Anticancer activity and mechanisms of diacetyldianhydrogalactitol on hepatoma QGY-7703 cells

Xue Yan Zhang, Xue Lian Yan, Wei Guo, Bo Xu, Min Li, Ying Zhou and Jing Rong Cui

Diacetyldianhydrogalactitol (DADAG) is a member of the hexitols which shows a significant anticancer effect. Despite the fact that the antitumor effects of DADAG have been studied in a number of cell lines, the mechanism of its action remains unclear. Herein, we explored antitumor effects of DADAG and the possible mechanisms by which it inhibited the growth of human hepatocellular carcinoma cell QGY-7703 and its derived xenograft tumors. Cell proliferation was evaluated with the sulforhodamine B assay *in vitro*. The results suggested that DADAG had mild antiproliferative activity on QGY-7703 cells. The antitumor effect of DADAG was assessed in nude mice xenografted with QGY-7703 cells. We found that DADAG significantly inhibited the tumor growth. Flow cytometry results indicated that the retarded cell proliferation is associated with increased G_2/M cell cycle arrest. Further studies showed that the induced G_2/M cell cycle arrest is, at least partially, attributed to an upregulation of cyclin B1, phospho-cell division cycle 2 (cdc2) (Thr^{15}), phospho-cdc2 (Thr^{161}), and cdc25c protein expression, and a decrease in cdc2 protein expression. Taken together, our data show that DADAG has mild proliferative effects on QGY-7703 cells *in vitro*, but it significantly inhibits the growth of QGY-7703 in a xenograft model *in vivo*. The modulation of several cell cycle progression regulation proteins responsible for G_2/M phase transition may account for its antitumor effects. *Anti-Cancer Drugs* 20:926–931 © 2009 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Hepatoma (hepatocellular carcinoma) is the fifth most common malignancy in the world and causes more than 1 million deaths each year [1]. Of the estimated 350 000 new cases/year in Asia, one third occur in China [2] and the incidence in China has been increasing in recent years, which has motivated a great deal of interest in finding more effective chemotherapeutic agents to treat this entrenched disease.

In 1957, Vargha *et al.* [3] initially applied sugar as the carrier of alkylating agent to synthesize mannomustine (BCM), which was effective for leukemic lymphadenosis and lymphadenoma. It led to the study of cytostatic sugar derivatives known as the hexitols. In this series, diacetyldianhydrogalactitol (DADAG) could easily penetrate the blood–brain barrier and exhibited higher antitumor activity and less toxicity and was more amphiphilic than other hexitols [4–6]. Therefore, DADAG was the most promising member of the hexitols. However, so far, no studies have reported the effects of DADAG on human hepatocellular carcinoma cell line QGY-7703. In this study, we investigated the antiproliferation effects of DADAG on QGY-7703 xenograft tumors using a proliferation assay *in vivo*. We also explored the possible mechanisms responsible for DADAG-induced growth inhibition on QGY-7703 cells.

Materials and methods

Cell culture

A hepatocellular carcinoma cell line (QGY-7703) was purchased from the Department of Genetics of Fudan University (Shanghai, China); the characteristics of QGY-7703 cells have been published elsewhere [7]. QGY-7703 cells were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL, Grand Island, New York, USA), supplemented with 10% heat-inactivated fetal calf serum and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). Cells were incubated at 37°C with 5% carbon dioxide at 95% humidity.

Drug preparation

DADAG was provided by the Guangxi Institute of Traditional Chinese Medicine (Guangxi, China) (Fig. 1). DADAG was dissolved in phosphate-buffered saline (PBS) to a concentration of 10 mmol/l as stock solution and was further diluted in PBS for in-vitro experiments under pathogen-free conditions. Cyclophosphamide (CPA) was

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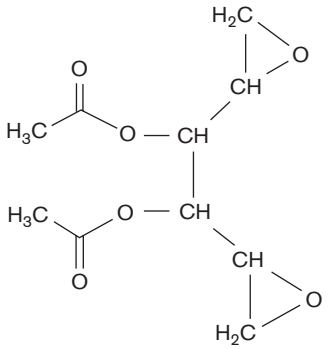
Fig. 1



The structure of diacetyldianhydrogalactitol. Molecular weight: 230.215.

purchased from the Shanghai Hualian Pharmaceutical Co. Ltd. (Shanghai, China). DADAG and CPA were then dissolved in normal saline for in-vivo experiments.

In-vitro proliferation assay

Growth inhibition was evaluated by using a sulforhodamine B (SRB) assay. SRB was obtained from Sigma-Aldrich (Sheboygen, Wisconsin, USA). Briefly, QGY-7703 cells were plated in 96-well microplates (2×10^4/well). After 24 h of incubation, the cells were treated with PBS and DADAG for 48 and 72 h. The cells were then fixed with 10% trichloroacetic acid at 4°C for 1 h. Plates were read at 540 nm for SRB staining by using a FLUOstar OPTIMA microplate multi-detection reader (BMG, Offenburg, Germany). Background control wells were included in each experiment. The morphology of QGY-7703 cells treated with 40 μg/ml DADAG for 72 h compared with untreated control group were observed under microscope.

Tumor model and treatment

Male nude mice (5–6 weeks old) were provided by Beijing Vital Laboratory Animal Technology (Beijing, China). Animal procedures were approved by the Department of Laboratory Animal Science of Peking University Health Science Center (Beijing, China). The mice were fed with sterile water and mouse chow. To the backs of 36 nude mice, 0.2 ml of QGY-7703 cell suspension (1.0×10^8/ml) was injected subcutaneously. After solid tumors developed, the mice were randomized into a normal saline control group (0.1 ml/10 g/day), a CPA group (30 mg/kg/2 days), and two DADAG groups (5 and 10 mg/kg/day), with nine mice in each group. Drugs were administered to the mice intraperitoneally for 15 days. Each mouse was weighed three times per week. At the end of the experiment, mice were euthanized and tumors were harvested and examined by hematoxylin and eosin staining. The tumor inhibition rate was calculated as $(1 - T/C) \times 100$, where T is the mean tumor weight of the treated group and C is the mean tumor weight of the normal control group [8].

Flow cytometry analysis

Cell cycle was determined by flow cytometry analysis. In summary, 2×10^6 cells treated with 10 μg/ml DADAG were harvested and washed twice with PBS. The cells were then fixed in 70% cooled ethanol at 4°C, until the beginning of the next procedure. The cells were centrifuged and ethanol was removed. Then the cells were incubated in PBS containing 10 μg/ml Rnase A at 37°C for approximately 30 min. Before examining, 50 μg/ml propidium iodide was added to the cells suspension. DNA content and cell cycle distribution were determined using a BECScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) equipped with an argon-ion laser set to excite at 488 nm. The analysis of cell cycle distribution was performed using the Cell-Quest and the Modfit software (Becton Dickinson).

Western blotting

QGY-7703 cells were treated with DADAG (10 μg/ml) for different time as indicated. After washing twice with cold PBS, equal amounts of cells were lysed with lysis buffer containing 62.5 mmol/l Tris–HCl (pH 6.8), 4 mol/l urea, 2% sodium dodecyl sulfate, 10% glycerol, 0.4% bromophenol, and 5% β-mercaptoethanol. Equal amounts of protein (40 μg/lane) from control cells and treated cells were subjected to electrophoresis in a 12.5% sodium dodecyl sulfate-acrylamide gel followed by electroblot transfer to Hybond C membranes (Amersham, Arlington Height, Illinois, USA). For immunodetection, membranes were blocked with 3% albumin fraction δ in Tris-buffered saline with 10% Tween-20 and were probed with primary antibodies specific to cyclin B1, β-actin, cell division cycle 2 (cdc2), phospho-cdc2 (Tyr^{15}), phospho-cdc2 (Thr^{161}) (Cell Signaling, Beverly, Massachusetts, USA), Cdc25c (Santa Cruz Biotech, California, USA) at 4°C for 16 h. After being washed three times with Tris-buffered saline–10% Tween-20 for 10 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich) for 1 h. The membranes were washed again and detection was performed using the enhanced chemiluminescence blotting detection system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Statistical analysis

Data are expressed as mean ± SD of three determinations. Differences between groups were assessed by using the Student's *t*-test. Differences were considered significant at P less than 0.05.

Results

Diacetyldianhydrogalactitol inhibits the proliferation of QGY-7703 cells *in vitro*

SRB assay was used to determine the effect of DADAG on the proliferation of QGY-7703 cells. As shown in Table 1, the inhibition rate of DADAG increased

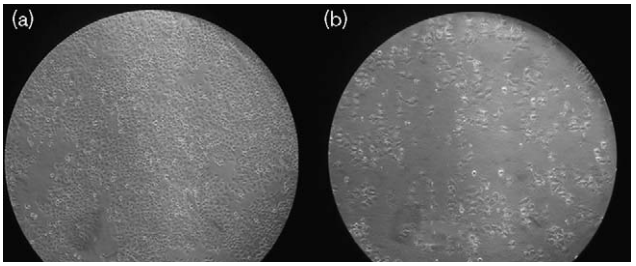
gradually with increasing concentration (0, 2.5, 5, 10, 20, 40 μg/ml), to a maximum of 47.9% for 72 h at the maximum concentration. These data suggested that DADAG exerted a mild inhibitory effect on QGY-7703 cells *in vitro*. The morphology of QGY-7703 cells treated with 40 μg/ml DADAG or vehicle control (PBS) was showed in Fig. 2. In the control group, cells grew well and arranged in regular size. In contrast, cells in the DADAG-treated group showed a disorder with enlarged size and unclear membrane. QGY-7703 cells were treated with DADAG in different concentrations (0, 2.5, 5, 10 μg/ml) for 48 and 72 h. The inhibition rate of DADAG was increased in both a time and concentration-dependent manner. The result is shown in Fig. 3.

Table 1 Effect of DADAG on QGY-7703 cells for 72 h

DADAG concentration (μg/ml)	OD value (mean ± SD)	Inhibition rate (%)
0	2.34 ± 0.63	0
2.5	2.25 ± 0.67	3.9
5	1.90 ± 0.71*	19.0
10	1.49 ± 0.42*	36.5
20	1.52 ± 0.27*	35.3
40	1.22 ± 0.37*	47.9

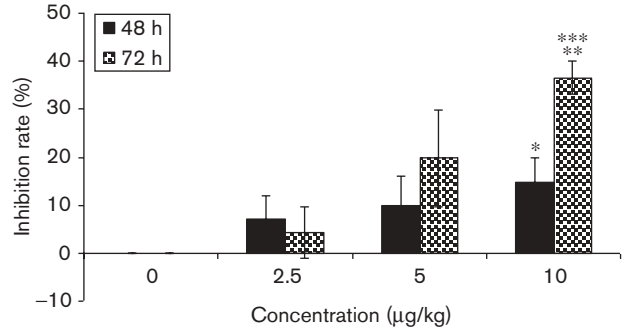
QGY-7703 cells were treated with DADAG in different concentrations for 72 h and the growth inhibition was determined by the sulphorhodamine B assay. The inhibition rate of DADAG was increased in a dose-dependent manner. These data suggested that DADAG had a mild inhibitory effect on QGY-7703 cell *in vitro*. Values are mean ± SD.
DADAG, diacetyldianhydrogalactitol; OD, optical density.
*$P<0.001$ versus phosphate-buffered saline group by Student's *t*-test ($n=3$).

Fig. 2



Morphology of QGY-7703 cells under a microscope (×40).
(a) Untreated control group; (b) diacetyldianhydrogalactitol (DADAG)-treated group. QGY-7703 cells were treated with 40 mg/kg DADAG for 72 h compared with untreated control group. In the control group, cells grew well and arranged in a regular size. Cells in the DADAG-treated group showed disorder with a larger size and unclear membrane.

Fig. 3



Effect of diacetyldianhydrogalactitol (DADAG) on QGY-7703 cells in different concentrations for 48 and 72 h. The inhibition of DADAG towards QGY-7703 cell proliferation occurred in both a time and concentration-dependent manner. *$P<0.05$; **$P<0.01$ versus PBS group; 72 h versus 48 h, ***$P<0.001$ by Student's *t*-test ($n=3$).

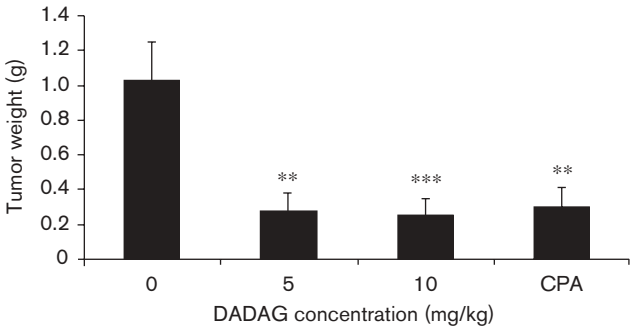
Inhibition of tumor growth by diacetyldianhydrogalactitol

Tumors in the DADAG-treated mice weighed significantly less than those in saline-treated mice ($P < 0.01$) (Fig. 4). It could also be observed by the size shown in Fig. 5. The inhibition rate of DADAG was close to 70.0% in the low-dose group. At the end of the experiment, all the mice were alive. Hematoxylin and eosin staining showed the cell morphology in different groups (Fig. 6). Tumor cells in the saline group were regularly arranged in similar size while cell size in DADAG-treated groups showed disorder; with the increase of DADAG concentration, the cells became incomplete and vacuoles were observed inside the cells.

Effect of diacetyldianhydrogalactitol on body weight of nude mice

Each mouse was weighed three times per week. All four groups of mice increased in weight after drugs were administered to the mice intraperitoneally for 15 days. However, the increase of body weight in the saline group was faster than in the other three groups. A significant difference was observed from day 7 in the CPA group and the DADAG5.0 group, as well as from day 13 in the DADAG10.0 group (Fig. 7).

Fig. 4



Effect of diacetyldianhydrogalactitol (DADAG) on QGY-7703 cells xenograft tumor. To the back of nude mice, 0.2 ml of QGY-7703 cell suspension (1.0×10^8/ml) was inoculated subcutaneously. After tumor formation, the mice were treated with normal saline (NS) (0.1 ml/10 g/day), DADAG (5 and 10 mg/kg/day), and cyclophosphamide (CPA) (30 mg/kg/2 days) for 15 days. At the end of the study, tumors were harvested and measured. DADAG significantly inhibited the growth of QGY-7703 cells xenograft tumor. The inhibition rate of both DADAG groups and the CPA group was 65.2, 68.3, and 62.4%, respectively. The results are presented as the mean tumor weight ± SD obtained from nine mice in each group. **$P<0.01$; ***$P<0.001$ versus NS group by Student's *t*-test ($n=9$).

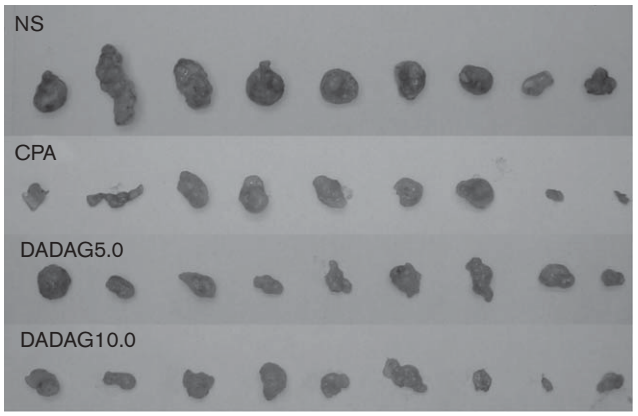
Effect of diacetyldianhydrogalactitol on cell cycle in QGY-7703 cells

On the basis of the results of the anticancer evaluation in both the in-vitro and the in-vivo mouse models, we then explored DADAG in our flow cytometry analysis. DADAG first caused S-phase arrest before the first 24 h and then showed significant G_2/M arrest with a concomitant decrease of the cell population in G_1 phase; the results showed a significant difference (Table 2).

Diacetyldianhydrogalactitol regulates the expression of G_2/M cell cycle-related proteins

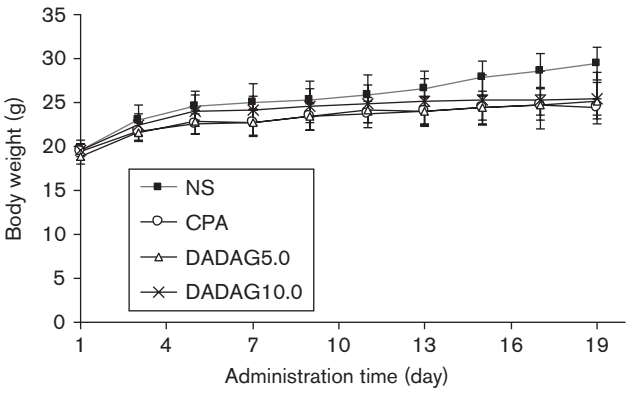
Next, we investigated the effect of DADAG on the level of several G_2/M-related proteins in QGY-7703 cells.

Fig. 5



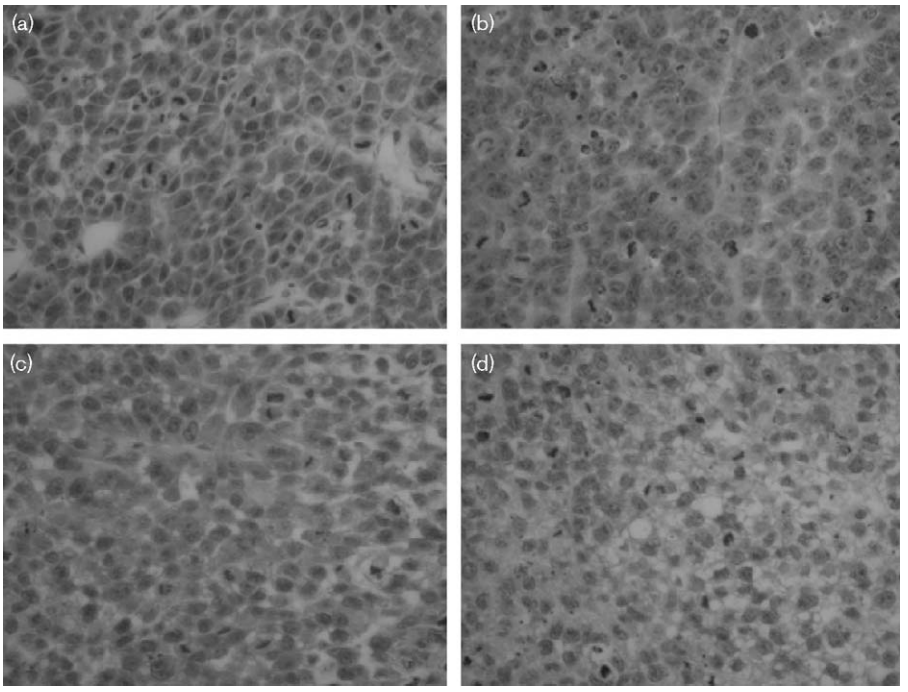
QGY-7703 xenograft tumors after 15 days therapy. Tumor size in the diacetyldianhydrogalactitol (DADAG)-treated group was obviously less than that in the saline-treated group. CPA, cyclophosphamide; NS, normal saline.

Fig. 7



The change of body weight in QGY-7703 cells xenograft tumor model. Significant difference began to show on the seventh day both in CPA groups and DADAG5.0 groups, on the thirteenth day in DADAG10.0 group versus the saline group. CPA, cyclophosphamide; DADAG, diacetyldianhydrogalactitol; NS, normal saline.

Fig. 6



Hematoxylin and eosin staining for liver cancer wax sample (×400). (a) Physiological saline group; (b) cyclophosphamide group; (c) DADAG5.0 group; (d) DADAG10.0 group. DADAG, diacetyldianhydrogalactitol.

Table 2 Effect of 10 μg/ml DADAG on cell cycle in QGY-7703 cell

DADAG treated time (h)	Cell cycle (%)		
	G_1 (mean ± SD)	S (mean ± SD)	G_2/M (mean ± SD)
0	54.4 ± 1.8	27.4 ± 2.0	18.3 ± 0.2
12	48.4 ± 3.1	34.9 ± 5.0	16.7 ± 1.9
24	30.7 ± 3.5*	42.4 ± 2.4*	27.0 ± 1.1
48	25.9 ± 0.4*	22.1 ± 0.2	52.0 ± 0.6**

Cell cycle (%) are the mean ± SD of three independent experiments.
DADAG, diacetyldianhydrogalactitol.
Student's *t*-test, $*P<0.05$, $**P<0.01$ compared with control (treated time 0).

Particularly, we focused on B-type cyclins (the key regulator of G_2/M progression) and D-type cyclin-dependent kinases (cdks), such as cdc2 and cdc25c. We found that DADAG (10 μg/ml) treatment decreased the expression of cdc2 and increased the expression of cyclinB1, phospho-cdc2 (Thr^{15}), phospho-cdc2 (Thr^{161}), and cdc25c in a time-dependent manner in QGY-7703 cells (Fig. 8a). We also found that the effects of DADAG on these protein regulation occurred in a dose-dependent manner when the treatment persisted for more than 48 h (Fig. 8b).

Taken together, these data provide evidence that the molecular events involved in DADAG-induced QGY-7703 cells G_2/M cycle arrest include the cdc2 downregulation and cyclinB1, phospho-cdc2 (Thr^{15}), phospho-cdc2 (Thr^{161}), and cdc25c upregulation.

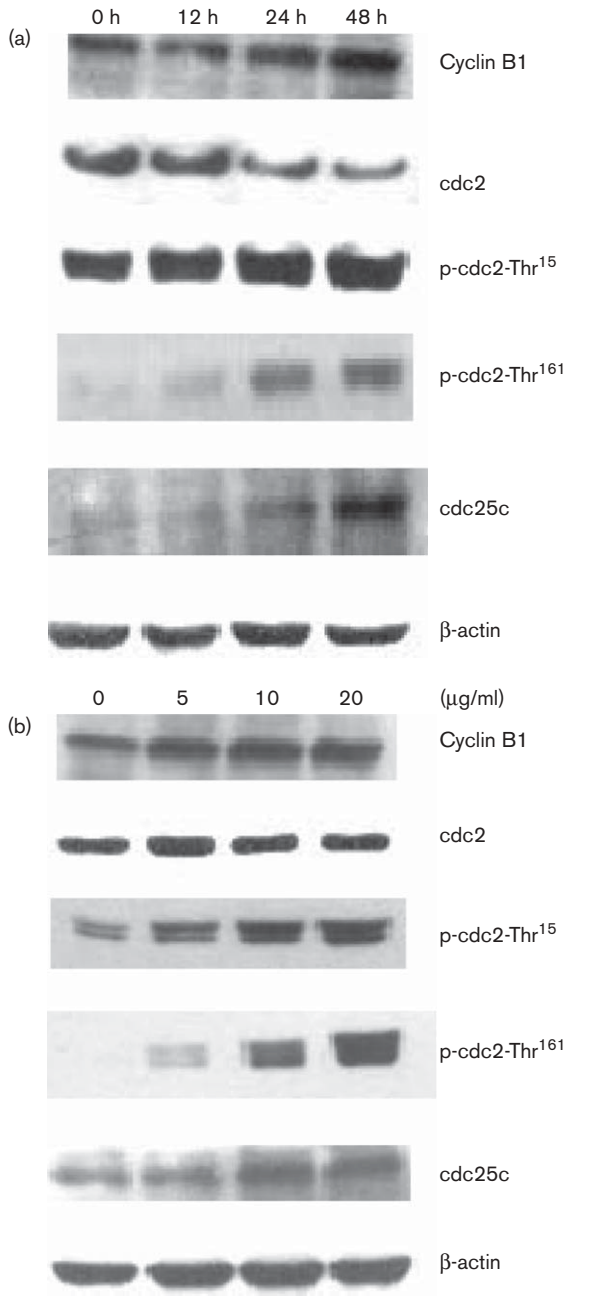
Discussion

DADAG, a promising member of the hexitols, exhibited antitumor effect on several tumor types [9]. Our study confirmed that DADAG exerted mild inhibitory effect on QGY-7703 cells *in vitro*, whereas it significantly inhibited the growth rate of tumor derived from QGY-7703 cells in a xenograft tumor model, with altered cell size and morphology.

DADAG was able to inhibit cell proliferation in a dose and time-dependent manner on QGY-7703 cells. Flow cytometric analysis showed that QGY-7703 cells treated with 10 μg/ml DADAG for different times showed increased accumulation in G_2/M phase.

Cell cycle regulation is a hallmark of tumor cells. Regulation of proteins that mediate critical events in the cell cycle can be a useful method for treating tumors [10]. The cell cycle is mediated by a highly conserved family of protein kinases, the cdks [10–12]. Activation of a cdk requires binding to a specific regulatory subunit, termed a cyclin. Together, these cyclin/cdk complexes are the cell cycle regulators. B-type cyclins, which are also associated with cdc2, control entry into mitosis. The cyclinB1/cdc2 complex was originally defined as the maturation-promoting factor or M-phase-promoting factor [13,14]. Regulation and the degradation of cyclinB1/cdc2 complexes at multiple levels ensure the tight regulation of the timing of mitotic entry and cell division. Without the synthesis of cyclin B1 before the G_2/M transition,

Fig. 8



(a) Regulation of cell cycle-related protein expression on QGY-7703 cells treated with 10 μg/ml diacetyldianhydrogalactitol (DADAG). Immunoblots were probed with antibodies specific for cyclin B1, cdc2, phospho-cdc2 (Thr^{15}), phospho-cdc2 (Thr^{161}), cdc25c. DADAG downregulated the expression of cdc2 and upregulated the expression of cyclin B1, phospho-cdc2 (Thr^{15}), phospho-cdc2 (Thr^{161}), cdc25c in a time-dependent fashion. Beta-actin level was examined as a loading control. (b) Regulation of cell cycle-related protein expression on QGY-7703 cells treated with DADAG. DADAG downregulated the expression of cdc2 and upregulated the expression of cyclin B1, phospho-cdc2 (Thr^{15}), phospho-cdc2 (Thr^{161}), cdc25c in a dose-dependent fashion. Beta-actin level was examined as a loading control. cdc, cell division cycle protein.

cdc2 remains inactive and the cell does not enter mitosis. The cell cycle will arrest at the G2 phase. If cyclin B1 is not degraded by ubiquitin [15], cells will not exit the mitosis. In addition, activation of cdc2 is controlled at several steps including cyclin B1 binding, phosphorylation of cdc2 at Thr^{161} and dephosphorylation of cdc2 at Thr^{14}/Tyr^{15}. In this study, we found that DADAG arrested QGY-7703 cells in the G_2/M phase. Western blot analysis showed that the expression of cdc2 was decreased and cyclin B1 was accumulated, which suggests that DADAG arrests the cells at the G_2 phase and prevents them from entering the mitosis by regulating cyclinB1/cdc2 complex. Phosphorylation on Thr^{161} is necessary for the activation of cdc2 kinase. In contrast, phosphorylation on either Thr^{14} or Tyr^{15} dominantly inhibits its activation. Cdc25c can activate cyclinB1/cdc2 complexes through hydrolyzing the phosphate group on Thr^{14}/Tyr^{15} [16]. Our results implied that the increase of Tyr^{15} led to the inactivation of cdc2, and therefore the combination of cdc2 and cyclin B1 was prevented, which contributed to the arrest of the cell cycle in the G_2/M phase.

In summary, DADAG displays a mild antiproliferative effect on QGY-7703 cell proliferation *in vitro*, whereas it significantly inhibits the growth of xenograft tumors derived from QGY-7703 cells. DADAG treatment also was able to alter QGY-7703 cell morphology. The mechanism by which DADAG inhibits tumor growth is related to cell cycle G_2/M phase arrest, which is because of the deactivation of cdc2 and cyclin B1, with concomitant accumulation of phospho-cdc2 (Thr^{15}), phospho-cdc2 (Thr^{161}) and cdc25c protein. This preliminary study indicates that DADAG may be a candidate for clinical therapeutic protocols especially for treating hepatoma. Its merits await further investigation.

Acknowledgements

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