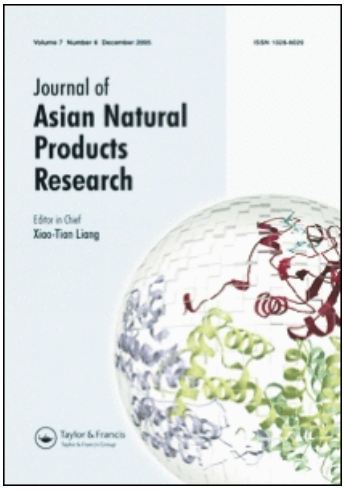

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Inhibitory effect of anti-hepatitis drug bicyclol on invasion of human hepatocellular carcinoma MHCC97-H cells with high metastasis potential and its relative mechanisms

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Inhibitory effect of anti-hepatitis drug bicyclol on invasion of human hepatocellular carcinoma MHCC97-H cells with high metastasis potential and its relative mechanisms

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To assess the anti-invasion effect of bicyclol (**1**) and its mechanism in human hepatocellular carcinoma (HCC) MHCC97-H cells with high metastatic potential. MTT assay was performed to evaluate the cytotoxicity of **1** to MHCC97-H cells and its inhibitory effect on the adhesion of these cells to laminin (LN) and fibronectin (FN). The anti-invasion effect of **1** was detected in an experiment using a transwell chamber. Transcription of vascular endothelial growth factor (VEGF), nm23-H1, and urokinase-type plasminogen activator receptor (uPAR) mRNAs was determined by an RT-PCR assay. The secretion and expression of α-fetoprotein (AFP) were analyzed by ELISA and flow cytometry, respectively. At concentrations of 10, 50, and 100 μmol/l, **1** obviously inhibited the adhesion of the MHCC97-H cells to LN and FN. The rates of inhibition of MHCC97-H cell invasion by 50 and 100 μmol/l for **1** were 37.3 and 50.2%, respectively. Drug **1** also decreased the expressions of VEGF, nm23-H1, and uPAR mRNA and the secretion of AFP in MHCC97-H cells. At low cytotoxic concentrations, the anti-hepatitis drug **1** demonstrated a significant anti-invasive effect in human HCC MHCC97-H cells with high metastatic potential. The inhibition of the expressions of VEGF, nm23-H1, and uPAR should contribute, at least in part, to the anti-invasion property of **1**.

Keywords: bicyclol; human hepatocellular carcinoma; MHCC97-H cell; invasion; metastasis

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors that develops rapidly and is associated with a high mortality rate [1]. The main causes for poor prognosis of HCC and high mortality rates associated with it are its invasiveness and metastasis. An effective method of blocking the advancement of HCC and reducing mortality is to inhibit its invasion and metastasis.

Bicyclol(4,4′-dimethoxy-2,3,2′,3′-dimethylene-dioxy-6-hydroxymethyl-6′-carbonyl-biphenyl, **1**) is a novel anti-hepatitis drug developed by Chinese scientists (Figure 1) [2]. Clinical trials revealed that **1** is effective in improving abnormal liver function and inhibiting the replication of hepatitis B virus in chronic hepatitis B patients [3]. Pharmacologically, **1** has an anti-fibrotic effect on CCl_4-induced liver fibrosis and inhibitory effect on

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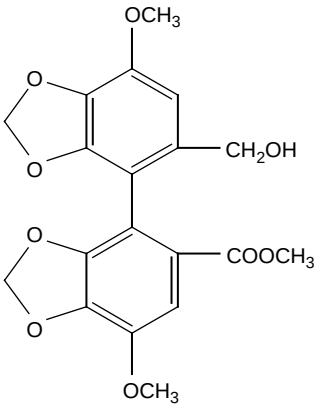

Figure 1. The chemical structure of bicyclol.

hepatotoxin-induced liver injury in mice and rats; it also has an anti-virus action in duck viral hepatitis and the 2.2.15 cell line [2,4,5]. Furthermore, it was found that **1** induced the differentiation of human hepatocarcinoma HepG2 cells and reduced AFB1 hepatotoxicity by increasing the detoxifying metabolism of AFB1 in rat liver [6].

In the present study, we found that **1** has a significant anti-carcinogenic effect in a model of two-stage chemical oncogenesis induced by 3-methylcholanthrene and 12-*O*-tetradecanoyl phorbol 13-acetate tissue plasmogen activator in rat liver epithelial cells (WB-F344 cells) *in vitro* [7]. The results revealed that **1** has a potential chemopreventive effect on hepatocarcinogenesis. It is known that chronic viral hepatitis and HCC are closely associated. About 90% of the human HCCs are associated with hepatitis virus infection [8]. At present, the therapy for chronic viral hepatitis generally has a long course. An anti-hepatitis drug that inhibits or suppresses the effects of hepatocarcinogenesis and the invasion of HCC besides improving abnormal liver function would be of great clinical value. This paper reports the inhibitory effect of bicyclol on the invasion of human HCC MHCC97-H cells with high metastatic potential and the mechanism underlying it.

2. Results and discussion

2.1 Cytotoxicity of bicyclol to MHCC97-H cells

After 96 h of incubation, the IC_{50} of bicyclol for MHCC97 cells was 192.8 ± 26.1 μmol/l. Bicyclol had a little cytotoxicity to the MHCC97-H cells. The experiments were performed five times, and the same results were obtained.

2.2 Anti-adhesion effect of bicyclol

The inhibitory effect of bicyclol on the adhesion of MHCC97-H cells to fibronectin (FN) and laminin (LN) is shown in Figure 2. At the concentrations of 10, 50, and 100 μmol/l, bicyclol markedly inhibited the adhesion of MHCC97-H to FN and LN. The rates of inhibition of adhesion to FN and LN by 50 and 100 μmol/l bicyclol were 24.6 and 39.1% and 23.5 and 25.7%, respectively.

2.3 Anti-invasion effect of bicyclol

The number of MHCC97-H cells in the control group was 26.3 ± 7.6 per visual field, and those in the groups treated with 10, 50, and 100 μmol/l bicyclol were 23.5 ± 6.0, 16.5 ± 5.8, and 13.1 ± 4.2 per visual field, respectively. The cells treated with bicyclol showed significantly

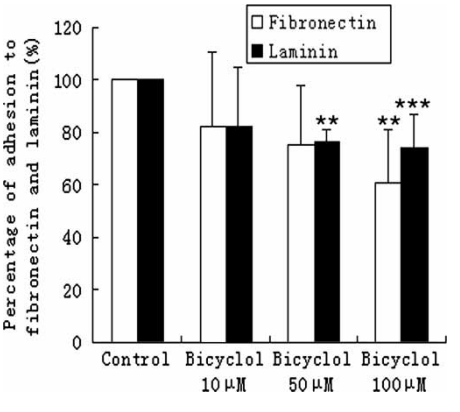

Figure 2. Inhibitory effect of bicyclol on the adhesion of human HCC MHCC97-H cells to FN and LN. $^{**}P < 0.01$, $^{***}P < 0.001$, versus the control group.

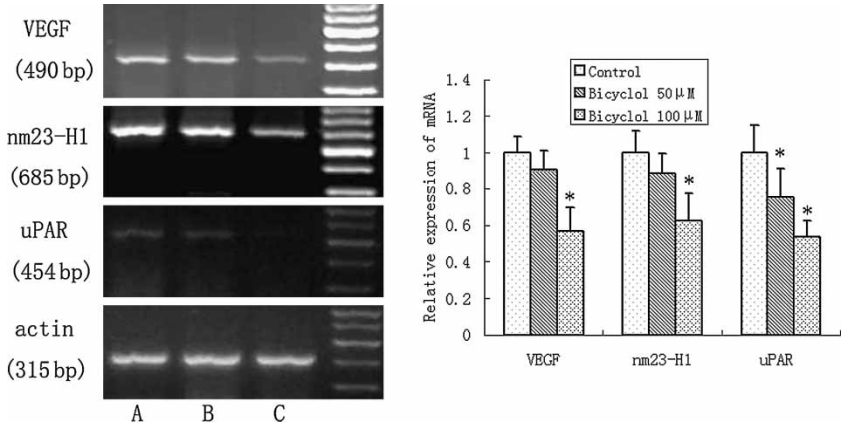

Figure 3. Inhibitory effect of bicyclol on the expression of VEGF, nm23-H1, and uPAR mRNA in MHCC97-H cells as detected by RT-PCR. A, Control; B, 50 μM bicyclol; C, 100 μM bicyclol. $*P < 0.05$, versus the control group.

lower invasive capacity than the control cells ($P < 0.05$), and the inhibitory effect was concentration dependent.

2.4 Inhibitory effect of bicyclol on vascular endothelial growth factor (VEGF), nm23-H1, and urokinase-type plasminogen activator receptor (uPAR) mRNA expressions

As shown in Figure 3, the expression of VEGF, nm23-H1, and uPAR mRNA in the MHCC97-H cells was apparent. When the cells were pretreated with 50 and 100 μmol/l bicyclol, respectively, for 24 h, the expression of VEGF, nm23-H1, and uPAR mRNA decreased markedly. Bicyclol at 100 μmol/l demonstrated a higher inhibitory effect on VEGF, nm23-H1, and uPAR mRNA than bicyclol at 50 μmol/l.

2.5 Inhibitory effect of bicyclol on the secretion and expression of α-fetoprotein (AFP)

The secretion of AFP by MHCC97-H cells exposed to 10, 50, 100, and 200 μmol/l bicyclol is shown in Table 1. At the concentrations of 10, 50, 100, and 200 μmol/l, bicyclol inhibited the secretion of AFP at the rate of 42.7, 53.1, 64.8, and 94.8%, respectively, in a dose-dependent fashion. The results of flow cytometry showed a high level of AFP expression in MHCC97-H cells. When the cells were exposed to 50 and 100 μmol/l of

Table 1. The inhibitory effect of bicyclol on the secretion of AFP in MHCC97-H cells with high metastatic potential.

Group	Bicyclol concentration (μmol/l)	AFP (μg/l)	Inhibitory rate (%)
Control	–	8.66 ± 2.70	
Bicyclol	10	4.96 ± 1.00	42.7
	50	4.07 ± 1.93	53.1
	100	3.05 ± 2.67	64.8
	200	0.45 ± 1.16**	94.7

Data are means ± SD values ($n = 4$). $**P < 0.01$, versus the control group.

bicyclol for 24 h, the AFP expression was inhibited significantly (Figure 4).

2.6 Discussion

The MHCC97-H cell is a human HCC cell line with high metastatic potential that is established through cloning and *in vivo* selection. This cell line shows typical epithelial appearance and malignant biological behaviors similar to those seen during clinical metastasis [11]. These cells have been approved for clinical and experimental studies on the mechanism of metastasis, screening for new predictor of metastasis, and the development of novel preventive and therapeutic strategies.

Tumor invasion and metastasis is a very complicated multiple-step process, and it is one of the greatest obstacles in clinical treatment. The transition from *in situ* tumor growth to metastatic advanced disease involves the ability of the tumor cells to invade the local tissue and to cross tissue barriers. To initiate this process, carcinoma cells must penetrate the epithelial basement membrane. The adhesion of tumor cells to components of the extracellular matrix (ECM), such as FN, LN, and matrigel, is crucial for metastasis. This study showed that bicyclol (**1**) could inhibit the adhesive activity of MHCC97-H cells to FN and LN. In the assay performed using a transwell cell chamber assay, **1** also showed a strong anti-invasion effect on MHCC97-H cells, which was expressed in terms of reduction in the number of tumor cells invading through the matrigel and reaching to the reverse side of filters.

It is well known that tumor metastasis is a fairly complicated procedure involving changes in many oncogenes and tumor suppressor genes. Non-metastatic gene 23 (*nm23*) is a tumor suppressor gene that was first identified by differential hybridization of a cDNA library with total RNA extracted from metastatic melanoma cell lines [12]. Further studies demonstrated that the *nm23* gene has multiple effects, and the significance of its expression in human tumors is organ-specific. In some tumors, the main role of the gene is

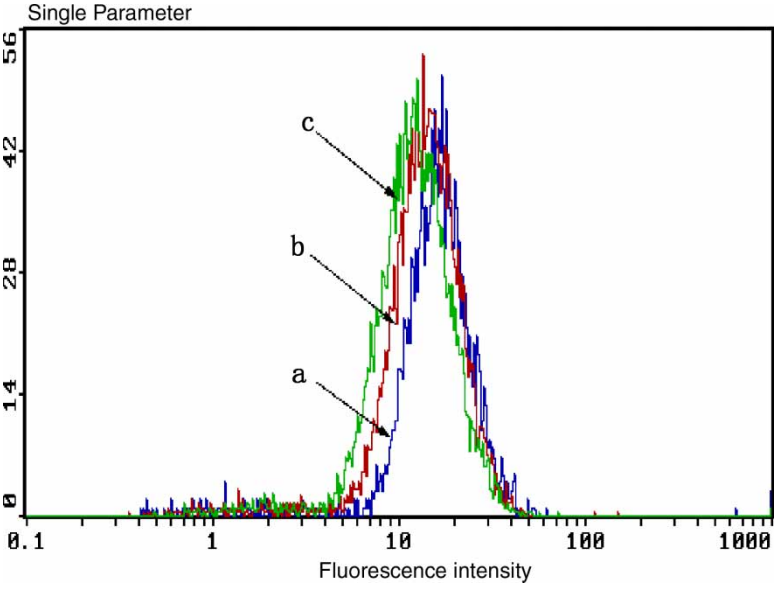

Figure 4. Effect of bicyclol on AFP expression in MHCC97-H cells, as detected by flow cytometry. (a) Control group; (b) 50 μM bicyclol; (c) 100 μM bicyclol.

metastasis suppression, while in others, it may induce cell differentiation and propagation. Wei *et al.* [13] reported that the rate of expression of the *nm23-H1* gene was higher in stomach cancer cells with infiltrative growth and metastasis to lymph nodes than in those with expansive growth but no metastasis to lymph nodes. Further, the expression-positive rate of the *nm23-H1* gene was higher during the progression stage of the stomach cancer than during the early stage. Wei *et al.* suggested that the function of *nm23-H1* gene might not exhibit a suppressive effect on metastasis but can affect infiltrative metastasis of stomach cancer. Lu *et al.* [14] indicated that the rate of expression of the *nm23-H1* gene in liver cancer cells with intrahepatic metastasis was 100%, which was significantly higher than that in those with no intrahepatic metastasis. Tian *et al.* [15] also reported that in the MHCC97 cell line, the *nm23-H1* gene exhibited positive expression, indicating that it might be related with cell propagation. The reason for the different levels of expression of the *nm23-H1* in various tumor cells is still not known. The results of this study showed that at lower cytotoxic concentrations, **1** could reduce the expression level of the *nm23-H1* gene in the MHCC97-H cells, suggesting that **1** may inhibit MHCC97-H cell metastasis by mediating the down-regulation of the *nm23-H1* gene. It is necessary to further explore the action of the *nm23-H1* gene in the MHCC97-H cells and the mechanism of inhibition of *nm23-H1* gene expression by **1**.

Tumor angiogenesis plays a significant role in multiple processes at various stages of tumor metastasis. Blocking tumor angiogenesis may prevent the original tumor from invading into the vascular system and thus stop tumor metastasis as well as inhibit propagation and advancement of tumor loci which has formed the minor metastasis. VEGF is the most active and most specific soluble factor promoting angiogenesis. HCC belongs to the group of typical tumors that are rich in blood vessels and whose growth, invasion, and metastasis are closely associated with VEGF. Our studies demonstrated that the VEGF gene was positively expressed in the human HCC cell line MHCC97-H. This finding is consistent with that reported by Yao *et al.* [16]. **1** was shown to reduce the expression of VEGF mRNA, suggesting that it may have the potential to inhibit tumor angiogenesis.

Apart from angiogenesis, ECM degradation is also a critical factor in the growth, invasion, and metastasis of malignant tumors. It is known that uPA is a metastasis-related factor that initiates the fibrinolytic system to increase degradation of stroma around the tumor cells; it also plays an important role in tumor angiogenesis [17]. It is well known that uPA must bind to its receptor uPAR. In this study, we detected uPAR expression in the MHCC97-H cells by performing RT-PCR. The results showed that the expression of uPAR mRNA was positive in the MHCC97-H cells, and **1** may decrease its expression.

AFP is one of the most important serum tumor markers used for diagnosing HCC in clinics [18]. In the present study, we found that **1** can also decrease the expression and secretion of AFP.

In summary, **1** at low cytotoxic concentrations showed significant anti-invasion effect in the human HCC cell line MHCC97-H. The mechanism underlying its action is mainly related to the decrease in the expression of VEGF, uPAR, and nm23-H1 mRNAs. Moreover, **1** exhibited a significant inhibitory effect on the malignant transformation of WB-F344 rat liver epithelial cells that is induced by chemical carcinogens [7]. Taken together, the results indicate that **1** has potential in preventing hepatocarcinogenesis and inhibiting the HCC invasion. It may be especially effective in preventing relapse of HCC following early-stage operation or

intervention therapy. Drug **1** is a novel, effective, and safe anti-hepatitis drug. It is very important to further explore the effects and the mechanisms of **1** on hepatocarcinogenesis and the invasion of HCC and the mechanisms underlying them.

3. Materials and methods

3.1 Cell lines and bicyclol

The human HCC cell line MHCC97-H, which has a high metastatic potential, was purchased from the Liver Cancer Institute of Fudan University, Shanghai, China. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 + 95% air, and passed using 0.25% trypsin containing 0.02% EDTA.

Bicyclol (**1**), a white crystalline solid, of 99% purity was provided by Peking Union Pharmaceutical Factory (Beijing, China). It was dissolved in dimethyl sulfoxide (DMSO) and diluted at least 1000-fold with a culture medium for *in vitro* experiments.

3.2 Reagents

DMEM was purchased from Gibco BRL (Carlsbad, CA, USA); LN and FN were from Peking University Health Science Center (Beijing, China); ethidium bromide (EB) and matrigel were from Sigma Chemical Co. (Spruce, NC, USA); Trizol reagent kit was from Gibco BRL; RT-PCR reagent box was from Promega (Madison, WI, USA); monoclonal mouse anti-human AFP was from Zymed Laboratories, Inc. (South San Francisco, CA, USA); and AFP ELISA kits were from Shanghai Senxiong Biotech Industry Co., Ltd (Shanghai, China). PCR primers were synthesized by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd (Shanghai, China). The primer sequences are shown in Table 2.

3.3 MTT assay

The cytotoxicity of **1** was determined by performing an MTT assay according to the method of Mosmann [9]. MHCC97-H cells (3×10^3 cells per well) were plated on 96-well plates and bicyclol was added at various concentrations (1, 5, 10, 50, 100, 200, and 300 μmol/l) 24 h later. The cells were cultured at 37°C in a CO_2 incubator for 72 h. The culture supernatant was sucked out and 0.5 mg/ml MTT stock solution was added to each well. After 4 h of incubation, 150 μl of DMSO was added. The mixture in the plates was mixed gently until the blue sedimentation was completely dissolved. The optical density of each well was determined using a microplate reader at a wavelength of 570 nm. The concentrations required to inhibit growth by 50% (IC_{50} values) were calculated

Table 2. Primers for VEGF, nm23-H1, β-actin, and uPAR and the size of amplified fragments.

Gene	Primer	Size (bp)
VEGF	5′-TTGCTTGCTCTACCTCCAC-3′ 5′-AATGCTTTCTCCGCTCTG-3′	490
nm23-H1	5′-GCAGCCCGGAGTTCAAACCTAA-3′ 5′-GCTGGGAGGAAGCATTTTAATC-3′	685
uPAR	5′-GCTTAGAGAAGACGTGCAGGGA-3′ 5′-TTCACCTTCCTGGATCCAGT-3′	454
β-Actin	5′-TGGAGGGGCCGGACTCGTCA-3′ 5′-CTTCCTTCCTGGGCATGGAG-3′	315

VEGF, vascular endothelial growth factor and uPAR, urokinase-type plasminogen activator receptor.

using TWODRUGS program based on a median effect equation.

3.4 Adhesion assay

An adhesion assay was performed according to a previously described method [10]. Briefly, FN or LN (2.0 μg) was applied on 96-well plates, and dried at room temperature. The plate was coated with 20 μl DMEM containing 2% bovine serum albumin (BSA) at 37°C for 1 h and then rinsed with phosphate-buffered saline (PBS; three times). We added 8×10^4 MHCC97-H cells pre-treated with 10, 50, and 100 μmol/l bicyclol for 24 h to each well and incubated the plate for 1 h. PBS was then used to rinse the non-adhering cells. MTT (0.5 mg/ml) was added to each well and the plate was incubated for 4 h. The absorbance value was then measured at 570 nm on a microplate reader.

3.5 Invasion assay

The invasive activity of tumor cells was determined using a transwell chamber. Briefly, polyvinyl pyrrolidone-free polycarbonate filters with a pore size of 8.0 μm were pre-coated on the outer side with 5 μg/10 μl FN and dried at room temperature. Matrigel diluted to 500 μg/ml with cold PBS and 10 μl of the solution was applied to the inner side of the filter and dried at room temperature. MHCC97-H cells at log phase were harvested and resuspended to a final concentration of 1×10^6/ml in DMEM containing 0.1% BSA. The cell suspension (100 μl) was added to the upper compartment, and 10, 50, and 100 μmol/l bicyclol were also added simultaneously. The filter chamber was incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. The filters were fixed with methanol for 1 min and then stained with hematoxylin for 3 min and eosin for 30 s. The cells on the upper side of the filter were removed by wiping the surface with a cotton swab. The number of cells that had invaded through the matrigel and reached the reverse side was counted under a microscope in five fields at a magnification of × 200. Each assay was performed in triplicate.

3.6 RT-PCR analysis

The MHCC97-H cells were pre-treated with 50 or 100 μmol/l bicyclol and DMSO for 24 h. Total RNA was isolated using TRIzol and RT-PCR was performed using an Access RT-PCR Introductory System (Promega) as described in the manufacturer's manual. The reaction mixture (50 μl) contained 1–2 μg of total RNA, 0.1 U/μl AMV reverse transcriptase, 0.1 U/μl Tfl DNA polymerase, 0.2 mM of each dNTP, 1 mM $MgSO_4$, and 1 μM of each primer. First-strand cDNA synthesis completed after 45 min at 48°C and then PCR was processed. The reaction conditions were as follows: nm23-H1, denaturation at 94°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1 min; VEGF, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 90 s; uPAR, denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 90 s; β-actin, denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. All cycles were performed 30 times. The PCR-amplified products were analyzed on 2% agarose gels in Tris/borate/EDTA buffer and visualized by staining with EB. The band absorbency of the aim mRNA and β-actin was quantitatively analyzed through the gel imaging instrument, and the ratio of them was regarded as the half-quantified number.

3.7 ELISA

MHCC97-H cells (1×10^6 cells per well) were plated on 24-well plates. The primary medium containing serum was sucked out

24 h later, and fresh medium without serum was added. The cells were incubated for 1 h at 37°C in a humidified 5% CO_2 atmosphere, and the medium was sucked out again. The medium without serum (400 μl) and 10, 50, 100, and 200 μmol/l bicyclol were simultaneously added to each well. After 24 h of incubation, the culture supernatant was collected and centrifuged at 2000*g* for 5 min. ELISA was performed using 100 μl of the supernatant according to the manufacturer's manual for the AFP ELISA kit.

3.8 Flow cytometry analysis

MHCC97-H cells were treated with 50 or 100 μmol/l bicyclol, or DMSO for 24 h and collected for the detection of AFP expression by flow cytometry. The cells were washed once with ice-cold PBS and then fixed with 4% paraformaldehyde (1 ml) for 40 min at room temperature. The fixed cells were washed once and resuspended with PBS containing 0.2% Triton X-100 and 5% serum. Monoclonal mouse anti-human AFP antibody (dilution, 1:50) or PBS was added to the cell suspensions. The cells were stored at 4°C for 40 min, washed with PBS, and FITC-labeled anti-mouse antibody (dilution, 1:100) was added. The cells were then allowed to stand for an additional 40 min at 4°C in the dark and filtered using a nylon net. Ten thousand cells were analyzed by flow cytometry, and the mean fluorescence intensity of AFP was calculated.

3.9 Statistical analysis

Data were presented as mean ± SD, and the *t*-test was used for inter-group comparison. A *P* value less than 0.05 was considered to be statistically significant.

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References

[1] P.P. Michielsen, S.M. Francque, and J.L. van Dongen, *World J. Surg. Oncol.* **3**, 27 (2005).
[2] Y. Li, G.W. Dai, Y. Li, and G.T. Liu, *Acta Pharm. Sin.* **36**, 723 (2001).
[3] G.T. Liu, C.Z. Zhang, Y. Li, H.L. Wei, and W. Hu, *Chin. Med. Sci. J.* **14**, 51 (1999).
[4] M. Li and G.T. Liu, *World J. Gastroenterol.* **10**, 1775 (2004).
[5] D.M. Zhao and G.T. Liu, *Natl Pharm. Assoc. J.* **81**, 844 (2001).
[6] H. Lu and Y. Li, *Acta Pharmacol. Sin.* **23**, 942 (2002).
[7] H. Sun and G.T. Liu, *Cancer Lett.* **236**, 239 (2006).
[8] L.M. Yerian, R.A. Anders, M. Tretiakova, and J. Hart, *Am. J. Surg. Pathol.* **28**, 357 (2004).
[9] T. Mosmann, *J. Immunol. Methods* **65**, 55 (1983).
[10] R. Han, *Research and development of anticancer drugs and experimental techniques* (Beijing Medical University and Chinese Xiehe Medical University Press, Beijing, 1997), pp. 353–356.
[11] J. Tian, Z.Y. Tang, and S.L. Ye, *Chin. J. Oncol.* **20**, 405 (1998).
[12] P.S. Steeg, G. Bevilacqua, L. Kopper, U.P. Thorgeirsson, J.E. Talmadge, L.A. Liotta, and M.E. Sobel, *J. Natl Cancer Inst.* **80**, 200 (1988).
[13] Z.B. Wei, Y.L. Wu, and Z.K. Zhai, *Chin. J. Oncol.* **19**, 378 (1997).
[14] Y.F. Lu, J.L. Lin, and M.Y. Wei, *Chin. J. Bases Clin. Gen. Surg.* **7**, 241 (2000).
[15] J. Tian, Z.Y. Tang, and Q. Cai, *Natl Pharm. Assoc. J.* **79**, 470 (1999).
[16] D.F. Yao, X.H. Wu, Y. Zhu, G.S. Shi, Z.Z. Dong, D.B. Yao, W. Wu, L.W. Qiu, and X.Y. Meng, *Hepatobiliary Pancreat. Dis. Int.* **4**, 220 (2005).
[17] F. Blasi, *Bioassays* **15**, 105 (1993).
[18] D. Bader, A. Riskin, O. Vafsi, A. Tamir, B. Peskin, N. Israel, R. Merksamer, H. Dar, and M. David, *Clin. Chim. Acta* **349**, 15 (2004).