Redifferentiation of human hepatoma cells (SMMC-7721) induced by two new highly oxygenated bisabolane-type sesquiterpenes

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Bisabolane-type sesquiterpenes are a class of biologically active compounds that has antitumour, antifungal, antibacterial, antioxidant and antivenom properties. We investigated the effect of two new highly oxygenated bisabolane-type sesquiterpenes (HOBS) isolated from *Cremanthodium discoideum* (*C. discoideum*) on tumour cells. Our results showed that HOBS induced morphological differentiation and reduced microvilli formation on the cell surface in SMMC-7721 cells. Flow cytometry analysis demonstrated that HOBS could induce cell-cycle arrest in the G1 phase. Moreover, HOBS was able to increase tyrosine-α-ketoglutarate transaminase activity, decrease α-foetoprotein level and γ-glutamyl transferase activity. In addition, we found that HOBS inhibited the anchorage-independent growth of SMMC-7721 cells in a dose-dependent manner. Taken together, all the above observations indicate that HOBS might be able to normalize malignant SMMC-7721 cells by inhibiting cell proliferation and inducing redifferentiation.

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1. Introduction

Substances derived from plants are used to develop new medicines because of their various bioactivities. Novel natural drugs and biologically active materials from herbs can thus help in pharmaceutical research. The genus *Cremanthodium* (Asteraceae) consists of about 64 species from all over the world, but is widespread in the mountains of the Himalayas and in contiguous areas. Several plants of this genus have been used in Tibetan traditional herbal medicine to treat fever, inflammation, pain and apoplexy (Guo 1987; Yang 1991). However, there are few data available in the literature regarding their phytoactive agents, chemical composition and structure–activity relationships. Zhu *et al* have reported the isolation two new highly oxygenated bisabolane-type sesquiterpenes (HOBS) from *Cremanthodium discoideum* (*C. discoideum*) (Zhu *et al* 1999). Bisabolane-type sesquiterpenes are a class of the Sesquiterpene family with antitumour, antifungal, antibacterial, antioxidant and antivenom activities (Isaac 1979; Itokawa *et al* 1985; Ingber *et al* 1990; Denyer *et al* 1994).

Cancer is an abnormal accumulation of cells that exhibit deregulated proliferation, abnormal differentiation and apoptosis (Kotnis *et al* 2005). Most tumour cells appear to be relatively undifferentiated or dedifferentiated; thus, differentiation therapy is an approach for the treatment of malignant tumour cells (Pan *et al* 2004). A number of studies have shown that malignant tumour cells could be induced

Keywords. Oxygenated bisabolane-type sesquiterpenes (HOBS); proliferation; redifferentiation; tumour

Abbreviations used: AFP, α-foetoprotein; DMSO, dimethysulphoxide; ELISA, enzyme-linked immunosorbent assay; FBS, foetal bovine serum; γ-GT, γ-glutamyl transferase; HOBS, highly oxygenated bisabolane-type sesquiterpenes; PBS, phosphate buffered saline; PI, propidium iodide; TAT, tyrosine-α-ketoglutarate transaminase

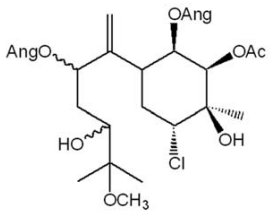
to differentiate by some substances such as ascorbic acid, cytokinins, retinoids, valproic acid (Short *et al* 2004; Catalano *et al* 2005; Ishii *et al* 2005; Sun *et al* 2006; El-Metwally *et al* 2007), among others. Moreover, human hepatoma cells have been successfully induced to differentiate in some laboratories by using different compounds such as ascorbic acid, DL-*α*-tocopherol, coumarin, diethyldithiocarbamate, besides some plant glycosides (Odashima *et al* 1989; Kang *et al* 1999; Kang *et al* 2000; Kang *et al* 2001; Zheng *et al* 2002; Pan *et al* 2004).

In our work, the effect of HOBS on the proliferation and differentiation of human hepatoma SMMC-7721 cells was examined. Our results showed that SMMC-7721 cells could be induced to redifferentiate not only phenotypically but also functionally. These results could provide the experimental bases for the development of new antitumour drugs from *C. discoideum.*

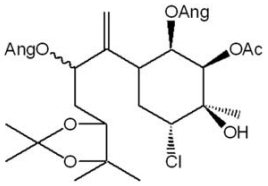
2. Materials and methods

2.1 *Reagents*

Two new compounds, namely: (1). 1*β*, 8-diangeloyloxy-2*β*-acetoxy-4*α*-chloro-11-methoxy-3*β*, 10-dihydroxybisabola-7(14)-ene: (HOBS1) and (2). 1*β*, 8-diangeloyloxy-2*β*-acetoxy-4*α*-chloro-3*β*-hydroxy-10, 11-o, o-isopropylidenebis-aboia-7 (14)-ene: (HOBS2) were kindly provided by Professors Drs Jia and Zhu (The State Key Laboratory of Applied Organic Chemistry, Lanzhou University, China). Their chemical structures are presented below:



1.



2.

HOBS was initially dissolved in dimethysulphoxide (DMSO) and further diluted in culture medium. The final concentration of DMSO in culture was always less than 0.4% (v/v) and did not cause any toxicity by itself.

RMPI-1640 medium was purchased from Gibco Co., USA. Foetal bovine serum (FBS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. (Hangzhou, China). All other reagents used were of analytical grade.

2.2 *Cells*

SMMC-7721 cells were obtained from the Second Military Medical University, Shanghai, China. Cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 37 ºC in a humidified atmosphere of 5% CO_2.

2.3 *Morphological analysis*

After treatment with 20 μg/ml HOBS1 and HOBS2 for 5 days, cells were grown on 5 mm glass coverslips and fixed in 0.1 M phosphate buffered saline (PBS), 2.5% glutaraldehyde (pH 7.4) at 4 ºC for 1 h, and then 1% osmic acid for 20 min, dehydrated with ethanol and dried in a critical point drier (Balzers Union). Finally, the coverslips were coated with gold using a sputter coater (Balzers Union). The specimens were examined with a Hitachi S-520 scanning electron microscope.

2.4 *Cell-cycle analysis*

SMMC-7721 cells at a density of 1×10^4 were treated with HOBS1 and HOBS2 (10 μg/ml, 20 μg/ml, 30 μg/ml). After treatment for 48 h, cells were prepared as a single cell suspension in 200 μl PBS, fixed with 2 ml of ice-cold 70% ethanol, and maintained at 4 ºC overnight. The cells were harvested by centrifugation at 500 × *g* for 10 min, resuspended in 500 μl PBS supplemented with 0.1% Triton X-100 and RNaseA (100 μg/ml), incubated at 37 ºC for 30 min, and stained with 50 μg/ml propidium iodide (PI) in the dark at 4 ºC for 30 min. The red fluorescence of individual cells was measured with a flow cytometer (EPICS XL, U.S COULTER), and the data were analysed using Cellquest version 1.2.2 software (B-D).

2.5 *α-foetoprotein (AFP), γ-glutamyl transferase (γ-GT) and tyrosine-α-ketoglutarate transaminase (TAT) assays*

SMMC-7721 cells at a density of 1×10^6 were treated with HOBS1 and HOBS2 (10 μg/ml, 20 μg/ml, 30 μg/ml). After 48 h, the content of AFP was determined by using an AFP reagent kit (Biological Reagent Research Institute, Lanzhou, China) by means of an AFP enzyme-linked immunosorbent assay (ELISA). The activity of *γ*-GT was determined by a *γ*-GT reagent kit (Chemical Agent Research Institute, Shanghai, China) using an azo-coupling reaction. TAT activity was detected by the method of Diamondstone (Diamondstone 1966). Protein content was determined by the Lowry method (Lowry *et al* 1951).

2.6 *Colony formation rate assay in soft agar*

After treatment with HOBS1 and HOBS2 (10, 20, 30 μg/ml) for 48 h, the SMMC-7721 cells (1×10^3) were cultured on

a plate containing 0.5% base agar and 0.17% top agar in RPMI-1640 with 10% FBS. After 21 days of incubation at 37ºC in a 5% CO_2 humidified atmosphere, colonies with >50 cells were counted.

2.7 *Statistical analysis*

Means were compared by a two-tailed *t*-test, *: $P<0.05$, **: $P < 0.01$.

3. Results

3.1 *HOBS induces morphological changes on the surface of SMMC-7721 cells*

Generally, the tumour cell surface contains a larger number of microvilli and their occurrence on the cell surface might reflect the higher metabolic activity of proliferative cells and may play an important role in the processes of tumour growth (Ghadially 1988). Therefore, evaluating the morphological changes in the microvilli is important in determining the effect of exotic substances (xenobiotics) on tumour cells. We observed the morphological changes of SMMC-7721 cells treated with HOBS by scanning electron microscopy. As shown in figure 1A, on the surface of control cells, several microvilli were present, with many long and thin microfilamentous pseudopodia that extended around the cell. After 5 days of treatment with HOBS1 and HOBS2, the microvilli and microfilaments on the cell surface were reduced significantly and shortened considerably (figure 1B and C). This finding demonstrates that HOBS could reverse the morphological features of SMMC-7721 cells towards those of normal hepatocytes.

3.2 *HOBS induces G1 cell-cycle arrest*

The connection between the cell cycle and cancer is obvious: cell-cycle machinery controls cell proliferation, and cancer is a disease of inappropriate cell proliferation. Inappropriate cell proliferation is linked to a vicious cycle with a reduction in sensitivity to signals that normally prompt a cell to adhere, differentiate or die (Collins *et al* 1997). Many anticancer agents can inhibit the proliferation of cancer cells by blocking one or more stages of the cell cycle. Subsequently, we examined the cell cycle by flow cytometry. After treating with HOBS1 and HOBS2 for 48 h, cell-cycle analysis showed that SMMC-7721 cell proliferation was arrested mainly at the G1 phase. As shown in figure 2, when compared with that of the control, the percentage of cells in the G1 phase among SMMC-7721 cells treated with 30 μg/ml HOBS1 and HOBS2 increased from 58.99% to 82.45% and 79.36%, respectively, and the

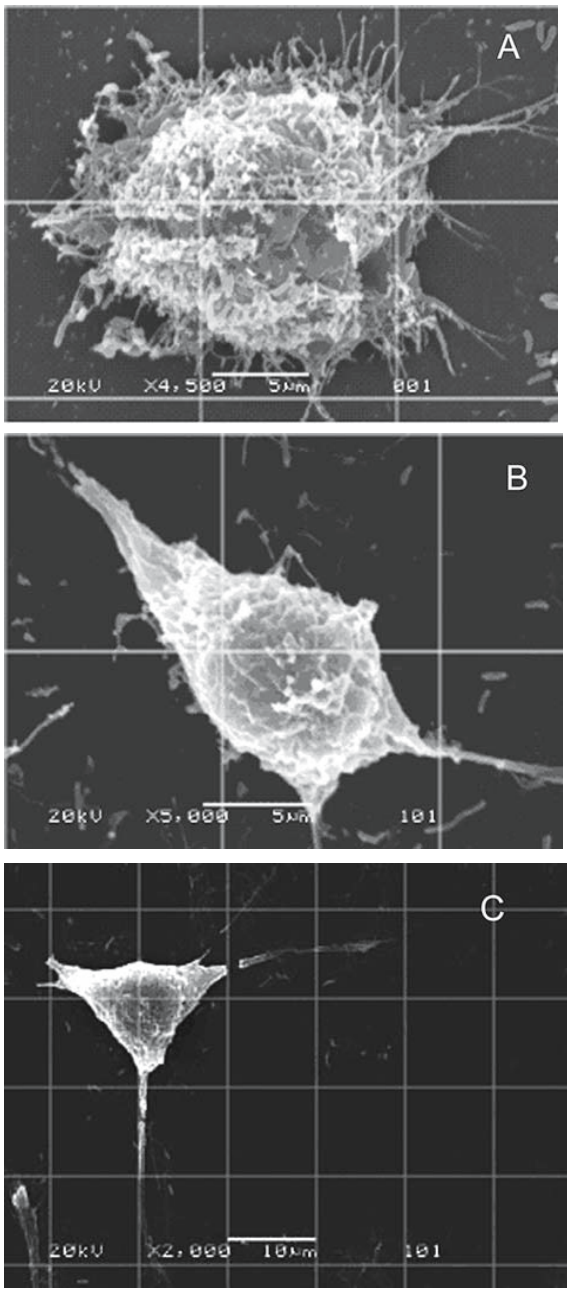


Figure 1. Morphological changes in SMMC-7721 cells treated with HOBS1 and HOBS2. (**A**) Control; (**B**) 20 μg/ml HOBS1 for 5 days; (**C**) 20 μg/ml HOBS2 for 5 days. The specimens were examined with a Hitachi S-520 scanning electron microscope.

percentage of cells in the S phase decreased from 27.13% to 3.83% and 4.62%, respectively. Interestingly, we found that the percentage of cells arrested at the G1 phase decreased gradually as the HOBS concentration dropped. In addition, the percentage of subG1 phase cells increased in a dose-dependent manner. This finding indicates that a higher dose of HOBS could induce apoptosis of SMMC-7721 cells. The percentage of cells in each phase of the cell cycle is shown

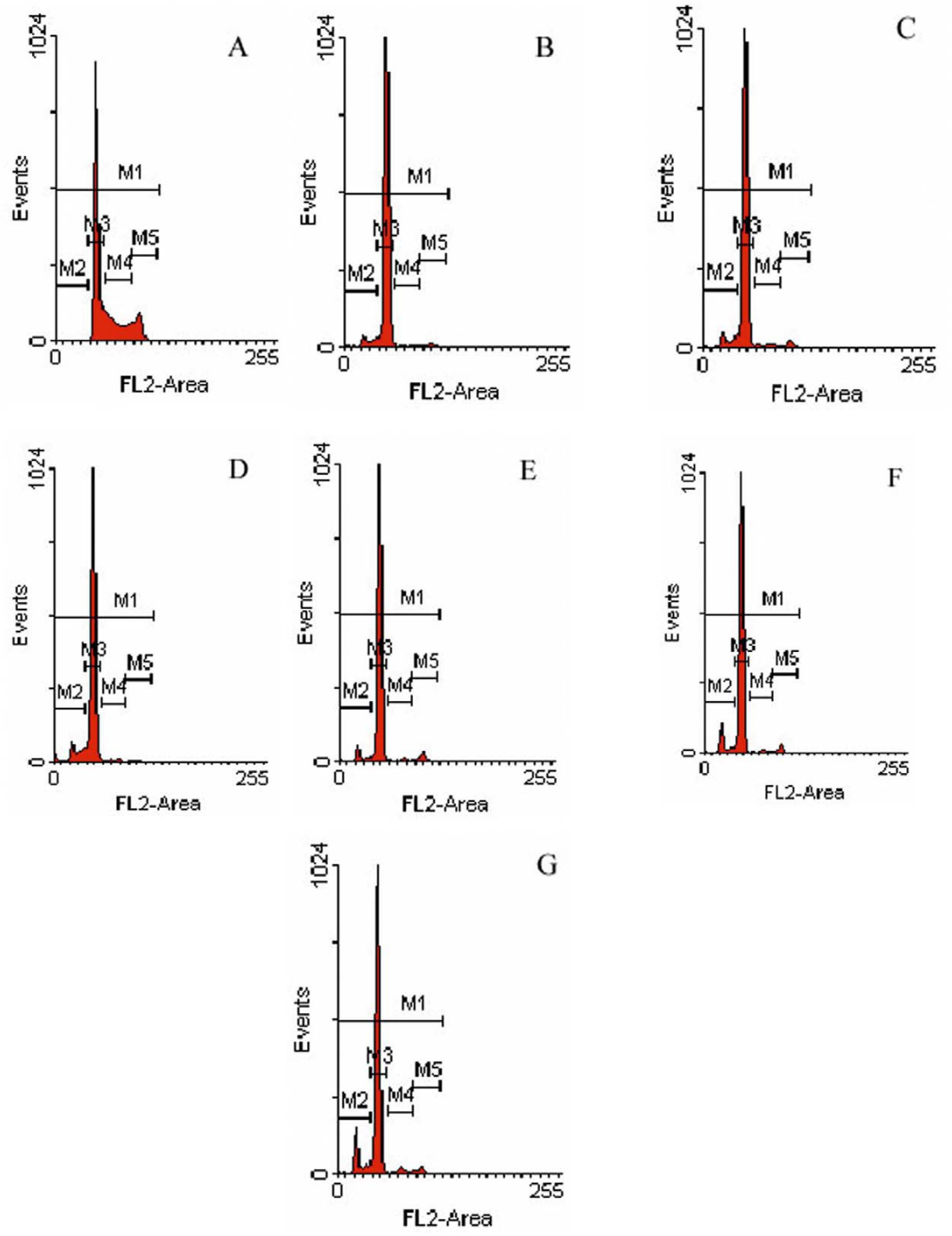


Figure 2. Flow cytometric analysis. SMMC-7721 cell lines were treated with different concentrations of HOBS for 48 h. (**A**) SMMC-7721 control; (**B**) SMMC-7721 treated with 10 μg/ml HOBS1; (**C**) SMMC-7721 treated with 20 μg/ml HOBS1; (**D**) SMMC-7721 treated with 30 μg/ml HOBS1; (**E**) SMMC-7721 treated with 10 μg/ml HOBS2; (**F**) SMMC-7721 treated with 20 μg/ml HOBS2; (**G**) SMMC-7721 treated with 30 μg/ml HOBS2. M1, total number of cells in cycle; M2, the number of cells in the sub-G1 phase; M3, the number of cells in the G1/M phase; M4, the number of cells in the S phase; M5, the number of cells in the G2 phase. FL2 area is a measurement of total propidium iodide (PI) fluorescence.

Table 1. Cell-cycle analysis by flow cytometry. SMMC-7721 cell lines were either not treated or treated with increasing concentrations of HOBS1 and HOBS2 (10 μg/ml, 20 μg/ml, 30 μg/ml, each) for 48 h. Results represent mean ± SE of data from at least three individual experiments.

Concentration	HOBS1				HOBS2			
(μg/ml)	subG1(%)	G1 (%)	S (%)	G2 (%)	subG1(%)	G1 (%)	S (%)	G2 (%)
0	1.12	58.99	27.13	14.98	1.12	58.99	27.13	14.98
10	7.52	87.12	3.39	2.58	6.24	87.15	2.61	4.41
20	8.19	84.8	4.38	3.45	9.25	84.1	3.37	3.76
30	12.62	82.45	3.83	1.94	13.1	79.36	4.62	3.57

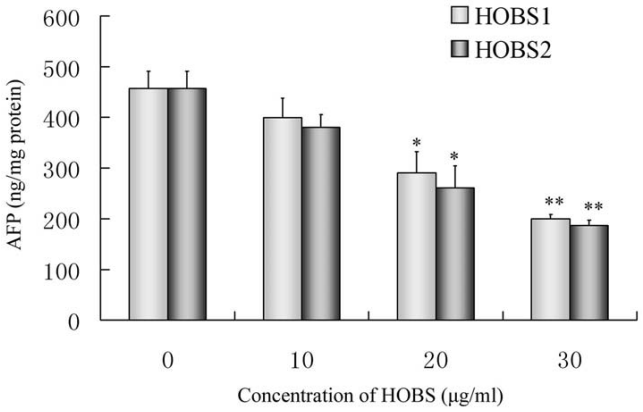


Figure 3. HOBS1 and HOBS2 reduce the level of alpha-foetoprotein (AFP). The AFP level was determined by ELISA without any treatment and after treatment with different concentrations of HOBS1 and HOBS2 (10 μg/ml, 20 μg/ml, 30 μg/ml, each) for 48 h. Results represent mean ± SE of data from at least three individual experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

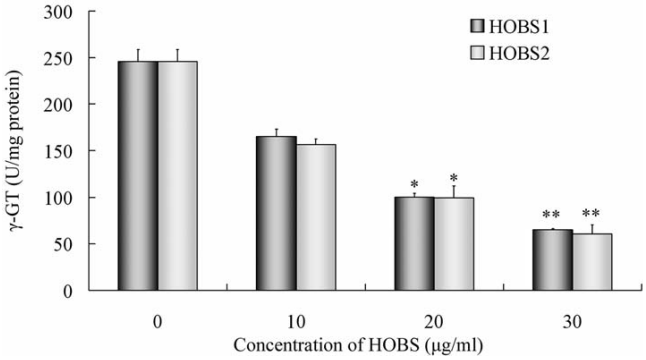


Figure 4. HOBS1 and HOBS2 inhibit the level of γ-GT. The level of γ-GT was determined by azo-coupling reaction without any treatment and after treatment with different concentrations of HOBS1 and HOBS2 (10 μg/ml, 20 μg/ml, 30 μg/ml each) for 48 h. Results represent mean ± SE of data from at least three individual experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

in table 1. From these results, we can infer that HOBS2 is not more efficient than HOBS1 in arresting the cell cycle at the G1 phase.

3.3 *HOBS decreases α-foetoprotein content in SMMC-7721 cells*

Based on the experimental evidence from the morphological changes and cell-cycle analysis, we further investigated the effect of HOBS on some biochemical markers of hepatoma. One marker of hepatic damage is AFP, which is highly expressed in hepatoma. AFP has been shown to be induced in hepatogenesis; it can be reactivated during liver regeneration and in hepatocellular carcinogenesis (Li and Wang 1990). As shown in figure 3, a significantly higher level of AFP was observed in control cells. However, after treating with HOBS1 and HOBS2 for 48 h, the AFP level decreased markedly. On treatment with 30 μg/ml HOBS1 and HOBS2, the levels of AFP decreased by 56.2% and 59.2%, respectively, when compared with that of the control. Moreover, HOBS2 is more efficient in reducing cellular AFP levels compared with HOBS1. These results demonstrated that HOBS has the ability to transform malignant SMMC-7721 cells towards normalcy.

3.4 *HOBS reduces γ-glutamyl transferase activity in SMMC-7721 cells*

γ-GT is another biochemical marker related to hepatocyte malignancy. It is highly expressed in embryonic livers and decreases rapidly to very low levels after birth (Yao and Dong 2007). Its activity increases markedly during the process of malignant transformation to hepatoma formation, so an abnormal γ-GT level is a sensitive tumour marker for the diagnosis of hepatocellular carcinoma (Pitot *et al* 1985). As shown in figure 4, a higher level of γ-GT was observed in the control cells. After treating with HOBS1 and HOBS2 for 48 h, the level of γ-GT decreased considerably. Treating the cells with 30 μg/ml HOBS1 and HOBS2 decreased the levels of γ-GT by 73.4% and 75.4%, respectively, in the test cell lines, and HOBS2 exerted stronger inhibition than HOBS1. This finding indicates that HOBS has the tendency

to restore malignant human hepatocarcinoma cell lines to the normal phenotype.

3.5 *HOBS inhibits tyrosine-α-ketoglutarate transaminase activity in SMMC-7721 cells*

TAT represents an excellent enzymatic marker for hepatoma differentiation, which is not synthesized before birth but is rapidly activated early in the neonatal period (Yeoh *et al* 1979). The activity of TAT in well-differentiated human hepatocytes is high, but decreases during malignant transformation (Ren *et al* 1998). As shown in figure 5, a lower level of TAT was observed in control cells. After treating the cell lines with HOBS1 and HOBS2 for 48 h, the level of TAT increased significantly. On treatment with 30 μg/ml HOBS1 and HOBS2, the level of TAT rose 3.5- and 4.9-fold, respectively, when compared with the control, and HOBS2 was more efficient than HOBS1 in increasing the level of TAT. These results indicate that HOBS is able to induce redifferentiation in SMMC-7721 cells.

3.6 *HOBS reduces colony-formation rate in SMMC-7721 cells*

Based on the findings described above, we next examined the effect of HOBS on the anchorage-independent growth ability of SMMC-7721 cells, which is a property of hepatoma differentiation (Ye *et al* 2004). As shown in figure 6, HOBS1 and HOBS2 both reduced the colony-formation rate in a dose-dependent manner. The colony-formation rate in the control group was taken as 100%. After treatment with

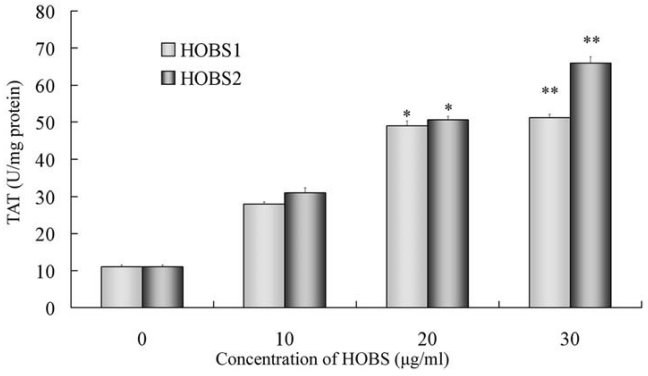


Figure 5. HOBS1 and HOBS2 increase the level of TAT. The level of TAT was detected in the whole cells by the method of Diamondstone without any treatment and after treatment with different concentrations of HOBS1 and HOBS2 (10 μg/ml, 20 μg/ml, 30 μg/ml each) for 48 h. Results represent mean ± S.E. of data from at least three individual experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

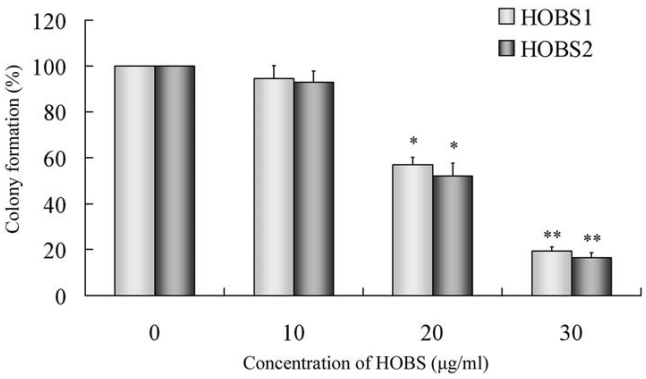


Figure 6. HOBS1 and HOBS2 reduce colony-formation rate in SMMC-7721 cells. Cells were routinely plated and cultured for 21 days either without treatment or after treatment with different concentrations of HOBS1 and HOBS2 (10 μg/ml, 20 μg/ml, 30 μg/ml, each) for 48 h. One colony was defined to be an aggregate of >50 cells. Results represent mean ± SE of data from at least three individual experiments. *, $P \leq 0.05$; **, $P \leq 0.01$.

30 μg/ml HOBS1 and HOBS2 for 48 h, the colony-formation rate of SMMC-7721 cells was reduced to 19.4% and 16.6%, respectively. These results demonstrated that HOBS was able to inhibit the anchorage-independent growth of SMMC-7721 cells and, as previously seen, HOBS2 exerted stronger inhibition than HOBS1.

4. Discussion

Conventional cancer therapies such as surgery, chemotherapy and radiotherapy have a number of limitations such as relatively high cost, development of resistance as well as many adverse effects. Therefore, there is a pressing need to search for new drugs and establish new therapeutic strategies that are safe and effective for cancer treatment. Differentiation therapy is a novel approach for the treatment of malignant tumour cells. In recent years, some substances that have the ability to induce differentiation have been identified, including ascorbic acid, cytokinins, retinoids, valproic acid (Short *et al* 2004; Catalano *et al* 2005; Ishii *et al* 2005; Sun *et al* 2006; El-Metwally and Pour 2007).

Among a number of substances isolated from plants, bisabolane-type sesquiterpenes represent one of the most important classes of biologically active compounds. Zhu *et al* isolated two new HOBS from *C. discoideum* (Zhu *et al* 1999), which showed strong antiproliferative and differentiation-inducing effects on SMMC-7721 cells.

SMMC-7721 cells have the morphology of typical malignant cells. From the results of scanning electron microscopy, we guess that HOBS could reduce the morphological features of cancer cells by inducing cell redifferentiation.

Generally, cell growth and differentiation are tightly controlled and coordinated to maintain normal tissue homeostasis. The commitment of cells to differentiate is generally made in the G1 phase of the cell cycle, and induction of differentiation is believed to require cell-cycle arrest (Pardee 1989). In this study, HOBS induced cell-cycle arrest at the G1 phase in SMMC-7721 cells. This result further confirms that HOBS participate in inducing cell redifferentiation. The number of SMMC-7721 cells in the subG1 phase increased when the HOBS concentration increased. Therefore, we consider that HOBS induces differentiation of SMMC-7721 cells and finally apoptosis as a physiological fate.

Based on the above investigations, we next decided to extend the series of experiments to validate the effect of HOBS on SMMC-7721 cells. It has been reported that AFP, γ-GT and TAT are biochemical markers of hepatocarcinoma (Pitot *et al* 1985; Li 1990; Ren *et al* 1998). AFP is an oncofoetal antigen normally produced by the liver and yolk sac during embryogenesis but not in normal adult tissues. AFP increases in the adult liver in response to liver regeneration or tumorigenesis, and is thus used for identifying liver disorders and cancers in adults. The level of γ-GT is extremely low in the adult liver, but its level gradually increases during the development of liver cancer. Thus, γ-GT is another marker for hepatocarcinoma. Contrary to γ-GT activity in the hepatocytes, TAT levels in normal hepatocytes is high, but decreases during the course of hepatic carcinogenesis. Therefore, TAT is also considered as a marker of hepatoma differentiation (Ren *et al* 1998). Therefore, evaluating the changes in these three substances in hepatoma cells is important for determining the effects of HOBS on SMMC-7721 cells. These results were consistent with our predictions: HOBS is able to inhibit AFP levels and γ-GT activity, and increase TAT activity. Thus, our findings indicate that HOBS is able to reverse the phenotype of human hepatocarcinoma and induce redifferentiation.

Soft agar culture is closer to the *in vivo* situation than normal culture, and anchorage-independent growth in soft agar has been used extensively in clinical and experimental oncology as an *in vitro* indicator of malignancy (Ye *et al* 2004). We observed that the malignant potential of SMMC-7721 cells was reduced after treatment with HOBS. The results confirmed that HOBS could induce differentiation of SMMC-7721 cells.

We also found an interesting phenomenon; HOBS2 exhibits stronger antiproliferative activity than HOBS1. It is expected that their different chemical structures may contribute to their differential effects on the cells and this phenomenon suggests that we can improve the activity of HOBS by optimizing the structures in further studies.

Normalizing tumour cells rather than killing them with highly cytotoxic chemicals or other agents that have side-effects is a new strategy for combating cancer. Differentiation inducers are such a class of antitumour agents that facilitate the differentiation of malignant neoplastic cells towards normalization. Our present work shows that HOBS1 and HOBS2 can inhibit proliferation of SMMC-7721 cells and induce redifferentiation. To our knowledge, this is the first report on the effects of HOBS on proliferation and differentiation of SMMC-7721 cells *in vitro*. HOBS and perhaps other bisabolane-type sesquiterpenes may be useful for the treatment of liver cancer. The molecular mechanisms by which HOBS exerts its antiproliferative effect on human hepatoma cells will be further studied in our laboratory. A limitation of this study is that the effect of HOBS has been shown on SMMC-7721 cells only. More work is required to find out the precise effect of HOBS on different types of cancer cells to understand whether the effect of HOBS is cell-line specific or via a general mechanism. These observations will provide a foundation for future studies *in vivo* and in clinical trials.

Acknowledgements

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