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Effect of polypeptides in bee venom on growth inhibition and apoptosis induction of the human hepatoma cell line SMMC-7721 in-vitro and Balb/c nude mice in-vivo

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Abstract

Polypeptides in bee venom (PBV) produced a significant growth inhibition against SMMC-7721 human hepatoma cell line. Analysis of the mechanisms of cell death indicated that PBV induced an apoptotic cell death. SMMC-7721 cells exposed to PBV (10.0 μg mL^{-1}) produced an insignificant morphological change. Analysis of the cytotoxicity with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay confirmed that the cytotoxic effects of PBV were dose- and time-dependent. The result of Ki67 immunohistochemistry demonstrated that the proliferation of SMMC-7721 cells treated with PBV (10.0 μg mL^{-1}) was inhibited. The apoptotic cell death was then confirmed by annexin V, propidium iodide staining and DNA fragmentation analysis. In in-vivo experiments, treatment with PBV (1.5 or 3 mg kg^{-1}) resulted in a significant retardation of SMMC-7721 cell growth in Balb/c nude mice. These findings suggested that PBV could be used as a chemotherapeutic agent against tumours.

Introduction

Polypeptides in bee venom (PBV), a traditional Chinese medicine, have been used for the treatment of inflammatory diseases such as rheumatoid arthritis. During the past two decades, peptides in bee venom containing melittin, as the PBV major protein component, have attracted considerable attention for their potential use in cancer therapy (Kang et al 1998; Shin et al 1999; Leuschner et al 2003; Liu et al 2002; Orsolic et al 2003; Ling et al 2004). Chueng (1982) showed that melittin was capable of binding to calmodulin, which played a key role in cellular proliferation. Gest & Salomon (1987) found that melittin inhibited the melanotropin receptor in M2R melanoma cell membranes. Studies demonstrated that melittin could induce an apoptotic cell death (Vento et al 2000; Arioka et al 2005; Hong et al 2005) and possessed anti-tumour effects (Winder et al 1998). However, other results suggested that the pore-forming agents killed malignant cells by necrosis (Duke et al 1994; Shaposhnikova et al 1997).

Apoptosis, a process of central importance in the prevention of tumour development (Kerr et al 1994; Czene et al 2002), is a programmed cell death mechanism serving homeostatic functions (Woodle & Kulkarni 1998). Apoptosis has been characterized by morphological changes (Romeroa et al 2003), DNA fragmentation, chromatin condensation and fragmentation of the nuclear membrane, plasma membrane blebbing and phosphatidylserine residue exposure. It has been shown in several studies that the process of apoptosis is regulated by the expression of several proteins such as the bcl-2 and caspase-3 (Korsmeyer 1999; Wooa et al 2004; Yang et al 2004). Apoptosis is related to cell immortality and carcinogenesis, thus, the induction of apoptosis in neoplastic cells is important in cancer treatment (Kamesaki 1998).

We have studied the cytotoxicity and Ki67 proliferation index on SMMC-7721 cells. The presence of apoptotic cell death was studied with FITC-annexin V, propidium iodide staining and DNA fragmentation analysis. The therapeutic effect of PBV in-vivo was studied using Balb/c nude mice.

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Materials and Methods

Reagents

Polypeptides in bee venom (PBV, melittin = 90%) were extracted in our laboratory. Anti-Ki67 monoclonal antibody (MIB-1) was obtained from Zymed Ltd (USA). FITC-annexin V was obtained from Calbiochem (Darmstadt, Germany). Nude Balb/c mice were purchased from Institute of Biochemistry and Cell Biology (Shanghai, China). Chemicals for cell culture were obtained from Gibco BRL Technologies Ltd (Paisley, UK). Propidium iodide (PI), RNase A, Vectastain Elite ABC peroxidase mouse IgG kit and diamino-benzidine (DAB) were purchased from Sigma Chemical Co. (St Louis, MO, USA), respectively.

Cell culture

The human hepatoma cell line SMMC-7721 was cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum at 37 °C (5% CO_2, 95% O_2) in a humidified cell incubator. The cells were seeded every other day and used at the logarithmic phase.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay

PBV was extracted from bee venom on a Sephadex G50 column (1.6 × 90 cm, eluted with 0.1 M formate buffer, pH 4.5) and a SP Sephadex C-25 column (3 × 30 cm, eluted with 10 mM Tris buffer, pH 7.5, 0–0.3 M NaCl). The content of melittin in PBV was evaluated with Nova Park C_{18} column (3.9 mm × 150 mm, 5 μm i.d. Waters). The mobile phase consisted of solution A (H_2O, 0.1% TFA, pH 2, 90%~0%) and solution B (CH_3OH, 0.1%TFA, 10%~100%). The flow rate was 1 mL min^{-1} and the detection wavelength was 214 nm. The limit of detection was 1 $\mu g\,mL^{-1}$. The cytotoxicity of PBV on SMMC-7721 cells was determined with the MTT assay six times. SMMC-7721cells (1×10^4) were seeded in 96-well plates for 24 h, and then incubated with the desired amount of PBV and phosphate-buffered saline (PBS) of an equal volume for 24 h. At the end of the incubation, PBV was removed, and cells were incubated for a further 4 h with 50 μL MTT (5 mg mL^{-1}). After incubation, 200 μL dimethylsulfoxide was added to dissolve the crystals. The plates were read immediately with a test wavelength of 490 nm.

Morphological changes induced by PBV

SMCC-7721 cells (1×10^5) were incubated with PBV (10.0 $\mu g\,mL^{-1}$) for 8 h. The supernatant was carefully aspirated, and SMCC-7721 cells were fixed with ethanol/acetone (1:1, v/v) and stained with haematoxylin and eosin (HE assay). The stained cells were observed on a Nikon ECLIPSE E400 microscope (Nikon Corporation, Tokyo, Japan).

Immunohistochemistry for Ki67

SMCC-7721 cells (1×10^5) were incubated with PBV (10.0 $\mu g\,mL^{-1}$) for 8 h. The supernatant was carefully aspirated, and SMCC-7721 cells were fixed with ethanol/acetone (1:1, v/v). Cell proliferation was evaluated by an immunohistochemistry assay as described by Lopez-Beltrana et al (2004). Briefly, cells were incubated for 1 h with an anti-Ki67 monoclonal antibody. The staining was visible using the Vectastain Elite ABC peroxidase mouse IgG kit and diaminobenzidine (DAB). The stained cells were evaluated on an ECLIPSE E400 microscope (Nikon Corporation, Tokyo, Japan). All nuclei with homogeneous staining, or only a nucleolar staining, were interpreted as positive. The proliferation index was evaluated by estimating the percentage of positive neoplastic nuclei within the area of 1000 cells, the prognostic value in cancer.

Cell cycle analysis

SMMC-7721 cells (1×10^6) were treated with PBV (10.0 $\mu g\,L^{-1}$) for 8 h. Cell cycle was analysed on a flow cytometer. Briefly, after washing with cold PBS twice, cells were fixed in 70% cold ethanol for 30 min. PI solution (100 μL 50 $\mu g\,mL^{-1}$ PI, 0.05 mg mL^{-1} RNase A) was added, and the cells were incubated at room temperature for 2 h in darkness. DNA analysis was performed on an ELITE ESP flow cytometer (Beckman-Coulter Inc., USA).

Annexin V binding

SMMC-7721 cells (5×10^5) were treated with PBV (10.0 $\mu g\,L^{-1}$) for 8 h. Cells were fixed in 70% cold ethanol for 30 min, and then washed with PBS. Labelling of cells with FITC-annexin V was performed as described by Liao & Lieu (2005). After centrifugation, SMMC-7721 cells were incubated with 0.5 $\mu g\,mL^{-1}$ FITC-annexin V and 50 $\mu g\,mL^{-1}$ PI for 25 min at room temperature in darkness. Apoptotic cells were evaluated on an ELITE ESP flow cytometer (Beckman-Coulter Inc., USA).

DNA fragmentation assay

SMMC 7721 cells (1×10^6) were treated with PBV (10.0 $\mu g\,mL^{-1}$) for 12 h and collected by centrifugation at 800 *g* for 5 min. The cells were suspended in 10 mM Tris ((pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton X-100), and kept at 4°C for 30 min. The supernatant was incubated with 20 mg mL^{-1} RNase A (2 μL) and 20 mg mL^{-1} proteinase K (2 μL) at 37 °C for 1 h, and then centrifuged at 12 000 *g* for 15 min. DNA was dissolved in TE buffer (10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0)), and then subjected to 1.5% agarose gel electrophoresis.

In-vivo therapeutic studies

Twenty-five nude Balb/c mice were bred in the laboratory of SPF class 2, and used when they were six-weeks old. SMMC-7721 cells (200 μL, 5×10^6) in log phase growth were implanted into the backs of the animals. When tumour

diameter reached 0.5 cm, the mice were randomly divided into five groups of five mice each. Mice were administered with PBV (0.75, 1.5, 3 mg kg^{-1}, i.p.), doxorubicin (1.5 mg kg^{-1}, i.p.) or PBS each day. Tumour diameters were measured each day. Tumour volume was determined using the method of Williams et al (1993). The mice were killed on the twelfth day after treatment, and the local tumours were removed carefully. Tumour diameter and net weight were measured. Relative tumour inhibition was calculated.

Statistical analysis

The IC50 value of PBV (the concentration required for 50% inhibition) was obtained using the nonlinear Hill equation with program Kaleida Graph (Synergy Software, Reading, PA, USA). Comparison of cytotoxicity and therapeutic efficacies were performed using a one-way analysis of variance (SPSS 12.0). Data was presented as mean values with the standard deviation (mean ± s.d.), and *P*-values less than 0.05 were considered significant.

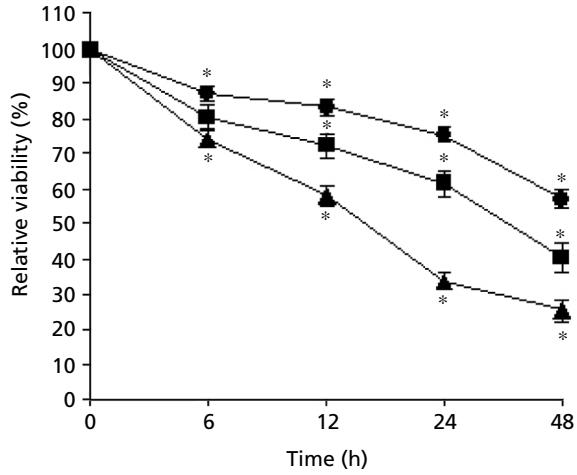


Figure 2 Time-dependent effect of PBV (●) 1; (■) 5; (▲) 10 (μg mL^{-1}) on SMMC-7721 cell viability. Viability was determined via the MTT assay. Data were expressed as mean ± s.d., n = 6. *$P < 0.05$, compared with the control group.

Results

In-vitro cytotoxicity of PBV

SMMC-7721 cells were treated with various concentrations of PBV and the cell viability was determined with the MTT assay. After 24-h incubation, PBV caused concentration-dependent growth inhibition in SMMC-7721 cells with an IC50 value of 6.68 μg mL^{-1} (Figure 1). A trend of increasing cytotoxicity with increasing incubation time was then observed (Figure 2). The growth of SMMC-7721 cells was significantly inhibited by PBV ($P < 0.05$). The viabilities of cells incubated with PBV 10.0 μg mL^{-1} for 8 h was 67.3% of the control value.

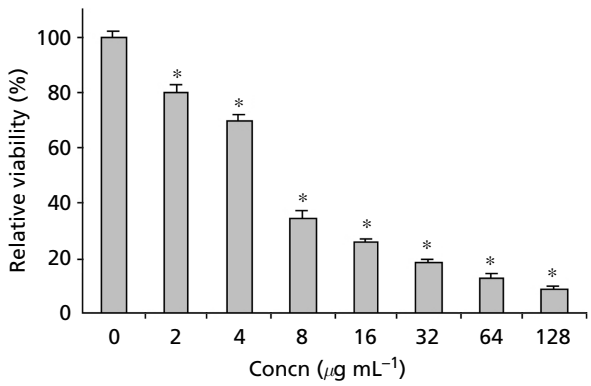


Figure 1 Concentration-dependent effect of PBV on SMMC-7721 cell viability. Viability was determined via the MTT assay. Data were expressed as mean ± s.d., n = 6. *$P < 0.05$, compared with the control group.

Morphological changes induced by PBV

To observe the effect of PBV on cell morphology, SMMC-7721 cells were stained with HE assay and examined via microscopy. Normal SMMC-7721 cells showed a subconfluence and formed a sheet of polygonal cells (Figure 3A). Morphological changes of SMMC-7721 cells were induced by treating with PBV (10.0 μg mL^{-1}) for 8 h (Figure 3B). SMMC-7721 cells lost their original shape and there was an increase in the percentage of rounded cells with progressive nuclear shrinkage. SMMC-7721 cells treated for 24 h showed an even greater loss of their original morphology, and the density of chromosomes increased (Figure 3C).

Immunohistochemistry for Ki67

Cell proliferation was evaluated by immunohistochemistry assay. Ki67 was overexpressed in SMMC-7721 cells, and the mean proliferation index was 97.0% (Figure 4A). PBV could inhibit the proliferation of SMMC-7721 cells significantly, and a significant decrease in the value of the proliferation index was observed (Figure 4B). The mean proliferation index of the treated cells was 10.2%, and only a few nucleoli were stained.

Cell cycle analysis

SMMC-7721 cells were incubated with PBV (10.0 μg L^{-1}) for 8 h, and the cell cycle was analysed on a flow cytometer. As shown in Table 1, there was a significant decrease in the size of cell populations in the G2/M phase and S phase (from 15.5% to 12.5% and from 18.4% to 2.9%, respectively). There was a marked increase in the size of cell populations in the G0/G1 phase (from 66.2% to 84.6%). Thus, the antiproliferative effect of PBV was partly attributable to the induction of cell cycle arrest at the G0/G1 phase of the cell cycle.

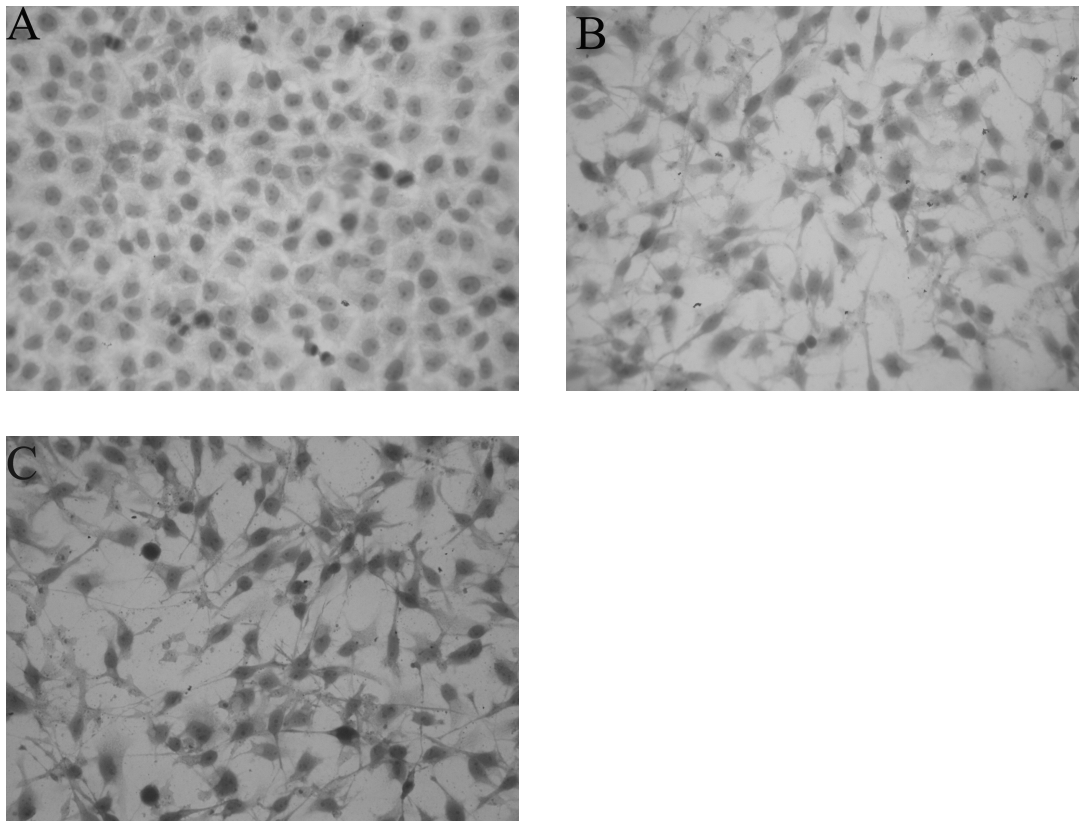


Figure 3 Morphological changes in SMMC-7721 cells stained by HE assay (100×). A. Control cells; B. SMMC-7721 cells treated with PBV (10.0 μg mL^{-1}) for 8 h; C. SMMC-7721 cells treated with PBV (10.0 μg mL^{-1}) for 24 h.

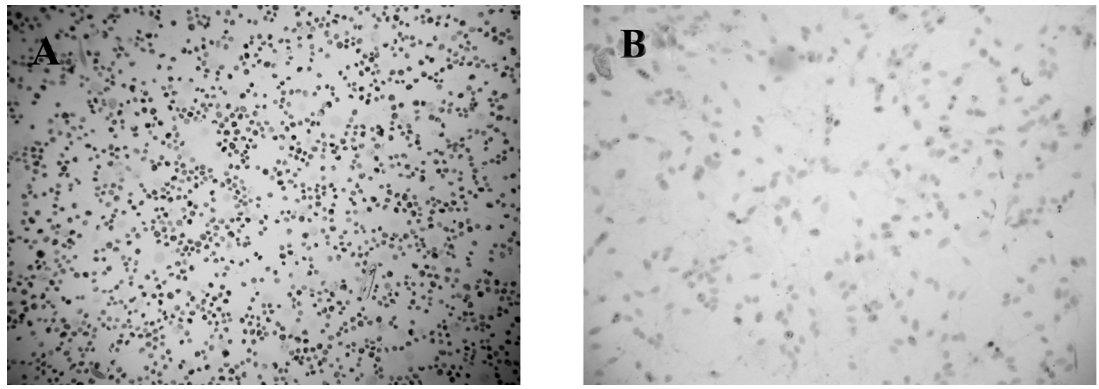
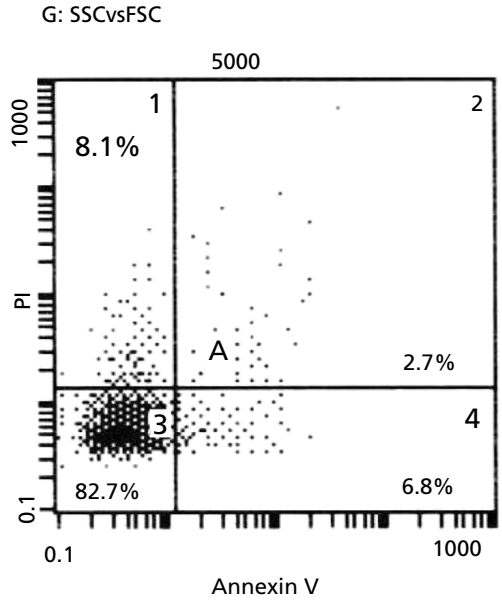


Figure 4 Immunohistochemistry for Ki67 in SMMC-7721 cells (40×). A. Control cells; B. SMMC-7721 cells treated with PBV (10.0 μg mL^{-1}) for 8 h.

Table 1 Effect of PBV (10.0 μg L^{-1}, incubated for 8 h) on the cell cycle of SMMC-7721 cells

	G0/G1 (%)	G2/M (%)	S (%)
Control	66.2 ± 1.4	15.54 ± 1.1	18.4 ± 1.2
PBV	84.6 ± 1.7*	12.5 ± 0.8*	2.9 ± 0.4*

Data were expressed as mean ± s.d., n = 3. *P < 0.05 compared with control.



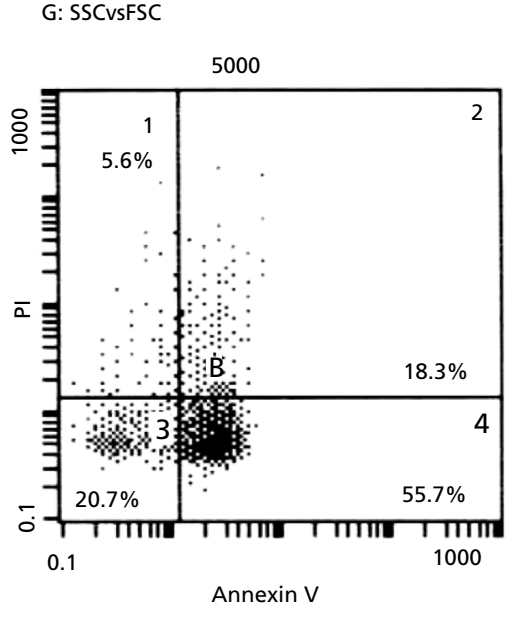


Figure 5 Effect of PBV on the apoptotic population of SMMC-7721 cells discriminated by FITC-annexin V and propidium iodide. A. Control; B. SMMC-7721 cells treated with PBV (10.0 μg mL^{-1}) for 8 h.

Annexin V binding

An in-vitro model of apoptosis was set up by SMMC-7721 cells treated with PBV and analysed for FITC-annexin V and PI binding on a flow cytometry. Flow cytometry analysis of SMMC-7721 cells revealed that an apoptotic population in PBV-treated cells (Figure 5B) was significantly greater than that in untreated cells (Figure 5A). The amount of apoptotic cells in treated cells was 8.1-times greater compared with untreated cells.

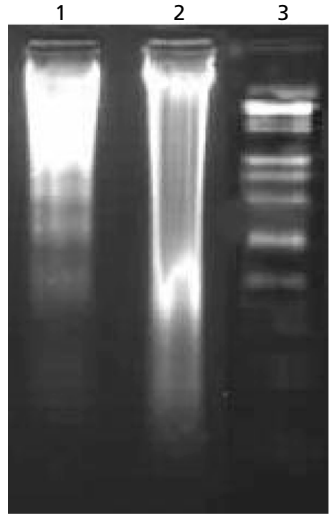


Figure 6 Electrophoretic analysis of genomic DNA of SMMC-7721 cells treated with PBV (10.0 μg mL^{-1}). Cells incubated for 12 h without PBV served as control. The genomic DNA was analysed by electrophoresis on a 1.5% agarose gel containing ethidium bromide. 1, PBV treated cells; 2, control; 3, marker.

DNA fragmentation analysis of apoptosis

To confirm the induction of apoptosis by PBV on SMMC-7721 cells, DNA fragmentation was analysed. As seen in Figure 6, treatment with PBV (10.0 μg L^{-1}) for 12 h resulted in the formation of definite fragments that could be seen via electrophoretic examination as a characteristic ladder pattern.

In-vivo therapeutic studies

Using the implanted SMMC-7721 cells, the anti-tumour efficacy of PBV was evaluated. Treatment with PBV or doxorubicin inhibited primary tumour growth compared with control group (Table 2). The relative tumour inhibition obtained with PBV (0.75, 1.5, 3 mg kg^{-1}) was 18.7%, 31.4% and 48.2%, respectively. There was no significant difference in tumour inhibition between PBV and doxorubicin at the same concentration, or between the PBV groups of 0.75 and 1.5 mg kg^{-1} ($P > 0.05$, post hoc tests). Furthermore, the tumour volume after treatment with PBV (3 mg kg^{-1}) was significantly reduced compared with other groups ($P < 0.05$, post hoc tests), as shown in Figure 7. During the experiment, physiological behaviour, appetite and weight of mice were not disturbed.

Discussion

The purpose of this study was to find the molecular mechanism and the anti-tumour effects of PBV on the hepatoma cell line SMMC-7721, simultaneously. It was demonstrated that PBV had a cytostatic effect on SMMC-7721 cells, inhibited proliferation of SMMC-7721 cells and induced apoptotic cell death.

Analysis of cytotoxicity with the MTT assay confirmed that the cytotoxic effects of PBV were dose- and time-dependent. As the concentration of PBV increased, the

Table 2 The effect of PBV on the growth of SMMC-7721 cells on Balb/c mice

PBV (mg kg^{-1})	Number of mice		Body weight (g)		Tumour weight (g)	Inhibition (%)
	Beginning	End	Beginning	End		
Control	5	5	20.14 ± 1.2	22.81 ± 0.82	0.58 ± 0.11	—
0.75	5	5	20.11 ± 1.14	21.28 ± 1.98	0.47 ± 0.10*	18.7 ± 2.50
1.5	5	5	19.34 ± 1.58	20.14 ± 2.63	0.40 ± 0.073*	31.4 ± 1.32
3	5	5	19.51 ± 1.23	21.41 ± 0.79	0.30 ± 0.089*	48.2 ± 1.66
Doxorubicin (1.5 mg kg^{-1})	5	5	20.42 ± 1.47	21.17 ± 1.04	0.46 ± 0.069*	20.7 ± 1.24

Data were expressed as mean ± s.d. *$P < 0.05$ compared with untreated control group (one-way analysis of variance test, post hoc tests).

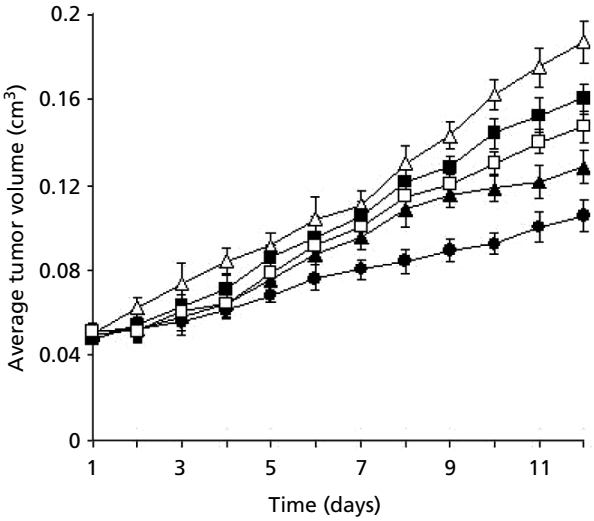


Figure 7 Therapy efficacy of PBV in a human SMMC-7721 hepatoma model. PBV was administered 12-times each day, at a total PBV dose of 0.75, 1.5, 3.0 mg kg^{-1} as indicated in the text. The control group was treated with PBS at the same volume. (△) The control group; (■) the group treated with PBV 0.75 mg kg^{-1}; (□) the group treated with doxorubicin 1.5 mg kg^{-1}; (▲) the group treated with PBV 1.5 mg kg^{-1}; (•) the group treated with PBV 3.0 mg kg^{-1}. Data were expressed as mean tumour volumes (cm^3) ± s.d.

relative viability decreased, and a significant inhibition was observed over the concentration of PBV ranging from 2 to 128 μg mL^{-1} ($P < 0.05$). The IC50 value of PBV in SMMC-7721 cells was 6.68 μg mL^{-1}. At the same concentration of PBV with the time lapsing, the relative viability also decreased. Other tumour cells such as Hela cells could be killed by PBV. When the concentration of PBV was 6.68 μg mL^{-1}, the relative viability of Hela cells was 20.3%.

Ki67 was a nuclear protein expressed in proliferating cells. It was expressed in all phases of the cell cycle except G0, with highest expression seen in G2/M (Key et al 1993; Scholzen & Gerdes 2002). A review of Ki67 showed mixed results regarding its prognostic value in cancer (Brown & Gatter 2002). The Ki67 labelling index had been used as an easy and reproducible method to determine the growth fraction of malignant and normal tissue (Gerdes et al 1984; Gerdes 1990; Duchrow et al 1994). Results showed that the expression of Ki67 was inhibited significantly by PBV, the proliferation index decreased from 97.0% to 10.2%. Nuclear proliferation as determined by DNA cell cycle analysis correlated with the expression level of Ki67. Analysis of the cell cycle also showed that the percentage of cell cycle in G0/G1 phase increased from 66.2% to 84.6%.

Apoptosis is a form of cellular suicide characterized by morphological and biochemical changes, which are different from necrosis. Morphological changes are distinct hallmarks during apoptosis. To examine this more thoroughly, SMMC-7721 cells were treated with PBV and stained with HE assay. Results showed that PBV-treated SMMC-7721 cells displayed a distinct morphological change. The morphology of induced-SMMC-7721 cells turned to rounding; importantly, the density of chromosomes increased. In addition, the results in this study showed that PBV induced alterations in the cell cycle (Table 1). Flow cytometric analysis revealed a prominent cell arrest in G0/G1, which is a hallmark of cells undergoing apoptosis (Cohen 1997). Accumulation of PBV-treated cells in G0/G1 phase seemed to be responsible for cytostatic effects.

Annexin V banded phosphatidylserine residues that become exposed on the surface of the plasma membrane early during apoptosis. Annexin V positive staining had been used as an early marker of apoptotic cell death (van Heerde et al 2000). In this study, the apoptosis was confirmed by monitoring phosphatidylserine externalization using FITC-annexin V staining and flow cytometric analysis (Figure 5) and DNA fragmentation analysis. Other studies suggested that PBV induced the apoptotic cell death (Vento et al 2000; Liu et al 2002; Jang et al 2003).

Balb/c nude mice were used for the in-vivo studies, aiming to further promote the PBV into clinical applications. The results showed that treatment with 1.5 or 3 mg kg^{-1} PBV resulted in significant retardation of SMMC-7721 cell growth ($P < 0.05$), with the relative tumour inhibition of 31.4% and 48.2%, respectively. The result demonstrated that PBV had a beneficial therapeutic potential for treatment of hepatoma.

Conclusion

It was demonstrated that PBV could induce apoptosis in human hepatoma SMMC-7721 cells in-vitro. The cytostatic effect was dose- and time-dependent. Apoptosis of the tumour cells was found to be the possible mechanism by which PBV inhibited tumour cell growth. In-vivo experiments showed that PBV could significantly inhibit the growth of a solid tumour. These data suggested PBV was a promising anti-tumour agent. For a better understating of the mechanism of the PBV-induced hepatoma cell death, further studies are necessary on the detailed signal pathway

of PBV controlling the expression of apoptosis-associated genes.

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