticancer drugs. The microtubule，one of the fundamental structures comprising the cytoskeleton of eukaryotic cells，is involved in such diverse cellular processes as cell division，locomotion，intracellular transport，and maintenance of cell shape[1]. Taxol has been proved to be an inhibitor of microtubule depolymerization in tumor cells. Clinical investigations have also indicated that taxol exhibits a significant antineoplastic activity against various tumors，especially ovarian，pulmonary，and mammary carcinomas[2].

To date，however，only a few chemotherapeutic drugs hold a high place in the treatment of human primary hepatocellular carcinoma (PHC) and there is clearly a need for evaluation of new anti-hepatoma drugs. Particularly，it has still not been established whether taxol is effective against PHC. The previous experimental studies suggest that though taxol alone appears to be potently cytotoxic against hepatoma a combined application of taxol with other chemotherapeutic drugs might show a better hepatoma growth inhibitory effect[3,4]. At present，phase Ⅰ and Ⅱ clinical trials of taxol against liver cancer have also been carried out in patients suffering from unresectable PHC，demonstrating beneficial therapeutic efficiency with mild side-effects[5,6]. PHC is one of the most common lethal diseases in China. In the present study，we examined the effect of taxol on the growth of SMMC-7721 human hepatoma cells *in vitro* and its action mechanisms. Our results confirm and extend the previous studies.

MATERIAL AND METHODS

Cell line and *in vitro* culture SMMC-7721 human liver carcinoma cell line was obtained from Chinese Type Culture Collection (Shanghai Institute of Cell Biology，Chinese Academy of Sciences，Shanghai，China)[7]. The cells were grown on monolayer cultures in 37 ℃，5 % CO_2 incubator in RPMI-1640 media (Gibco，Grand Island，NY) supplemented with 15 % heat-inactivated new-born calf serum and passaged at intervals of 2－3 d.

Drug Taxol (Shanghai Hualian Pharmaceutical Factory，Shanghai，China) was dissolved in dimethyl sulfoxide (Me_2SO) to make a stock solution，which was then diluted as desired with the culture media. The Me_2SO concentration was kept under 0.001 % in all the experiments and did not exert any detectable effect on cell growth or apoptosis.

MTT-microculture tetrazolium assay For assay of cell growth，trypsinized cells were dispensed into each well of Costar flat-bottom culture plates (Costar，

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Charlotte，NC). After a 24-h culture，taxol was added into each well according to the desired concentrations. Following incubation，cell growth was measured by MTT microculture tetrazolium assay[8]. Briefly，the cells were incubated with MTT 500 mg·L^{-1} for 4 h. Then，the supernatant was carefully aspirated，150 μL of Me_2SO was added to each well to dissolve the MTT-formazan product. Absorbance at 570 nm (A_{570}) was measured with an enzyme-linked immunosorbent assay plate reader.

Microscopic observation Cells were fixed with 2.5 % glutaraldehyde，and postfixed with 2 % osmium tetroxide. After dehydration，the samples were embedded in Epon 812 epoxydic resin，and then sectioned. The sections were routinely stained and examined by electron microscope. When the cells were observed with fluorescence microscope，the cells were fixed with 70 % ethanol for 30 min. After wash，the cell pellets were gently resuspended in 100 μL of a solution containing propidium iodide (PI，50 mg·L^{-1}) and RNase A (50 kU·L^{-1}). Twenty minutes later，the cells were observed under the fluorescence microscope.

Flow cytometry analysis The cells were resuspended in a solution containing sodium citrate 40 mmol·L^{-1}，sucrose 250 mmol·L^{-1}，and 5 % Me_2SO. The suspension was stored at − 20 ℃ for 20 min，then thawed rapidly at room temperature and centrifuged to collect the cells. The cells were resuspended in a solution containing RNase A 30 kU·L^{-1}，0.1 % Triton X-100，and PI 50 mg·L^{-1} at 37 ℃ for 10 min. Finally，the cells were analyzed by flow cytometry[9].

Data analysis Growth data were expressed as $\bar{x} \pm s$ and compared by t-test.

RESULTS

Growth inhibition of SMMC-7721 cells SMMC-7721 cells at 1 × 10^8 cells·L^{-1} were incubated with different concentrations of taxol for 72 h and the effect of taxol on the cell growth was examined by MTT assay. The growth of SMMC-7721 cells was markedly inhibited by taxol with the IC_{50} value of 18.96 nmol·L^{-1} (Tab 1). Moreover，the cytotoxicity of taxol was concentration-dependent. With taxol 1 nmol·L^{-1} treatment，however，no inhibition was found. The time-effect analysis of taxol (10 nmol·L^{-1}) showed that its cytotoxicity against the human hepatoma cells (2 × 10^7 cells·L^{-1}) was in a time-dependent manner (Tab 2).

Tab 1. Effect of 72 h-taxol treatment on the growth of SMMC-7721 human liver cancer cells (1 × 10^8 cells·L^{-1}) *in vitro*. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$，$^bP < 0.05$，$^cP < 0.01$ *vs* 0 nmol·L^{-1} group.

Concentration/ nmol·L^{-1}	Cell growth/ A_{570}	Inhibition/ %
0	1.023 ± 0.054	
1	1.073 ± 0.045^a	− 5
3	0.896 ± 0.014^b	12
5	0.796 ± 0.023^c	22
10	0.576 ± 0.019^c	44
20	0.438 ± 0.038^c	57

Tab 2. Time-effect analysis of taxol (10 nmol·L^{-1})-mediated growth inhibition of SMMC-7721 human hepatoma cells (2 × 10^7 cells·L^{-1}). $n = 3$. $\bar{x} \pm s$. $^bP < 0.05$，$^cP < 0.01$ *vs* control group.

Group	Time/ d	Cell growth/ A_{570}	Inhibition/ %
Control	2	0.199 ± 0.021	
Taxol	2	0.164 ± 0.015^b	18
Control	4	0.616 ± 0.042	
Taxol	4	0.283 ± 0.021^c	54
Control	6	0.778 ± 0.027	
Taxol	6	0.096 ± 0.019^c	87

Effect on cell-cycle kinetics of SMMC-7721 cells Cell-cycle kinetics of SMMC-7721 cells was analyzed by flow cytometry. As demonstrated in Fig 1，taxol at 10 nmol·L^{-1} caused an accumulation of the cells in G_2/M phase after 4 h，which peaked at 12 − 24 h with about 55 % − 59 % of the cells in G_2/M phase compared with about 17 % at baseline，and decreased thereafter.

Morphologic observation further confirmed taxol-mediated cell-cycle arrest in SMMC-7721 cells. The results from light microscope indicated that taxol at 1 nmol·L^{-1} caused no obvious morphologic changes of SMMC-7721 cells when compared with untreated cells. But，significant changes were observed after treatment with taxol 3 nmol·L^{-1}. The cells became round，diopter-enhanced，detached，and blebbed (photos not shown). When the cells were exposed to taxol 10 nmol·L^{-1} for 24 h，significant cell-cycle arrest was also observed with fluorescence microscope (Fig 2b) as compared with the untreated cells (Fig 2a). Coexisting multinucleated cells were found as well (Fig 2c). Similar results were obtained by electron microscope (photos not shown). These findings indicate that taxol causes cell-cycle arrest and aberrant mitosis of SMMC-7721 human hepatoma cells.

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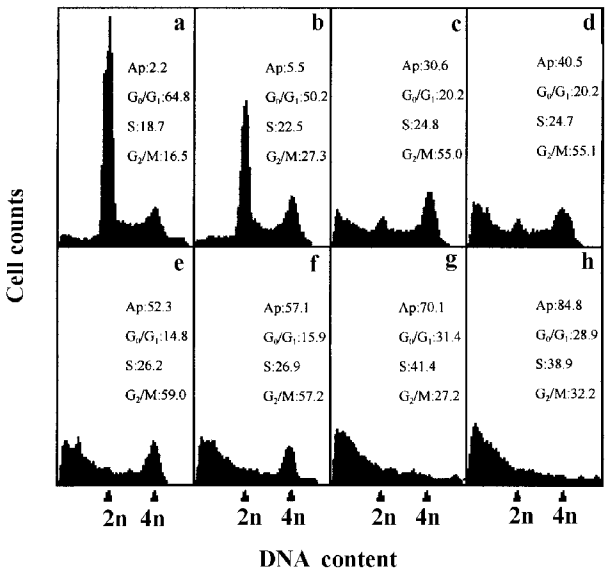

Fig 1. Cell-cycle changes and apoptotic induction in SMMC-7721 cells treated with taxol. Cells were treated with taxol 10 nmol·L^{-1} for 0 h (a), 4 h (b), 12 h (c), 16 h (d), 20 h (e), 24 h (f), 48 h (g), and 72 h (h). Ap: apoptotic cells.

Induction of SMMC-7721 cell apoptosis As analyzed by flow cytometry (Fig 1), taxol could induce the apoptosis of SMMC-7721 cells. Apoptosis appeared after the cells were exposed to taxol 10 nmol·L^{-1} for 12 h with 57.1 % of apoptotic cells appearing at 24 h and 84.8 % at 72 h compared with 2.2 % at baseline. Under the fluorescence microscope, the majority of the cells displayed shrinkage after treatment with taxol 10 nmol·L^{-1} for 48 h. The nuclei of some cells exhibited large hypercondensed chromatin masses, some cells formed apoptotic bodies (Fig 2d). On the contrary, the untreated cells did not show these apoptotic characteristics (Fig 2a). The results from electron microscopy were similar to those mentioned above. After treatment with taxol 10 nmol·L^{-1} for 48 h, the chromatin of SMMC-7721 cells was located along the nuclear envelope, or formed irregularly shaped crescents at the nuclear edges (photos not shown). Together with the above-mentioned observations, that 24-h treatment with the drug caused obvious cell-cycle arrest and aberrant mitosis, it is suggested that taxol-induced apoptosis occurs later than changes in cell-cycle kinetics.

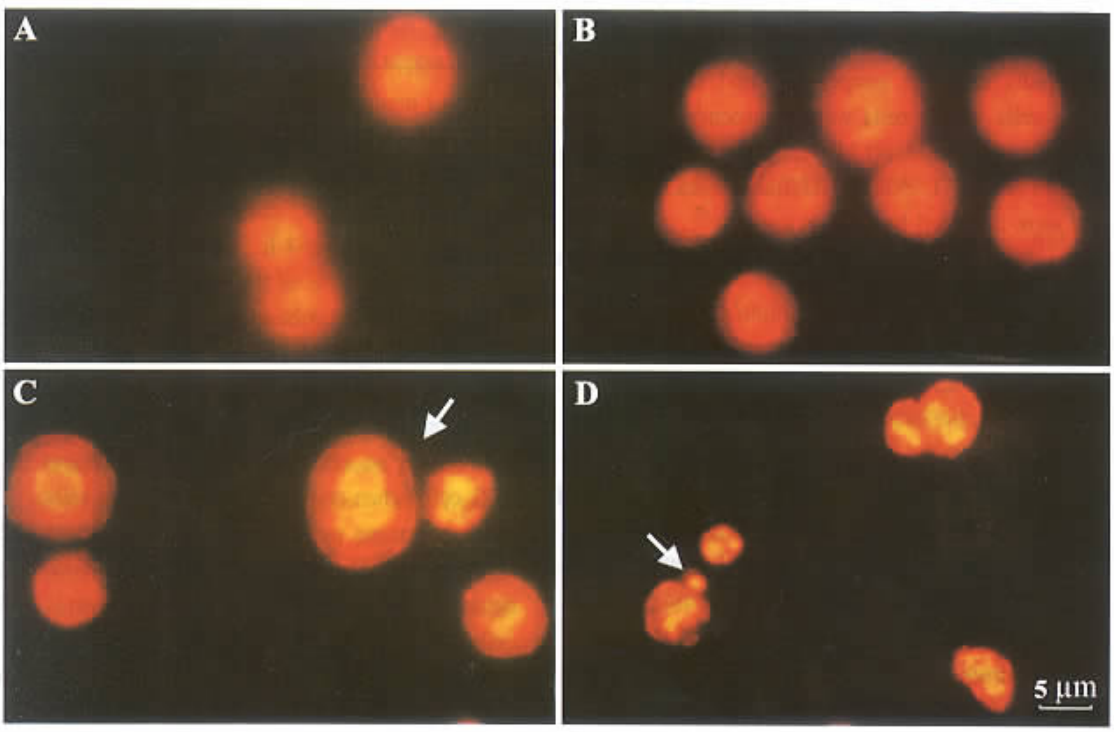

Fig 2. Effect of taxol on mitosis and apoptosis of SMMC-7721 cells. With the treatment of taxol 10 nmol·L^{-1} for 24 h, mitotic arrest (b) and coexisting multinucleated cells (c, arrow) were observed. With the treatment of taxol 10 nmol·L^{-1} for 48 h, chromatin condensation and apoptotic bodies (d, arrow) were observed. The taxol-untreated cells exhibited no obvious morphologic changes (a). ×400.

DISCUSSION

In the present study, MTT assay was used to observe the effect of taxol on the growth of SMMC-7721 human hepatoma cells *in vitro*, indicating that the drug could inhibit the hepatoma cell growth. Its concentration- and time-effect relationships were also significant. Moreover, regarding the MTT assay, measurements of taxol-induced human hepatoma cell growth inhibition were comparable to those obtained with viable cell count and [^{3}H]-TdR incorporation (data not shown). Previous studies have also demonstrated that taxol could be cytotoxic against other human hepatoma cell lines, such as HuH-7, HepG2, Hep3B, and BEL-7404[3,10,11], suggesting that the growth-inhibiting effect of taxol was not specific for SMMC-7721 human hepatoma cells.

In order to elucidate the mechanisms of the anti-hepatoma action of taxol, we examined its possible effect on cell-cycle kinetics of SMMC-7721 cells. Following the treatment with taxol, two major patterns of cell-cycle perturbation, ie, mitotic arrest and aberrant mitosis, were observed. The most striking feature was more than 3-fold accumulation of cycling cells in the G_2/M phase after taxol treatment. Moreover, coexisting multinucleated cells occurred as well when the cells were treated with taxol. Studies with non-hepatoma tumor cells treated with taxol also demonstrated that the drug could block cell-cycle traverse in the G_2/M phase and caused cell multinucleation, a typical morphological characteristics of aberrant mitosis[12]. In clinical practice, such information might be useful for themotherapy of liver cancer with taxol and other cytotoxic drugs which affect cell-cycle progression.

Recently, apoptosis, a genetically determined form of cell death, has forced a reevaluation of the mechanisms by which cytotoxic agents inhibit tumor growth[13]. Some researchers have demonstrated that the antitumor effect of taxol is associated with its apoptotic induction[13-15]. Apoptosis can be triggered by either premature or delayed cell-cycle progression[16]. However, there exists a controversy over the relationship between cell-cycle arrest and apoptosis caused by taxol[17-19]. Our studies indicate that taxol-mediated mitotic arrest and aberrant mitosis occur earlier than apoptosis in SMMC-7721 human hepatoma cells. As compared with the untreated cells (Fig 2a), treatment with taxol for 24 h caused obvious cell-cycle arrest (Fig 2b) and aberrant mitosis (Fig 2c), whereas until 48 h after taxol treatment, the majority of the cells did not display typical apoptotic changes (Fig 2d). The results from flow cytometry analysis also further demonstrated that appearance of apoptosis was a later event following mitotic blockade and aberrant mitosis (Fig 1). In addition, we also detected the DNA fragmentation by gel electrophoresis after taxol treatment of SMMC-7721 cells, however no positive DNA ladder was found. Although we can not offer any explanation for this, apoptosis occurred without DNA laddering[20].

It is at metaphase-anaphase cell-cycle transition that the spindle checkpoint monitors the assembly of the mitotic spindle and the bipolar attachment of chromosomes to spindle microtubules. If defects are apparent, a signal must be generated and transduced, bringing about a delay in cell-cycle progression and further apoptosis[21,22]. On the other hand, $p34^{cdc2}$-cyclin B complexes could catalyze chromosomal condensation and nuclear envelope breakdown during mitosis. Activation of $p34^{cdc2}$-cyclin B1 kinase is required for taxol-induced G_2/M arrest and apoptosis in breast cancer cells[23]. Thus, the molecules related to cell-cycle checkpoint including cyclins and cyclin-dependent kinases will be one of our research focuses in the near future.

In conclusion, the present *in vitro* study indicates that taxol exhibits marked anti-hepatoma activity against SMMC-7721 human hepatoma cells. Its G_2/M phase arrest and aberrant mitosis as well as apoptotic induction may contribute to the human hepatoma cell growth inhibition. Moreover, at least two distinct mechanisms, ie, cell-cycle arrest and aberrant mitosis, are responsible for taxol-induced human hepatoma cell apoptosis, although the exact mechanisms are still unclear. These *in vitro* results provide the bases of further experiments *in vivo*.

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紫杉醇体外抗肝癌活性

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关键词　紫杉醇；肝细胞癌；培养的肿瘤细胞；细胞分裂；细胞周期；细胞凋亡

目的：研究紫杉醇在体外对 SMMC-7721 人肝癌细胞的抑制作用及其机理. **方法**：用 MTT 比色法测定细胞生长. 用流式细胞仪及显微镜观察进行细胞周期动力学和凋亡的分析. **结果**：紫杉醇抑制人肝癌细胞生长的 IC_{50}为 18.96 nmol·L^{-1}，并有一定的浓度和时间依赖关系. 经紫杉醇 10 nmol·L^{-1}处理后细胞积聚在 G_2/M 期并发现明显的凋亡，形态学观察可见多个多核细胞. **结论**：紫杉醇通过引起细胞周期阻滞、异常的有丝分裂以及细胞凋亡抑制人肝癌细胞生长.

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