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Induction of Differentiation by Panaxydol in Human Hepatocarcinoma SMMC-7721 Cells *via* cAMP and MAP Kinase Dependent Mechanism

Ze-Jian Wang[a,‡], Li Song[a,‡], Lin-Chen Guo,[a] Min Yin,[a] and Yong Ning Sun[*,b]

[a]School of Pharmacy, Shanghai Jiaotong University, Shanghai 200240, China, and
[b]Department of Traditional Chinese Medicine, Sixth People's Hospital,
Shanghai Jiaotong University, Shanghai 200233, China

Panaxydol (PND) is one of the main non-peptidyl small molecules isolated from the lipophilic fractions of Panax notoginseng. The present study was carried out to demonstrate the potential effects of panaxydol on the induction of differentiation of human liver carcinoma cell lines SMMC-7721. Cell viability was evaluated by MTT method and Trypan blue exclusion assay respectively. The changes of morphology were detected by transmission electron microscope. Inhibitors were applied to detect the signaling pathway of differentiation. The level of intracellular cyclic AMP was determined by radioimmunoassay. The expression of p-ERK, Id1, and p21 were determined by Western blot. We found that panaxydol inhibit the proliferation of SMMC-7721 cells and caused the morphology and ultrastructure changes of SMMC-7721. Moreover, panaxydol dose-dependently increased the secretion of albumin and alkaline phosphatase activity, and decreased the secretion of AFP correspondingly. These changes of differentiation markers in SMMC-7721 can be reversed by the protein kinase A inhibitor RpcAMPS and by MAP kinase kinase 1/2 inhibitor U0126 or sorafenib. Intracellular cAMP was elevated by panaxydol in SMMC-7721 cells. Panaxydol dose-dependently decreased the expression of regulatory factors Id1 and increased the protein levels of p21 and p-ERK1/2 correspondingly. It suggested panaxydol might be of value for further exploration as a potential anti-cancer agent *via* cAMP and MAP kinase-dependent mechanism.

Key words——hepatocellular carcinoma; cell differentiation; signal transduction; Id

INTRODUCTION

Hepatic carcinoma is the most common digestive tumors with fatal outcome. Although a number of treatment strategies have been attempted, success in treating these tumors has been limited and the prognosis of hepatocellular carcinoma patients is poor. Traditional chemotherapy for patients with hepatic carcinoma has limited benefits and rarely increased the survival rate.[1] Up to now, Hepatic arterial infusion chemotherapy for hepatocellular carcinoma can double or triple the expected survival period for the patients. However, the long term survival of patients with hepatocellular carcinoma not only depends on how to control carcinoma proliferation but on how to preserve poor liver function. Epidemic studies suggest primary hepatic carcinoma had a strong association with hepatitis B virus (HBV) or hepatitis C virus (HCV) infection and liver cirrhosis.[2] Therefore, it seems more reasonable to induce the differentiation in hepatocellular carcinoma.

Panax notoginseng has the effects of stopping bleeding and relieving swelling to alleviate pain. It also has the action of regulating humoral and cellular immunity, protecting the liver. It is well known that polyacetylenes, especially panaxynol and panaxydol, among highly bioactive compounds that occur in many medicinal plants (*e.g.*, *Panax notoginseng*). They are likely to be responsible for at least a part of the beneficial of intake related plants. Panaxydol, a non-peptidyl small molecule showed stronger antiproliferation activity on several human cancer cell lines than that of panaxynol.[3,4] Our previous results suggested low doses of panaxynol can inhibit cell proliferation without killing cells directly.[5] Here we tested the hypothesis that panaxydol may induce the differentiation of hepatic carcinoma. Sorafenib (BAY 43–9006), an orally active multikinase inhibitor, is approved for the treatment of advanced renal cell carcinoma and hepatocellular carcinoma. Here, we chose it as a positive control to inhibit the proliferation of hepatocellular carcinoma cells. Our data suggest that panaxydol might be of value for further ex-

ploration as a potential anti-cancer agent *via* cAMP and MAP kinase-dependent mechanism.

MATERIALS AND METHODS

Materials   Panaxydol was isolated and purified as described previously from the roots of *P. notoginseng* and stored at $-20℃$. Its purity checked by GC is over 98％. Panaxydol stored at $-20℃$ and dissolved in DMSO before use. Final DMSO concentration in medium is $<0.1％$ (v/v). Culture medium DMEM (Dulbecco's modified Eagle's medium, high glucose) and fetal bovine serum were purchased from GIBCO (Grand Island, NY, USA); Rp-Adenosine 3′,5′,-cyclic monophosphothioate (RpcAMPS), H7, U0126, Methylthiazoletetrazolium (MTT) were products of the Sigma (St Louis, MO, USA); Sorafenib [N-(3-trifluoromethyl-4-chlorophenyl)-N′-(4-(2-methylcarbamoyl pyridin-4-yl)oxyphenyl)urea] was the product of Bayer Pharmaceutical (West Haven, CT, USA). Human alpha fetoprotein (AFP) ELISA kit was from ADI, (San Antonio, Texas, USA). Alkaline phosphatase (AKP) kit and albumin kit were bought from Jiancheng Institute of Biotechnology (Nanjing, China); BCA protein assay kit was from Pierce (Rockford, IL, USA). Rabbit polyclonal anti-phosphorylated-ERK 1/2 (p-ERK1/2) and mouse monoclonal anti-GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal anti-p21 and Id1 were offered from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). ECL Western blotting detection system was purchased from Amersham Biosciences (UK). All other reagents were of analytical grade. Sorafenib were also kept in DMSO solution and diluted with culture medium before the application.

Cell Culture and Cell Viability Assay   Human carcinoma cell line SMMC-7721 was bought from the cell bank of the Shanghai Institutes for Biological Sciences; Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10％ heat-inactivated fetal calf serum. Cell viability was evaluated by using a colorimetric assay with 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphe nyltetrazolium bromide (MTT), an indicator of mitochondrial respiratory chain activity. Briefly, logarithmically growing cells were grown at a density of $2×10^4$ cells/ml and treated with panaxydol or sorafenib for 5 days. The optical density of each well was measured at 490 nm (OD490) by using an enzyme-linked immunosorbent assay (ELISA) reader (Bio-Tek ELX800UV). The cells without panaxydol treatment served as negative controls, and the cells with sorafenib treatment served as positive controls. The proliferation of cells was determined by calculating the absorbance of the test wells as the percentage of the control well. Trypan blue exclusion assay was used to evaluate cytotoxic effects of panaxydol on SMMC-7721. After cells were removed from the bottom of the well, cell suspension was incubated with an equal volume of trypan blue dye (4％ W/V) for 5 min. 150 cells were counted per sample, and cells that failing to exclude the dye were considered nonviable.

Preparing Samples for Transmissional Electron Microscope   SMMC-7721 cells were seeded at the concentration of $2×10^5$ cells/ml. After cultured with 20 μM panaxydol for 5 d, the cells were prefixed with 2.5％ glutaraldehyde for 2 h, and then post-fixed with 1％ osmium tetroxide, dehydrated in graded alcohol, replaced in propene oxide. The samples were examined in a Philips CM 120 TEM microscope (Philips).

Assay for AFP, Albumin and Alkaline Phosphatase Activity   SMMC-7721 cells were seeded in 250 ml bottles $(1×10^6$ cells/ml) and allowed to grow for 24 h before being exposed to panaxydol or sorafenib. After incubation for 5 d, the culture supernatants were harvested for measuring the contents of AFP, albumin, and the activities of ALP. The protein concentrations in the lysates were detected by the BCA kit. The AFP level in culture medium was determined by ELISA according to the manufacture's instructions.[6] The albumin content was determined using a Bromocresol Green Assay kit according to the instructions prorided by the manufacturer.

The determination of ALP activity is based on the colorimetric assay. ALP hydrolyzes p-nitrophenyl phosphate into a yellow colored product (maximal absorbance at 405 nm). Enzyme activities are expressed in an international unit per liter (IU/L).

Inhibition of Signal Pathway   A 10 μM RpcAMP (antagonist of cAMP dependent protein kinase), 50 μM H7 (antagonist of protein kinase C), 5 μM U0126 (antagonist of MKK1 and MKK2) or 10 μM sorafenib (inhibitor of RAF/MEK/ERK pathway) were used respectively to observe their inhibitory effects on the differentiation induced by 20 μM panaxydol in SMMC-7721 cells. AFP, albumin and alkaline phosphatase activity were detected by commercial kits af-

ter 5 days incubation respectively.

cAMP Assay   SMMC-7721 cells were plated on Petri dishes (150000 cells/dish), preincubated for 30 min in Krebs-Ringer Hepes buffer with IBMX (10 μM) and incubated for 30 min in the presence of panaxydol. The incubation was stopped by the addition of $HClO_4$ (final concentration is 0.5 μM). The incubation medium was neutralized with KOH solution, centrifuged as required. cAMP was quantified as radioimmunoassay after acetylation as previously described.[7]

Western Blot   Cells were harvested after 5 days incubation with panaxydol. Immunoblotting analysis was carried out as previously described and the following antibodies were used: monoclonal antibody anti-p21 (Protein Tech, 1 : 1000), poly-anti-p-ERK1 /2 (Cell Signaling, 1 : 1000) or primary monoclonal antibody anti-Id.

Statistical Analysis   Data collected from 3 independent experiments were expressed as mean$\pm$ S.D. and analyzed by ANOVA for statistical significance test. Student-Newman-Keuls (SNK) post hoc testing was used to compare individual treatment groups.

RESULTS

Panaxydol Inhibited the Proliferation of SMMC-7721 Cells   The effects of panaxydol on proliferation were assessed by using MTT assay. Panaxydol inhibited the proliferation of SMMC-7721 cells in a dose dependent manner (Fig. 1). Since the MTT assay measures the metabolic activities of the cells, the reduction in the cell number could be a consequence of either cell death or the reduction in the cell proliferation. Therefore, we evaluated cytotoxic effects of panaxydol by Trypan blue assay. After 5 days incubation, the inhibition rate of 20 μM panaxydol was $46\pm$ 7.2% detected by MTT assay. Cell death rate of 20 μM panaxydol was only $4.8\pm1.2\%$ detected by trypan blue staining (Fig. 2). Ten μM sorafenib induced death rate by $30.4\pm4.2\%$ at 5 day post treatment.

Transmission Electron Microscopic Observation   Electron microscopic examination further revealed that untreated SMMC-7721 cells contained a large and irregular nucleus, loose chromatin, a distinct nucleolus and simple cytoplasmic organelles (Fig. 3A). By contrast, there was an increase in the amount of heterochromatin and in the number of mitochon-
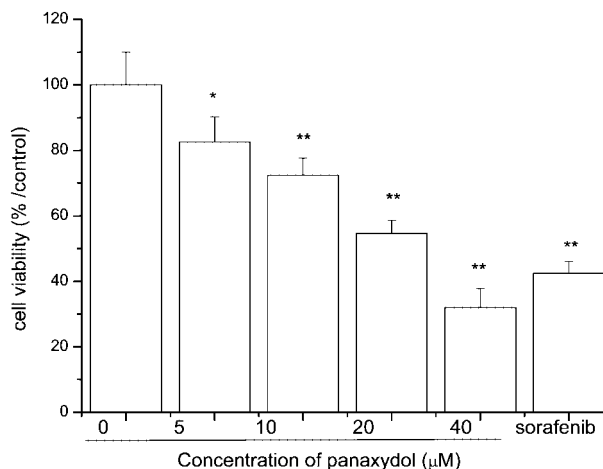

Fig. 1.   Effects of Panaxydol on the Proliferation of SMMC-7721 Cells after 5 Days Incubation

The error bars represent the standard error of the mean (S.E.M.) of at least three experiments ($n=6$). Cell concentration at 2×10^4 cell/ml.
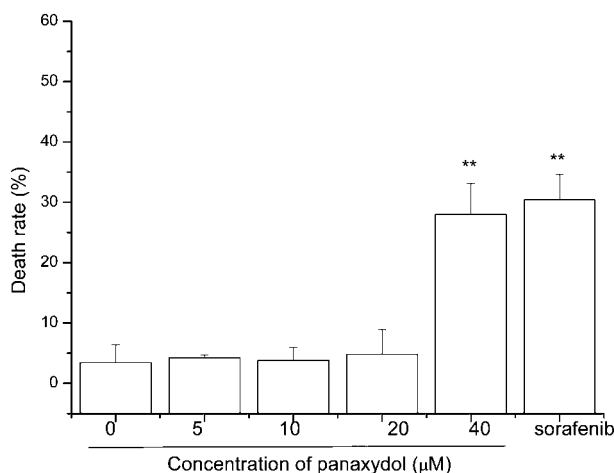

Fig. 2.   Effect of Panaxydol on Cell Cytotoxicity in SMMC-7721 Cells Determined by Trypan Blue Exclusion Assay

The error bars represent the standard error of the mean (S.E.M.) of at least three experiments ($n=6$).

dria in the panaxydol-treated cells (Fig. 3B). The Gogi complexes grew larger with an increase in the number of rough endoplasmic reticula. Additionally, the nucleus became smaller and the chromatin located along the nuclear edge became more condensed. However, no formation of apoptotic bodies was observed.

Panaxydol Induced Changes in AFP and Albumin Secretion and in Alkaline Phosphatase Activities in SMMC-7721 Cells   We further investigated the effects of panaxydol on the biochemical markers of SMMC-7721 cells. AFP is a single strand glycoprotein which shows a dramatic decline after birth. AFP ac-
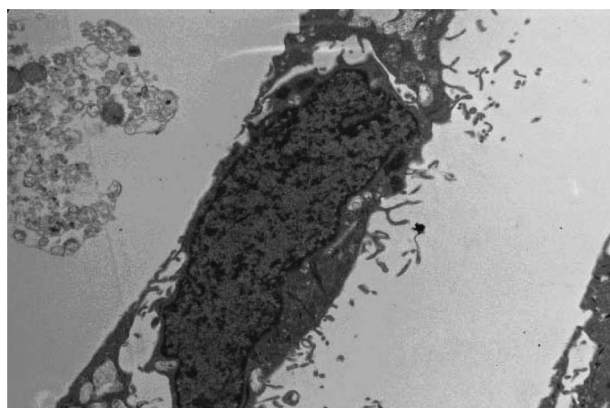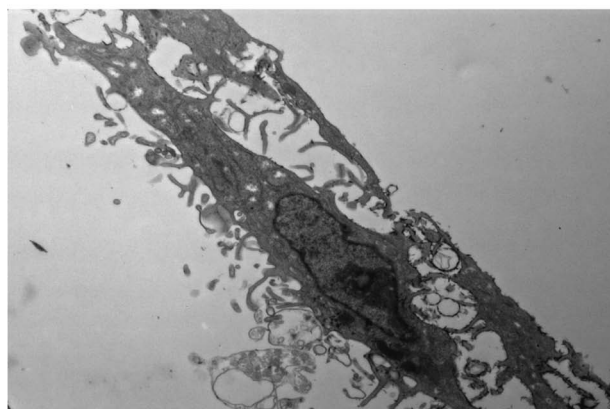
Fig. 3. Panaxydol Induces Morphological and Ultrastructural Changes in SMMC-7721 Cells

(A) Morphology of SMMC-7721 cells cultured without panaxydol. (B) Morphology of SMMC-7721 cells cultured with panaxydol (20 μM) for 5 days. Original magnification is 9700×.

Table 1. Effect of Panaxydol on AFP Secretion in SMMC-7721 Cells

groups	AFP (ng/10^6 cells)		
	Day 1	Day 3	Day 5
Control	12.9±0.7	15.2±2.3	16.6±1.5
Sorafenib (10 μM)	12.5±1.2	10.3±0.7**	7.2±1.3*
PND (10 μM)	12.7±1.7	12.9±1.9	10.5±0.3*
PND (20 μM)	11.4±1.3	9.4±1.7*	6.4±0.6*

Data are represented as mean±S.D. of three independent experiments ($n=3$). * $p<0.05$, ** $p<0.01$ *vs.* control group.

Table 2. Effect of Panaxydol on Albumin Secretion in SMMC-7721 Cells

Group	Albumin (g/l)		
	Day 1	Day 3	Day 5
Control	10.7±0.2	11.1±0.2	10.8±0.8
Sorafenib (10 μM)	11.2±0.5*	12.2±0.4**	11.7±1.1*
PND (10 μM)	13.6±0.2*	15.5±0.1*	18.2±0.6*
PND (20 μM)	15.8±0.4*	18.1±0.3*	22.5±1.1*

Data are represented as mean±S.D. of three independent experiments ($n=3$). * $p<0.05$, ** $p<0.01$ *vs.* control group.

Table 3. Effect of Panaxydol on Alkinailne Phosphatase (ALP) Activity in SMMC-7721 Cells

Group	ALP (U/g protein)		
	Day 1	Day 3	Day 5
Control	11.9±0.3	10.5±0.3	12.0±0.7
Sorafenib (10 μM)	12.6±1.1	11.6±2.4	12.7±1.7
PND (10 μM)	12.8±0.2	23.8±2.0**	37.9±0.9**
PND (20 μM)	17.7±0.5**	30.2±1.5**	57.7±4.4**

Data are represented as mean±S.D. of three independent experiments ($n=3$). ** $p<0.01$ *vs.* control group.

cumulation in serum is usually associated with liver regeneration or heptocarcinogesis.[8] Both panaxydol (20 μM) and sorafenib (10 μM) caused a markedly depression in the AFP level in the SMMC-7721 cells (Table 1). Additionally, panaxydol increased the level of albumin in the SMMC-7721 cells compared with that of controls (Table 2). The alkaline phosphatase activity in the panaxydol -treated groups was markedly increased compared with that in the control group. These effects appeared in dose-dependent manners. Ten μM sorafenib show no significant changes on the level of albumin and alkaline phosphatase activity compared with that in the control group (Table 3).

Inhibition of Signal Pathway The PKA inhibitor RpcAMPS as a non-selective cAMP antagonist competitively inhibits PKA and other enzymes associated with cAMP and U0126 or sorafenib an inhibitor of MKK1 and MKK2 in both inactive and active forms can serve as a powerful tool to investigate the activation of ERK1/2 by MKK1/2. As shown in Table 4, cotreatment of SMMC-7721 cells with RpcAMPS and with U0126 or sorafenib resulted respectively in significant inhibition in albumin secretion and ALP activity promoted by panaxynol, suggesting the association between both cAMP and ERK1/2 and the differentiation induced by panaxydol in SMMC-7721 cells. We observed also the effect of H7 (the relatively non-selective PKC inhibitor). Cotreatment of SMMC-7721 cells with 50 μM H7 in the presence of 20 μM panaxydol for 5 days resulted in only about 18% down-regulation of the ability of panaxydol to stimulate albumin secretion and ALP

Table 4.　Effects of Certain Inhibitors on the Differentiation in SMMC-7721 Cells Induced by Panaxydol

Group	AFP (ng/10^6 cells)	Albumin (g/l)	ALP (U/g protein)
Control	16.9±2.4	10.6±1.2	12.0±0.7
PND (20 μM)	6.8±0.5*	23.8±2.4**	56.4±4.5**
PND (20 μM) + RpcAMPS	15.4±2.8	12.8±2.3	12.1±1.8
PND (20 μM) + H7	8.6±2.1	20.8±3.2*	49.8±6.2**
PND (20 μM) + U0126	10.3±2.4*	18.2±3.2*	42.1±1.8*
PND (20 μM) + sorafenib	8.9±1.5*	17.2±3.8*	44.2±2.6*

Data are represented as mean±S.D. of three independent experiments ($n=3$). * $p<0.05$, ** $p<0.01$ *vs.* control group.
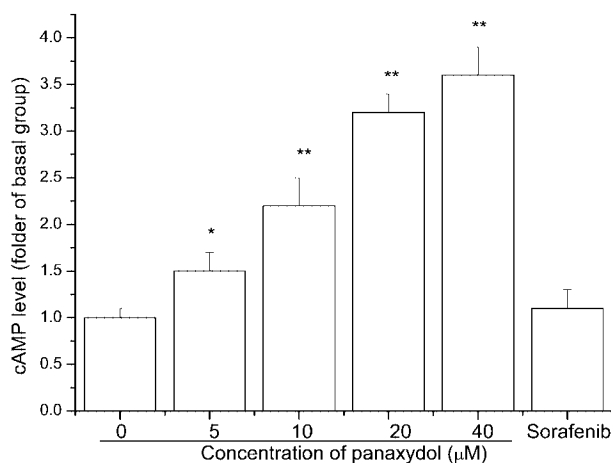

Fig. 4.　cAMP Levels in SMMC-7721 Cells after Panaxydol Treatment

　SMMC-7721 cells were incubated for 30 min in the presence of indicated concentration of panaxydol. The incubation was stopped by the addition of 250 μl of HClO$_4$. The solution was neutralized and centrifuged. cAMP was quantified by radioimmunoassay after acetylation. Results are expressed as mean±S.D. for three independent experiments. *$p<0.05$, **$p<0.01$ *vs.* control group.

activity for H7 group and about 19% down-regulation for AFP secretion (Table 4). The weak inhibitory effect of H7 on differentiation marker might be the result of diminution of panaxydol-induced ERK phosphorylation

Panaxydol Enhances Intracellular cAMP Accumulation　Elevation of intracellular cAMP alone has been reported to promote tumor differentiation.[9] We investigated the effects of panaxydol on cAMP accumulation in the cytoplasm. Panaxydol dose-dependently induced intracellular cAMP accumulation as it did for the reduction of multiplication in SMMC-7721 cells (Fig. 4).

Panaxydol Regulated the Expression of p-ERK1/2, Id1 and p21　ERKs are mainly involved in cell proliferation and differentiation processes. Id proteins were initially discovered as proteins involved in the negative control of cell differentiation.[10] We exa-

mined the effects of panaxydol on p-ERK, Id1 and P21. We found that the Id1 expression was significantly reduced after 5–20 μM panaxydol treatment for 5 days. By contrast, panaxydol exposure induced significantly increased of P21 and p-ERK expression (Fig. 5).

DISCUSSION

　Cell proliferation is delicately balanced with cell differentiation and cell death to maintain a constant cells number in adult tissues and organs. The imbalance signals which in favor of proliferative signals, often as a result of genetic mutations lead to malignancy. Therefore, cell death pathways are often considered to be a reasonable tumor-suppressor mechanism to maintain tissue homeostasis. In contrast to other cancers, the prognosis of patients with hepatocellular carcinoma (HCC) is not only related to tumor proliferation, but also to liver function.[11] In eukaryotic tissues, cell differentiation is associated with decreased proliferation. Although sorafenib prolongs overall survival and delays the time of progression in patients with advanced hepatocellular carcinoma, the survival benefit of sorafenib is still not satisfactory. In the Asia–Pacific trial, median overall survival was 6.5 months in the sorafenib arm compared to 4.2 months in the placebo arm.[12] Given this context, cell differentiation may be more suitable for the treatment of hepatocellular carcinoma.

　Hepatocellular carcinoma affects more than 500000 people in the world a year, more than 50% of whom are in China. SMMC-7721 is a poor differentiated primary human hepatocarcinoma cell line separated from an old Chinese patient. The cells possess high capacity for tumorigenicity and low capacity for metastasis.[13] Our results from the MTT assay showed that panaxydol exhibited a dose and time dependent inhibitory effect on SMMC-7721 cell prolifer-
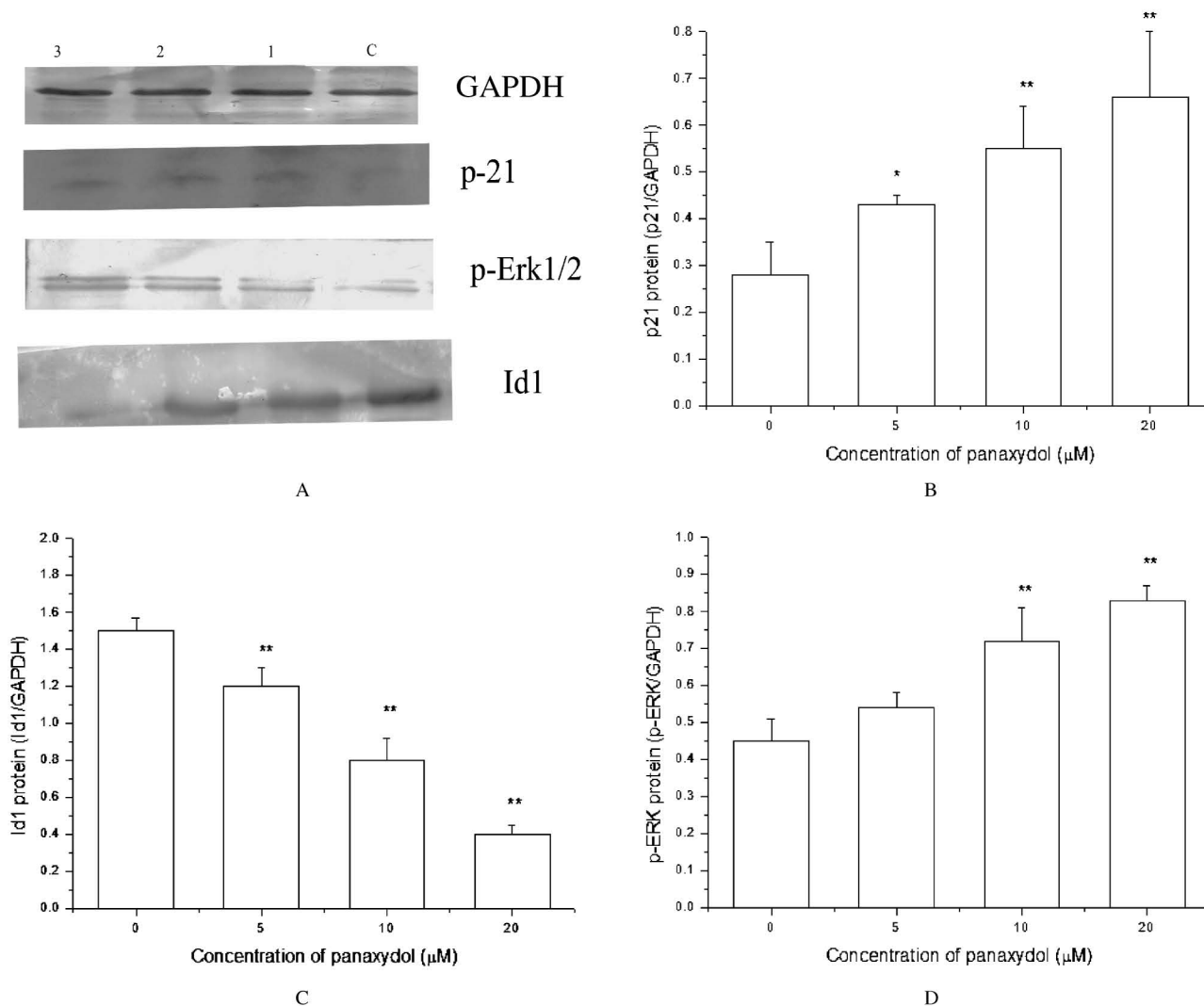
Fig. 5.   Panaxydol Regulates the Expression Levels of p21, p-ERK and Id1 in SMMC-7721 Cells

The levels of p21, p-ERK and Id1 were examined by immunoblotting analysis after 5 days incubation with 5, 10 and 20 μM panaxydol. Lane C: control group, Lane 1~3: SMMC-7721 cells treated with 5, 10, 20 μM panaxydol respectively. Data (mean±S.D.) were summarized from three independent experiments ($n=3$), *$p<0.05$, **$p<0.01$ vs. control group.

ation. Trypan blue exclusion assay suggest that the low dosage of panaxydol (5–20 μM) mainly inhibit cell proliferation instead of killing cells directly. Sorafenib (10 μM) significantly induced cell death after 5 days treatment in SMMC-7721. These results suggest that panaxydol and sorafenib inhibit the proliferation of SMMC-7721 cell through different mechanisms.

20 μM panaxydol induced typical morphological and ultrastructural changes in the SMMC-7721 cells associated with features of cellular differentiation, which were evidenced by reduction in cell volume and the size of the nucleus, chromosomal condensations, and cytoplasm augmentmentation accompanied by the proliferation of mitochondria, rough endoplas-

mic reticula and Golgi complexes. These results indicate that panaxydol can induce the differentiation of SMMC-7721 cells into more mature hepatic cells.

AFP synthesis is not closely linked with cell proliferation but undifferentiated parenchymal cells.[14] The mechanism of the reduction of AFP secretion by panaxydol may results from the inhibition of undifferentiated parenchymal cells or cell death. Albumin, on the other hand, is a protein secreted by mature hepatocytes and its secretion is one of the markers of hepatocyte differentiation. We found that panaxydol caused a marked reduction in AFP secretion and an increase in albumin secretion in the panaxydol-treated SMMC-7721 hepatocarcinoma cells. Alkaline phosphatase (ALP) is a hydrolase en-

zyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and alkaloids. Membrane-bound ALP is involved in the regulation of phosphorylation of plasma membrane-bound proteins.[15,16] Several lines of evidence suggest that the markedly elevation of membrane-bound ALP activity in tumor cells is the biochemical marker of differentiation.[17,18] Panaxydol increased the alkaline phosphatase activity in SMMC-7721 cells. These results suggest that panaxydol may reverse the phenotype of human hepatocarcinoma cells through the induction of their differentiation towards more mature forms of hepatocytes. Although sorafenib (10 μM) significantly inhibit the AFP secretion, it did not change the AFP and albumin secretion in SMMC-7721 cells. Taken together, the data suggests that sorafenib inhibit AFP secretion by the inhibition of undifferentiated parenchymal cells or cell death.

HCC cells differentiation is a complex process that includes both morphological and biochemical changes. The most obvious phenomena are the decrease in cell multiplication, and the emergence of normal functions. During differentiation, cells start to express some chemical coding genes that provide their functional identity, which is believed to involve participation of the mitogen-activated protein kinase (MAPK), cAMP-dependent protein kinase A (cAMP/PKA) and/or protein kinase C (DAG/PKC)-mediated signaling pathways.[7,19,20] Using the special antagonists of signal transduction, we found that the differentiation of HCC cells induced by panaxydol may be associated with the activation of cAMP dependent protein kinases and that of MAPK pathway. Bidirectional cross talk between Erk and cAMP signaling could explain this effect. cAMP could directly modulate MAPK pathway by binding to and activating EPAC, a specific GTP exchange-protein for the small GTPase Rap1.[21] Although the role of Rap1 in the MAPK pathway is not completely understood, it seems to act by binding to and activating B-Raf and/or inhibiting the Ras-Raf pathway. Sorafenib can inhibit the proliferation of SMMC-7721 cells by RAF/MEK/ERK pathway. When cells incubated with panaxydol and sorafenib simultaneously, sorafenib did reduce the differentiation effects by panaxydol in SMMC-7721 cells. These results further suggest that Erk/MAPK pathway plays an important part in the differentiation of SMMC-7721 cells induced by panaxydol.

We have found that panaxydol elevated cAMP might be not corresponded to its cytotoxicities potency. Panaxydol (5−40 μM)-induced growth inhibition in SMMC-7721 cells was consistent with the increase of intracellular cAMP level. Panaxydol (40 μM)-induced cytotoxicity in SMMC-7721 cells was inversely proportional to the intracellular level of cAMP. Therefore, the mechanism by which high doses of panaxynol treatment cause cell death still need further explore.

The ERK1/2 signaling pathway is preferentially activated in response to growth factors and phorbol ester, and regulates cell proliferation and differentiation.[22] The present data emphasize on the significant role of this ERK 1/2 in cell differentiation. The differentiation induced by 3-hydrogenkwadaphnin or 12-o-tetradecanoylphorbol 13-acetate showed the upregulation of p-ERK and p21 in k562 or HL-60 cells respectively.[23,24] It has been reported that sustained post-transcriptional p21 expression is dependent on ERK activation.[25] The inhibitors of differentiation/DNA binding proteins (Id proteins) have been shown to inhibit differentiation and to potentially stimulate proliferation. Overexpression of Id proteins are associated with poor clinical outcome in certain cancers.[26,27] p21WAF1/CIP1, one CKI of CIP/KIP family, binds to a number of cyclin/CDK complexes such as cyclin D1/CDK4, cyclin E/CDK2 and cyclin A/CDK2, inhibits kinases activities and induces cell cycle arrest and cell differentiation.[28] Overexpression of Id1 promotes cell proliferation potentially through inhibition of p21 expression.[28] These findings together indicated that panaxydol affected cellular differentiation in part by reducing the expression of Id1 and upregulating the expression of p-ERK and p21.

In conclusion, our results indicate that panaxydol might be of value for further exploration as a potential anti-cancer agent.

Conflicts of interest   The authors have no conflicts of interest to declare in relation to this article.

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