

Original Article

1,2:5,6-dianhydrogalactitol inhibits human glioma cell growth *in vivo* and *in vitro* by arresting the cell cycle at G₂/M phase

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Abstract

1,2:5,6-dianhydrogalactitol (DAG) is a hexitol epoxide with marked antitumor activity against multiple types of cancer cells, but the molecular mechanisms by which DAG functions as an antitumor agent is largely unknown. In this study, we investigated the inhibitory effects of DAG on human glioma cell growth *in vitro* and *in vivo* and uncovered the underlying molecular mechanisms. Treatment with DAG (120 μmol/L) dose-dependently inhibited the proliferation and colony formation in human glioma cell lines LN229, U251, and U87MG *in vitro*. DAG (1, 2, 5 μmol/L) induced cell cycle arrest at G₂/M phase in the 3 glioma cell lines in a dose-dependent manner. The signaling pathways involved in DAG-caused cell cycle arrest was further analyzed in LN229 cells, which revealed that DAG dose-dependently activated two parallel signaling cascades, *ie*, the p53-p21 cascade and the CDC25C-CDK1 cascade. DAG also significantly enhanced the radiosensitivity of LN229 cells as shown in the clonogenic assay. In nude mice bearing subcutaneously xenografted LN229 glioma, administration of DAG (5 mg/kg, *iv*, twice per week for 6 weeks) effectively suppressed the growth of xenografted tumors: the relative tumor growth rate (T/C) was reduced to 22.38%, and the tumor growth inhibitory rate (TGI) was 83.58% ($P < 0.01$). In addition, DAG administration significantly activated the CDC25C-CDK1 cascade in the xenografted tumors. In conclusion, DAG inhibited human glioma cell growth *in vitro* and *in vivo* by inducing cell cycle arrest at G₂/M phase. Two parallel cascades are activated and involved in the cell cycle arrest.

Keywords: 1,2:5,6-dianhydrogalactitol; human glioblastoma; cell cycle arrest; p53-p21 cascade; CDC25C-CDK1 cascade; radiosensitivity; xenografted tumors

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Introduction

Glioblastoma (GBM) is the most common malignant brain tumor and is classified as grade IV, the most aggressive grade in the World Health Organization classification of tumors of the central nervous system^[1]. The current standard of therapy combines maximal surgical resection and radiotherapy with concomitant and adjuvant temozolomide (TMZ)^[2]. Unfortunately, the median survival of patients after treatment is limited to 16 to 19 months, with approximately 25% to 30% of patients alive 2 years after diagnosis^[3]. Drug resistance mediated by MGMT and MDR1 greatly limits the application of

the first-line drug TMZ^[4]. Defects in DNA mismatch repair (MMR) or highly activated DNA base excision repair (BER) increase resistance to TMZ in GBMs^[5,6]. Because hematotoxicity rates are high and efficacy is modest, other traditional cytotoxic chemotherapies (carmustine, lomustine, or carboplatin) exhibit low efficacy^[7,8]. Combining bevacizumab, temozolomide and radiotherapy resulted in improved progression-free survival but not overall survival in patients with newly diagnosed GBM disease^[9,10]. Given the poor survival with currently approved treatments, new therapeutic options for GBM are badly needed^[2].

1,2:5,6-dianhydrogalactitol (DAG), a hexitol epoxide, was reported to be the most active of 177 agents tested against a mouse ependymoblastoma tumor^[11,12]. It is a highly water-soluble small molecule that readily crosses the blood-brain barrier^[11,13]. Several clinical studies investigating its therapeutic

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effect on primary brain tumors and other cancers were completed in the late 1970s^[11, 14-16]. Eagan and colleagues reported that in 42 patients with grade 3 and 4 supratentorial astrocytomas, those receiving DAG plus irradiation had a significantly longer median survival time than those receiving irradiation only (67 vs 35 weeks)^[11]. Thus, DAG has been shown to be a good candidate for brain tumor chemotherapy, including glioma. However, the molecular mechanism by which DAG functions as an anti-tumor agent is largely unknown. Several research groups studied the molecular mechanisms of DAG in the 70s. Most of them were focused on the direct interaction between DAG and DNA. A previous study showed that DAG is a bifunctional alkylating agent. When Yoshida sarcoma cells were treated with DAG, DAG interacted with DNA and yielded three alkylated products: 7-(1-deoxygalactit-1-yl)guanine, 7-(1-deoxyanhydrogalactit-1-yl)guanine and 1,6-di(guanin-7-yl)-1,6-dideoxygalactitol. The last product indicated that inter- or intra-strand crosslinks could be formed^[17]. The interaction between DNA and DAG was also confirmed *in vitro* by chemical approaches. Moreover, Dennis Brown and his colleagues reported that glioma cell lines with different MGMT gene expression levels had similar sensitivity to DAG, but cells highly expressing MGMT were more resistant to TMZ^[18]. These results indicated that DAG and TMZ probably function through different mechanisms, and DAG might be more effective than TMZ in tumors with high MGMT expression. However, whether DAG functions through mechanisms other than alkylation is unclear.

Chemotherapeutic agents usually cause DNA damage, which induces cell cycle arrest or apoptosis^[19, 20]. The G₂/M DNA damage signaling pathway plays a critical role in G₂/M phase cell cycle arrest. In this pathway, DNA damage activates several complex cascades that ultimately serve to inactivate the CDK1-cyclin B1 complex^[21]. p53 plays an essential role in this process, with its DNA binding and transcriptional regulatory activity induced by upstream signaling, followed by increased expression of proteins such as p21, GADD45 and 14-3-3 α . All of these proteins can interact with the CDK1-cyclin B1 complex, blocking its cell cycle activity^[22-24]. The efficiency of this CDK1-cyclin B1 suppression differs according to cell type, damage profile and related gene mutations^[25, 26].

To study the antitumor activity of DAG on glioma and uncover the underlying mechanism, we treated glioma cells with different doses of DAG both *in vitro* and *in vivo*, monitored the effects of DAG on cell cycle progression, and studied the signaling pathways participating in this process.

Materials and methods

Mouse experiments

All mouse experiments were subject to institutional approval by the WuXi AppTech IACUC, and the use and care of animals were according to AAALAC stipulations.

Cell culture

In our study, three human glioblastoma cell lines LN229,

U251, U87MG were cultured. They were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc, Waltham, MA, USA) and antibiotics (penicillin and streptomycin, 50 units/mL each) (Invitrogen, Carlsbad, CA, USA). Primary cultured astrocytes were isolated from P1 neonatal mouse cortex and were cultured in DMEM:F12 (1:1) medium (HyClone, Logan, UT, USA) with FBS and antibiotics, similar to glioma cells. Cells were grown in a humidified incubator with 5% CO₂ at 37°C.

Reagents and antibodies

1,2:5,6-dianhydrogalactitol was kindly provided by Guangxi Wuzhou Zhongheng Group Co, Ltd (Wuzhou, China) and was freshly dissolved in PBS before use. Phospho-ATM-S1981 polyclonal antibody, ATM antibody, phospho-CHEK2-T68 polyclonal antibody, CHK2 antibody, phospho-CDC25C-S216 polyclonal antibody, CDC25C antibody, CDKN1A and Tp53 monoclonal antibody were purchased from ABclonal Biotech Co, Ltd (Wuhan, China), and anti-phosphorylated CDK1 (tyrosine15 [Y15]), CDK1 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell growth assay

The effects of 1,2:5,6-dianhydrogalactitol on glioma cell and primary cultured astrocyte growth were examined. Cells were treated with 0 (PBS), 1, 2, 5, 10, or 20 μ mol/L DAG for 72 h. Cell proliferation was estimated by using a Beckman particle counter (Beckman Coulter, Brea, CA, USA) to count cell number. Cell number at individual doses was normalized to the PBS treated group (control). The data were obtained from three independent assays performed in duplicate.

Clonogenic assay

LN229 and U251 cells were seeded in six-well plates at a concentration of 400 cells per dish, in triplicate. Cells were cultured for 12 h before treatment with 0, 0.1, 0.5, 1, 2, or 5 μ mol/L DAG. After 10 d of incubation, clones were rinsed with PBS, fixed in formaldehyde for 15 min, and finally stained with 0.5% crystal violet for 30 min. Clones having more than 50 cells were counted.

Cell cycle distribution

Flow cytometry assays were used to study the effect of DAG on cell cycle distribution. After DAG treatment for 72 h, glioma cells were harvested and fixed with cold 75% alcohol overnight at 4°C. The cells were then washed with PBS, treated with RNase A (Sigma-Aldrich Co) at 0.2 mg/mL, and then stained with 10 μ g/mL propidium iodide at 37°C for 15 min. For each sample, 1 \times 10⁴ cells were assayed with a FACS-Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA), and cell cycle distributions were analyzed by CellQuest Pro software (Becton-Dickinson).

Apoptosis test

Glioma cells were harvested after DAG treatment for 72 h. Annexin V-FITC and PI were used to stain the cells. For each sample, 1×10^4 cells were analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Western blot

Total cell protein lysates were extracted from cells in SDS buffer and then separated by SDS-PAGE. Proteins were then transferred to PVDF membranes (GE Healthcare, Shanghai, China), membranes were blocked in 5% skim milk (dissolved in TBST) for 1 h. The membranes were then probed by primary antibodies: phospho-(Ser1981)-ATM, ATM, phospho-(Thr68)-CHK2, phospho-(Ser216)-CDC25C, phospho-(Tyr15)-CDK1, CHK2, CDC25C, CDK1, p53, p21, GAPDH, β -actin, α -tubulin overnight at 4°C. The membranes were then incubated with secondary antibodies [rat anti-rabbit IgG(H+L) antibody or sheep anti-mouse IgG(H+L) antibody] for 1 h at room temperature. Bound antibodies were detected by an ECL chemiluminescence detection system (GE Healthcare, Shanghai, China).

Quantitative real-time RT-PCR analyses

Total RNA was isolated from glioma cells with a UNIQ-10/TRIZOL total RNA extraction kit (Sangon, Shanghai, China) and reverse-transcribed to cDNA with PrimeScript RT Reagent Kit (Takara, Otsu, Shiga, Japan). Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis was performed using SYBR Premix Ex Taq (Takara). The primers for p21 mRNA were 5'-CCATGTGGACCTGTCACTGT-3' (forward) and 5'-AAGATGTAGAGCGGGCCTTT-3' (reverse), and p21 mRNA levels were normalized to human β -actin mRNA detected by primers 5'-GGCTACAGCTTCACCACCAC-3' (forward) and 5'-GAGTACTTGCCTCAGGAGG-3' (reverse). The qPCR conditions were as follows: 95°C, 10 s; (95°C, 5 s, 60°C, 34 s) 35 cycles, followed by a melting-curve analysis. The data were analyzed by using $2^{-\Delta\Delta Ct}$.

Radiation exposure

Clonogenic assays were performed to test the effects of DAG on the radiosensitivity of glioma cells. LN229 cells were seeded in six-well plates (400 cells per well). For the DAG treatment groups, cells were treated with DAG (0, 0.5, 1, or 2 μ mol/L) at 12 h post-seeding. For the radiation groups, cells were treated by X-ray (0, 0.5, 1, or 2 Gy) 13 h post-seeding. For the combination groups, 12 h post-seeding, cells were treated by DAG (0, 0.5, 1, or 2 μ mol/L), and 1 h later cells were treated by X-ray (0, 0.5, 1, or 2 Gy). Cells were then cultured for 10 d. Clones were stained by Giemsa stain, and clones that had more than 50 cells were counted.

In vivo studies

We established a subcutaneous glioma xenograft model to study the antitumor activity of DAG *in vivo*. LN229 cells were suspended in MEM, and 2×10^6 cells per mouse were subcutaneously injected into the flank of BALB/c nude mice (SLAC

Laboratory Animal, Shanghai, China) at 6–8 weeks old. The tumor volume was calculated as follows: $0.5 \times L \times W^2$. Tumor-bearing mice were divided into two groups ($n=8$) with similar average volumes (vehicle: 108 ± 4 mm³ vs DAG: 107 ± 4 mm³). Then, both groups underwent the following treatment: The DAG treatment group received DAG at 5 mg/kg or 10 μ L/g, *iv*, twice per week for 6 weeks. The vehicle group received saline at 10 μ L/g, *iv*, three times per week for 6 weeks. Tumor volumes were measured twice per week. All mouse experiments were subjected to institutional approval by the WuXi AppTech IACUC, and the use and care of animals were according to AAALAC stipulations.

Statistical analysis

All tests were performed using the Prism 5 software package. The mean and SEM were calculated, and significant differences were determined using one-way ANOVA and *t*-test. In this study, $P < 0.05$ was considered significant.

Results

DAG markedly inhibited glioma cell growth *in vitro*

1,2:5,6-dianhydrogalactitol was reported to show high antitumor activity against various types of cancer cells^[12,13,17]. In this study, we tested the inhibitory effect of DAG on the growth of glioblastoma cell lines. First, we examined whether DAG affected glioma cell proliferation. A dose-dependent inhibition of growth was observed 72 h post DAG treatment in LN229, U251 and U87MG glioma cell lines. The growth of U251 cells was reduced more rapidly than the other two cell lines (Figure 1A, 1B). Then, we tested the effect of DAG on glioma cell clonogenic ability. In the clonogenic assays, LN229 and U251 cells were cultured for 10 d after treatment with the indicated concentrations of DAG. Compared to the PBS treated control, we observed that the number of clones in the DAG treated groups was reduced dramatically in both LN229 and U251 cell lines (Figure 1C, 1D). A concentration of 5 μ mol/L DAG almost totally inhibited the formation of clones. To test the effect of DAG on normal astrocytes, we isolated primary astrocytes from P1 neonatal mouse cortex. DAG also inhibited the growth of primary cultured astrocytes. However, all these results point to the conclusion that DAG suppresses glioma cell growth *in vitro*, despite the possibility that it might have a ubiquitous function on normal cells.

DAG induced cell cycle arrest at G₂/M phase in a dose-dependent manner in glioma cells

Although high antitumor activity of DAG has been extensively reported, the underlying molecular mechanism, especially in GBM cells, has not been well addressed. Previous studies indicated that DAG is a bifunctional alkylating agent, causing DNA damage^[17]. As cell cycle arrest is usually induced by DNA damage, we investigated the cell cycle profiles of DAG-treated glioma cells. After 72 h exposure to different doses of DAG, LN229 cells were stained with PI and analyzed by flow cytometry. We observed that the G₂/M fractions of the treatment groups all increased in a dose-dependent manner (Ctrl

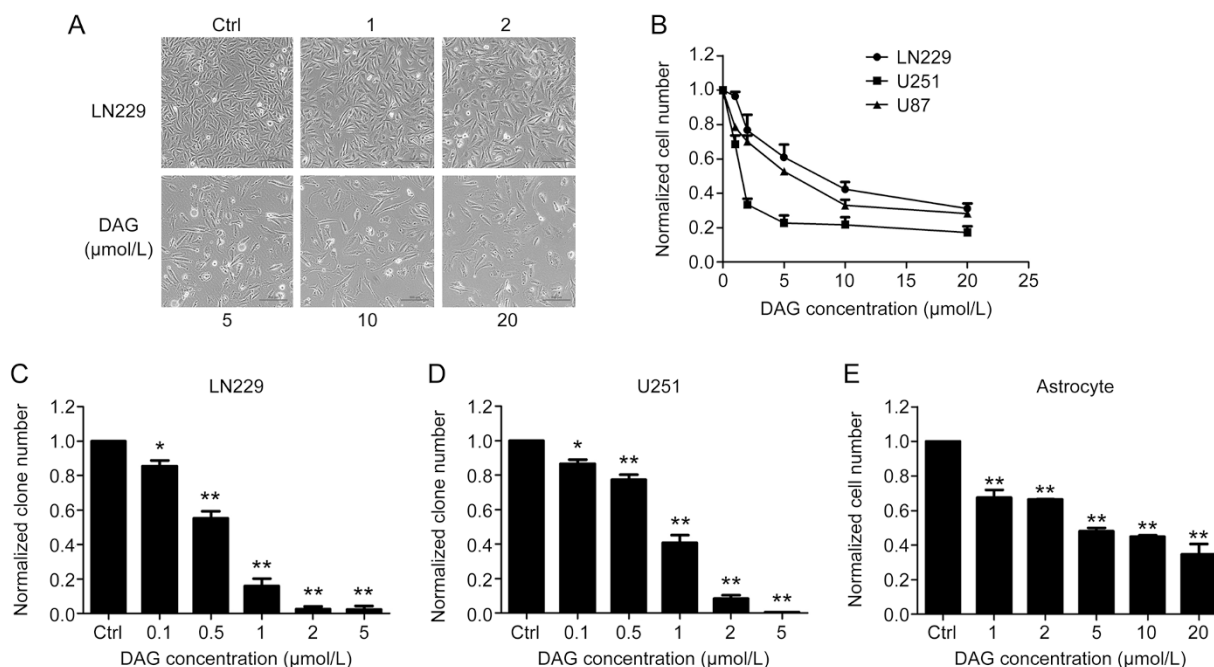


Figure 1. Growth inhibition of DAG on glioma cells. (A) Cell morphology of LN229 cells after treated by DAG (PBS, 1, 2, 5, 10, 20 μmol/L) for 72h. Cell numbers were counted by using a Beckman particle counter and normalized to the Ctrl (PBS) group. (B) Cell growth curve of three glioma cells after treatment with DAG for 72 h. (C, D) Quantification of LN229 and U251 clones by counting the clones which have more than 50 cells. Clones were stained with Giemsa staining after treatment with DAG for 10 d. The number of clones for DAG-treated groups was normalized to control group. (E) Cell number of primary cultured astrocyte after treatment with DAG for 72 h. The primary astrocytes were divided from neonatal mice (P1) cortex. Data are representative of 3 independent experiments, performed in duplicate. Significant difference ($P < 0.05$) compared with PBS control group and DAG treated groups by one-way ANOVA followed by Tukey's test for multiple comparisons. $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$. Ctrl=control.

vs 1, 2, or 5 μmol/L=13.2%±1.2% vs 20.8%±3.5%, 38.7%±5.3%, or 68.6%±2.1%, respectively). Conversely, a dose-dependent reduction of the percentage of cells in the G_0/G_1 fraction was observed. The fraction of cells in S phase was slightly affected (Figure 2A, 2B). The same accumulation of G_2/M phase cells was observed in U251 and U87MG cells after treatment with DAG (Figure 2A). We also tested whether DAG induced LN229 cell apoptosis. Treatment of LN229 cells with low doses of DAG (1 or 2 μmol/L) for 72 h did not increase the apoptotic fraction. Even treated with the highest dose of DAG (20 μmol/L), the apoptotic fraction of LN229 cells was just slightly increased (less than 15%) (Figure 2C). Thus, DAG inhibited cell growth effectively by arresting glioma cells in G_2/M phase.

p53-p21 cascade was activated in a dose-dependent manner in DAG treated LN229 cells

The tumor suppressor p53 plays a critical role in the G_2/M phase DNA damage signaling pathway by integrating various upstream signals and upregulating the expression of multiple downstream genes, some of which ultimately inhibit the activity of the CDK1-cyclin B1 complex^[23, 24, 27-29]. In our study, we investigated the effects of 72 h of DAG treatment on p53 and other related proteins in LN229 cells. p53 accumulated in a dose-dependent manner (Figure 3C). Two upstream proteins, ATM and CHK2, were activated by phosphorylation (ATM at

Ser1981 and CHK2 at Thr68) in a similar pattern (Figure 3A, 3B). Both of them are able to stabilize p53 by phosphorylating it at Ser15 and Ser20, respectively, which blocks the interaction between p53 and HDM2^[27, 29]. Both the mRNA and protein levels of p21 were upregulated following p53 accumulation (Figure 3D, 3E). Previous research reported that p53 could mediate the transcription of p21, which binds to the CDK1-cyclin B1 complex and inactivates it, leading to G_2/M phase cell cycle arrest^[30]. These results indicated that DAG markedly activated the p53-p21 cascade and contributed to G_2/M phase arrest.

The CDC25C-CDK1 cascade was activated in parallel

As mutations in the human *TP53* gene affecting p53 function are highly prevalent in glioblastomas^[7], we tried to find another cascade independent of p53-p21 activation. Interestingly, we observed the activation of the CDC25C-CDK1 cascade. The phosphorylation of CDC25C at Ser216 was upregulated in a dose-dependent manner, and CDK1 phosphorylated at Tyr15 significantly accumulated compared to the control (Figure 4A, 4B).

DAG enhanced the radiosensitivity of LN229 cells

As cells in G_2 are more sensitive to irradiation than cells in other phases^[21], we hypothesized that DAG treatment could sensitize LN229 cells to irradiation. We tested the inhibitory

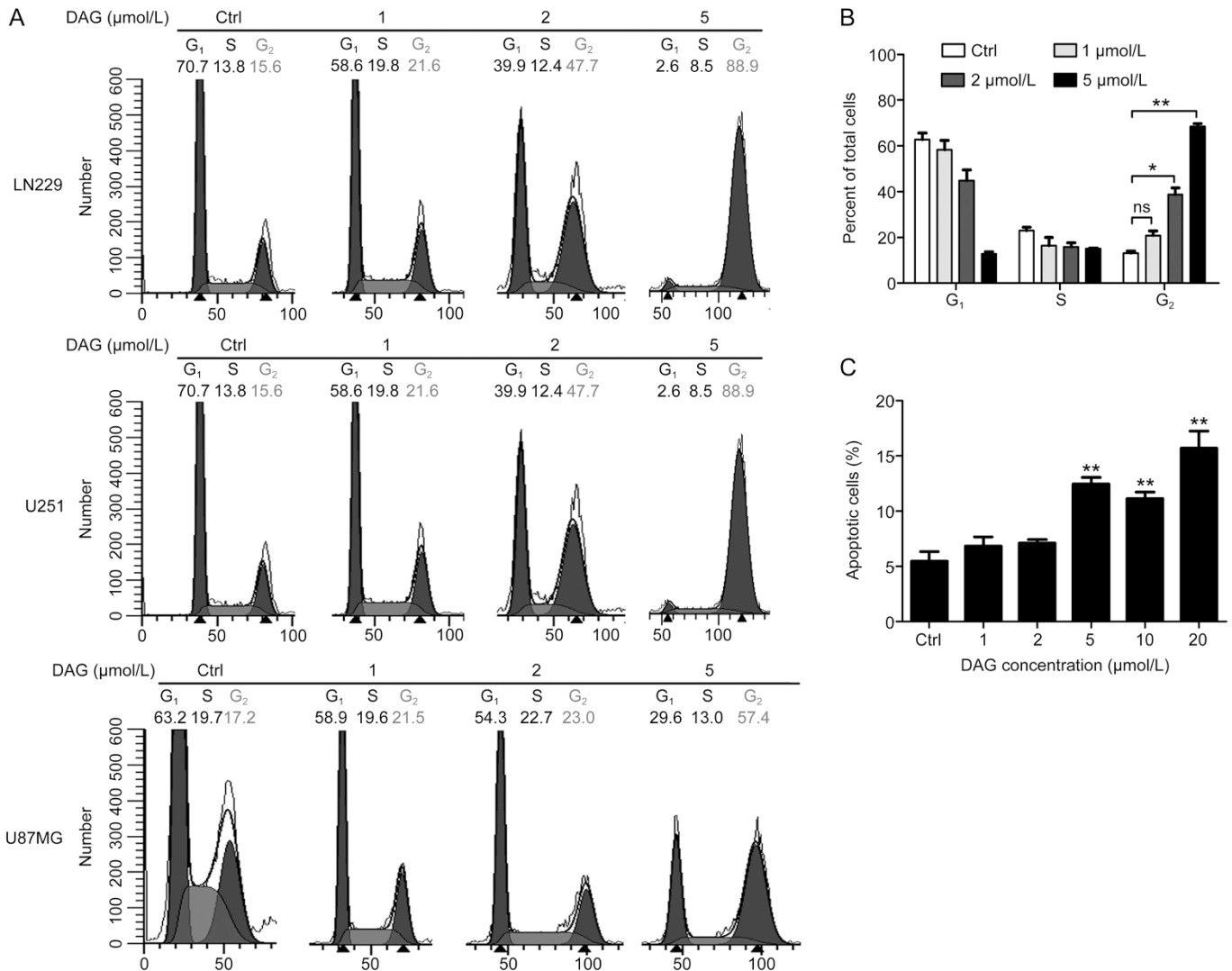


Figure 2. DAG induced cell cycle arrest in glioma cells. (A, B) Flow cytometric analysis of DNA content in glioma cells after treatment with DAG (PBS control, 1, 2, 5 μmol/L) for 72 h. Percentage of each cell phase was statistically analyzed after treatment with difference dosages of DAG. Accumulation of G₂ phase cells was indicated by comparing each treatment group with control. ^{ns}P>0.05, ^{*}P<0.05, ^{**}P<0.01. Ctrl=control. (C) Effect of DAG treatment on apoptotic levels of LN229 cells. Annexin V-FITC and PI were used for staining. Data are representative of 3 independent experiments, performed in duplicate. Significant difference (P<0.05) compared with PBS control group and DAG treated groups by t-test.

effects of different treatments (DAG only, radiation only, and DAG combined with radiation, in different doses) on LN229 cells by clonogenic assay. The treatments were: DAG only (0, 0.5, 1, or 2 μmol/L); radiation only (0, 0.5, 1, or 2 Gy); and DAG combined with radiation (0.5 μmol/L DAG+0.5 Gy, 1 μmol/L DAG+1 Gy, or 2 μmol/L DAG+2 Gy). The combination index (CI) and isobolograms were then analyzed by the Chou-Talalay method. Colony formation was inhibited in all treatment groups. The combined treatment groups were more effectively suppressed than the single treatment groups (Figure 5C). In the combined treatment, when the inhibitory effect reached 50%, 70%, and 90%, the combination index (CI) was 0.79, 0.87, and 0.97, respectively (Figure 5A, 5B). These results indicated that DAG and irradiation synergistically inhibited glioma cell growth.

DAG inhibited glioma development *in vivo*

To investigate the anticancer activity of DAG *in vivo*, we established a subcutaneous xenograft tumor model of LN229 cells. After DAG treatment, tumor volumes were measured twice per week. DAG significantly inhibited tumor development as early as 21 d post-treatment (Figure 6C). The relative tumor growth rate (T/C) was 22.38%, and the tumor growth inhibitory rate (TGI) reached 83.58% (P<0.001) 41 d post-treatment. The tumor volume of the treatment group was 346.6±50.7 (n=8), while the tumor volume of the vehicle group was 1566.7±242.6 (n=7) (Figure 6A, 6B). We examined protein expression in tumor tissues and found that the CDC25C-CDK1 cascade was significantly activated. p53 accumulated slightly, and there was no significant difference in p21 expression between the control and the DAG treatment groups

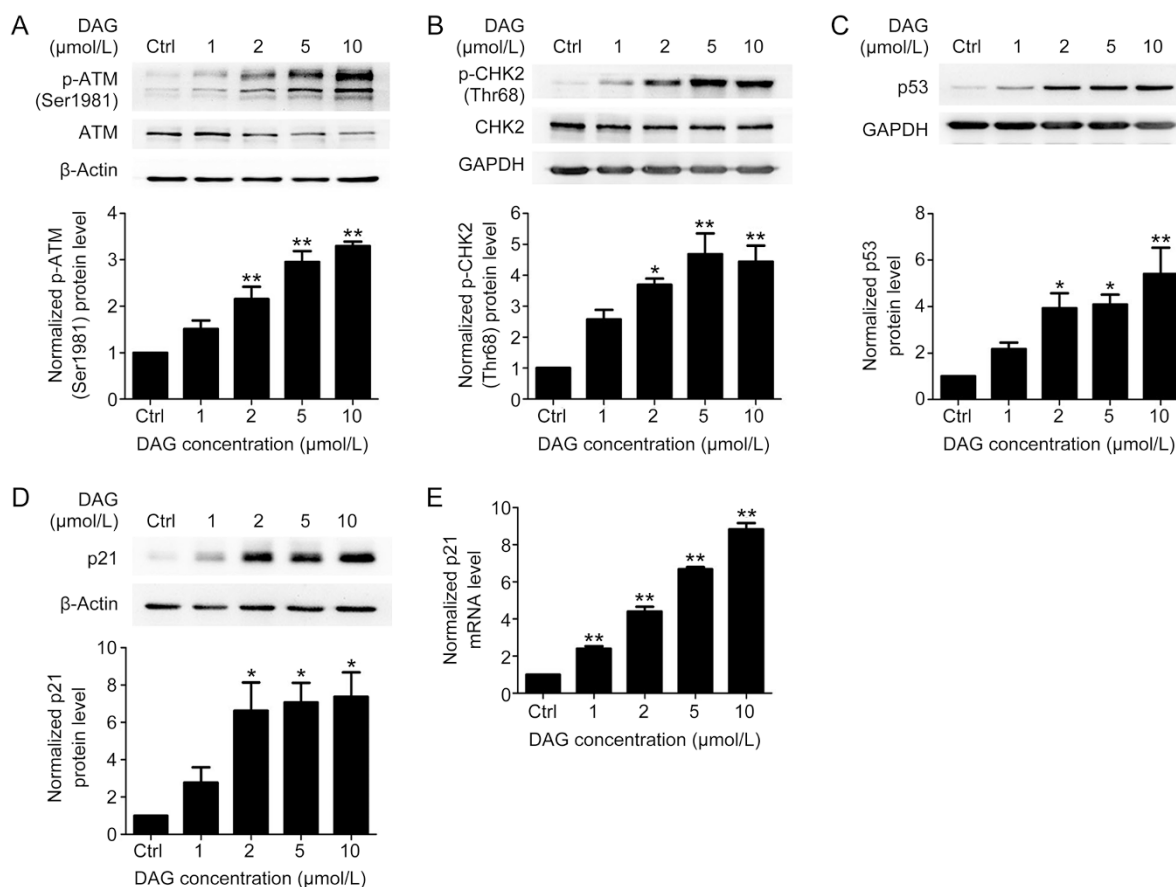


Figure 3. Effect of DAG exposure on G₂/M phase DNA damage signaling pathway in LN229 cells. (A–D) Immunoblotting for phosphor-(Ser1981)-ATM, phosphor-(Thr68)-CHK2, p53 and p21 using lysates from LN229 cells treated with the indicated doses of DAG for 72 h. Phosphorylation levels of treatment groups were normalized to control. (E) qPCR results showing increase in p21 mRNA levels in LN229 cells 72 h after treated by the indicated doses of DAG. p21 mRNA levels were normalized to the control. Data are representative of 3 independent experiments, performed in duplicate. Significant difference ($P < 0.05$) compared with PBS control group and DAG treated groups by one-way ANOVA followed by Tukey's test for multiple comparisons. ^{ns} $P > 0.05$, * $P < 0.05$, ** $P < 0.01$. Ctrl=control.

(Figure 6D–6F). This result suggested that DAG effectively suppressed the development of glioma and that the CDC25C-CDK1 cascade plays a more important role *in vivo*.

All the above results suggested that DAG has high antitumor activity in LN229 cells both *in vitro* and *in vivo*, arresting the cell cycle at G₂/M phase by activating two parallel cascades.

Discussion

Although many therapeutic schedules have been applied for glioblastoma treatment, the patient prognosis is still poor^[7]. New treatment approaches are badly needed. In the current study, we showed that 1,2:5,6-dianhydrogalactitol effectively inhibited glioblastoma cell growth both *in vitro* and *in vivo*. DAG has some potential advantages in treatment of glioma. First, a very important feature of DAG that would benefit the treatment is that it can readily cross the blood-brain barrier and accumulate in tumor tissue^[11,31]. Second, previous clinical trials reported that dose-limiting thrombocytopenia was the main side effect of DAG^[14]. Here we tested the effects of DAG on primary cultured mouse astrocytes and rat hepatocytes,

DAG did not affect the cell viability in primary cultured rat hepatocytes (data not shown), despite its growth inhibitory effects on primary mouse astrocytes *in vitro*, recent clinical study from DelMar Pharmaceuticals Inc showed that no serious adverse events were reported at doses up to 40 mg/m² per day, indicating a promising safety and clinical tolerance^[32]. Third, a glioma cell line, T98G, with high MGMT gene expression is resistant to temozolomide but is sensitive to DAG^[18]. It is suggested that for patients in with high MGMT-expressing glioma, DAG may be more effective than the first line drug TMZ. Finally, Egan and his colleagues reported that DAG plus irradiation effectively prolonged the survival time (67 vs 35 weeks) of patients with malignant supratentorial astrocytomas, compared to irradiation alone^[11]. All together, this suggests that DAG could be a promising chemotherapeutic agent for glioblastoma treatment, and further study will shed light on this possibility.

The G₂/M phase DNA damage signaling pathway is critical for the cellular response to DNA damage caused by chemotherapeutics and thus has a great impact on treatment effec-

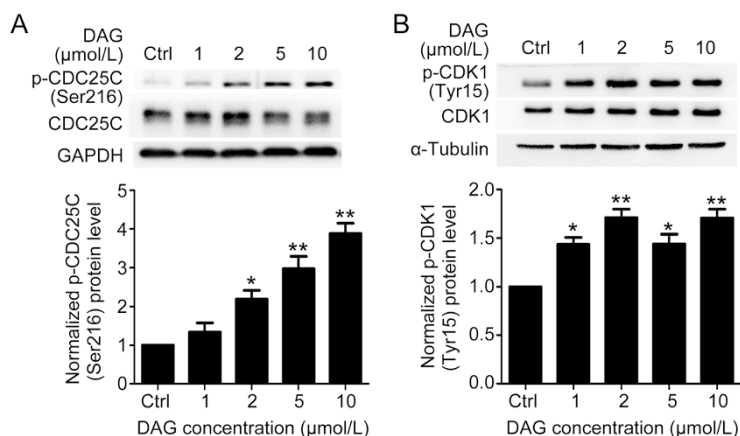


Figure 4. CDC25C-CDK1 cascade was also activated. (A, B) Immunoblotting for phospho-(Ser216)-CDC25C, CDC25C, phospho-(Tyr15)-CDK1 and CDK1 using lysates from LN229 cells, which were treated by DAG (PBS, 1, 2, 5, 10 μmol/L) for 72 h. Protein levels of treatment groups were normalized to control. Data are representative of 3 independent experiments, performed in duplicate. Significant different ($P<0.05$) compared with PBS control group and treatment groups by one-way ANOVA followed by Tukey's test for multiple comparisons. $^{ns}P>0.05$, $^*P<0.05$, $^{**}P<0.01$. Ctrl=control.

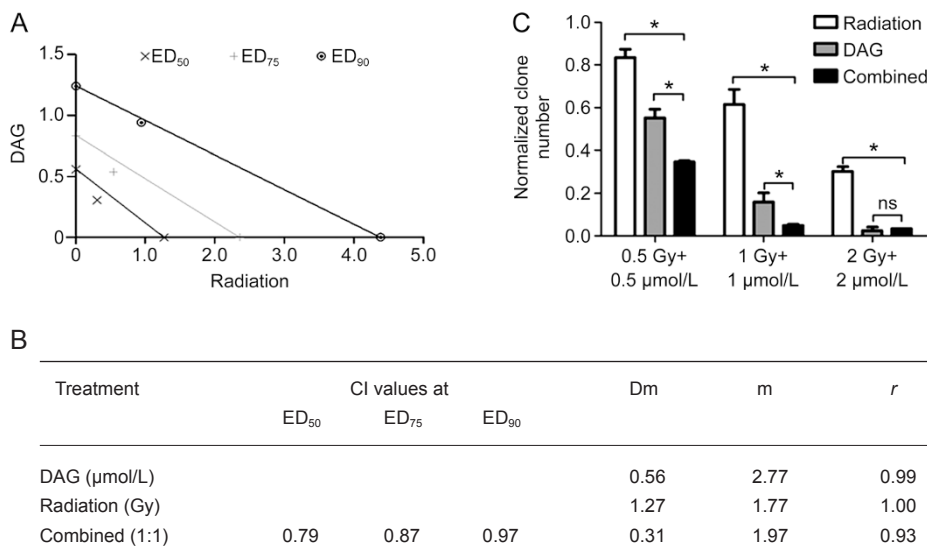


Figure 5. DAG enhanced radiosensitivity of LN229 cells. (A, B) Isobologram and the combination index (CI) results were analyzed by Chou-Talalay method. Dm value: The median-effect dose or concentration. m value: a measurement of the sigmoidicity of the dose-effect curve; $m=1$, >1 , and <1 indicates hyperbolic, sigmoidal, and negative sigmoidal shape, respectively. r value: the linear correlation coefficient of the median-effect plot. (C) Clone numbers of each treatment group. DAG only (0.5, 1, 2 μmol/L); radiation only (0.5, 1, 2 Gy); DAG combined with radiation (0.5 μmol/L DAG+0.5 Gy, 1 μmol/L DAG+1 Gy, 2 μmol/L DAG+2 Gy). Data are representative of 3 independent experiments, performed in duplicate. Significant difference ($P<0.05$) compared with DAG only, radiation only and combined groups by t test. $^{ns}P>0.05$, $^*P<0.05$, $^{**}P<0.01$. Ctrl=control.

tiveness. DNA damage activates several complex cascades in this pathway that ultimately serve to inactivate the CDK1-cyclin B1 complex, which causes G₂/M cell cycle arrest^[21]. Combining our own work and studies from the literature, we propose the following model to explain the mechanism of DAG-induced G₂/M phase cell cycle arrest in glioma cells (Figure 7). DAG-induced DNA damage activates ATM and CHK2 by phosphorylation, activating two parallel cascades. One is the p53-p21 cascade, in which activated ATM and CHK2 prevent p53 degradation by phosphorylating it. Accumulated p53

activates the transcription of p21, and p21 then binds to the CDK1-cyclin B1 complex, leading to G₂/M cell cycle arrest. At the same time, activation of the CDC25C-CDK1 cascade leads to CHK2 phosphorylating CDC25C at Ser216. This prevents CDC25C from dephosphorylating CDK1 at Tyr15. Accumulation of phosphorylated CDK1 then induces cell cycle arrest.

DAG inhibition of glioma cell growth by both p53-dependent and p53-independent pathways is important because mutations affecting p53 function are found in up to 85% of glioblastomas. Thus, the p53-independent function of DAG

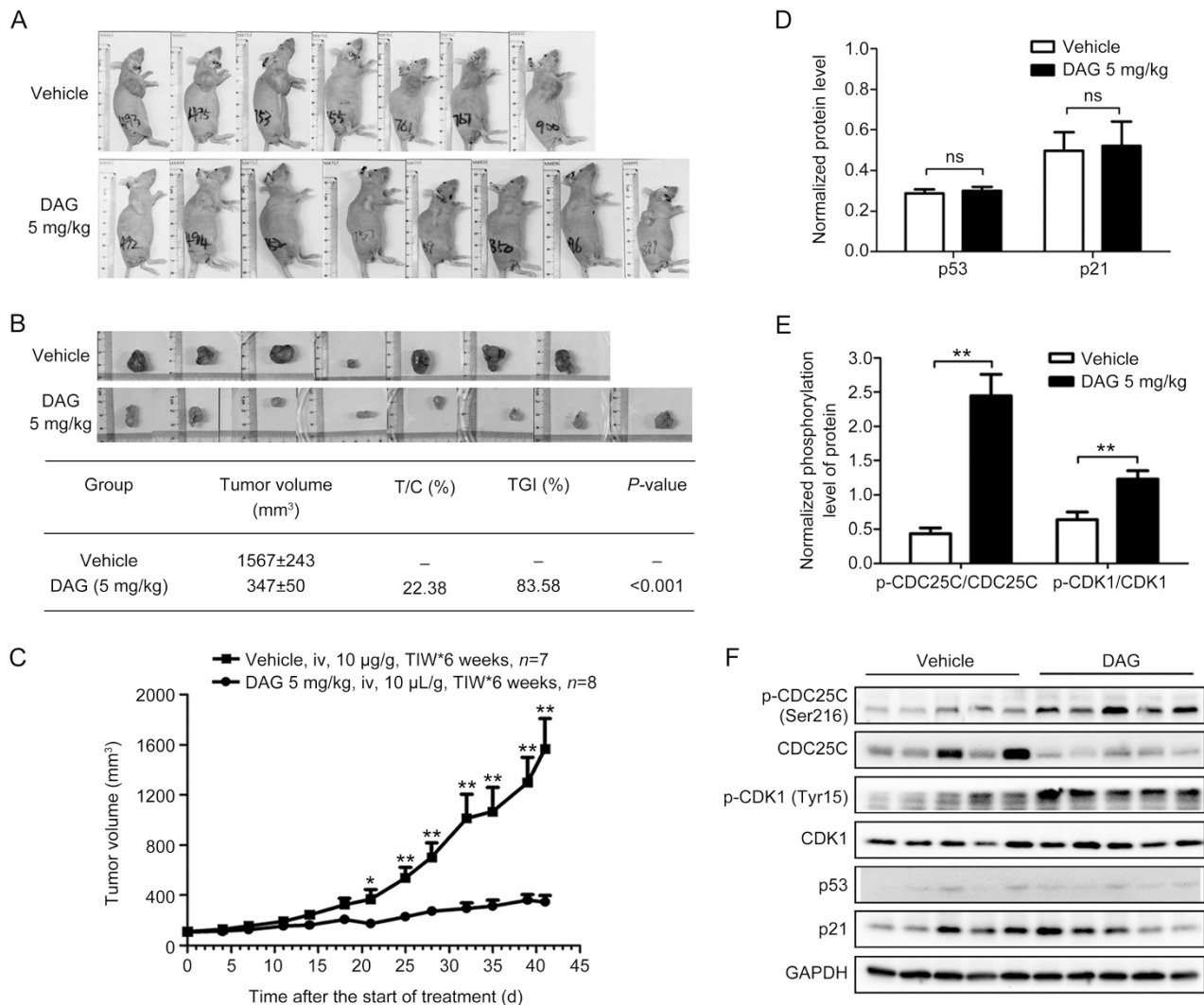


Figure 6. DAG inhibited subcutaneously LN229 xenografted tumor growth *in vivo*. (A, B) Tumor bearing mice and tumors 41 d post treatment. When the average tumor volume was about 107 mm³, tumor bearing nude mice were treated after separated to two groups ($n=8$) randomly. Tumor volumes were measured twice a week. One mouse was undertaken euthanasia, when the tumor it bearing was larger than 2000 mm³. The test was completed at the 41th d after the start of treatment. The tumor growth inhibition rate: $(TGI)=[1-(V_{DT}-V_{D0})/(V_{CT}-V_{C0})]*100\%$ and the relative tumor growth rate: $(T/C)=(V_{DT}/V_{D0})/(V_{CT}/V_{C0})*100\%$, V_{DT} : tumor volume of treatment group each time measured; V_{D0} : tumor volume of treatment group at d 0; V_{CT} : tumor volume of vehicle group each time measured; V_{C0} : tumor volume of vehicle group at d 0. (C) Tumor growth curve. Significant different ($P<0.05$) compared the tumor volumes of vehicle group and treatment group at the same day by *t*-test. (D–F) Protein expression and phosphorylation in the xenograft tumor. Significant difference ($P<0.05$) compared the protein expression and phosphorylation of vehicle group and treatment group by *t*-test. ^{ns} $P>0.05$, * $P<0.05$, ** $P<0.01$. Ctrl=control.

might allow its application in a wider glioma spectrum. Certainly, the importance of both the p53-p21 pathway and the CDC25C-CDK1 pathway will be examined in further work.

Although DAG powerfully inhibited the growth of glioma cells by causing cell cycle arrest, it also provided an opportunity for cells to repair damaged DNA, which promoted cancer cell survival. To further improve the treatment effect, we combined DAG and irradiation to treat LN229 cells. As we expected, DAG significantly increased the radiosensitivity of LN229 cells. This result is important for the clinical application of DAG for three reasons. First, the combination of DAG

and irradiation is significantly more effective than each alone. Second, while both high dose chemotherapy and radiotherapy have serious side effects, combinatorial treatment could allow for reduced dosage that can effectively improve quality of life and decrease drug resistance. Finally, DAG arrested LN229 cells at G₂/M phase, the most sensitive phase for irradiation, making DAG followed by radiotherapy a good strategy for clinical glioblastoma treatment.

Our study suggests that DAG inhibited LN229 cell growth both *in vitro* and *in vivo* by arresting cell cycle at G₂/M phase in both a p53-dependent and p53-independent manner.

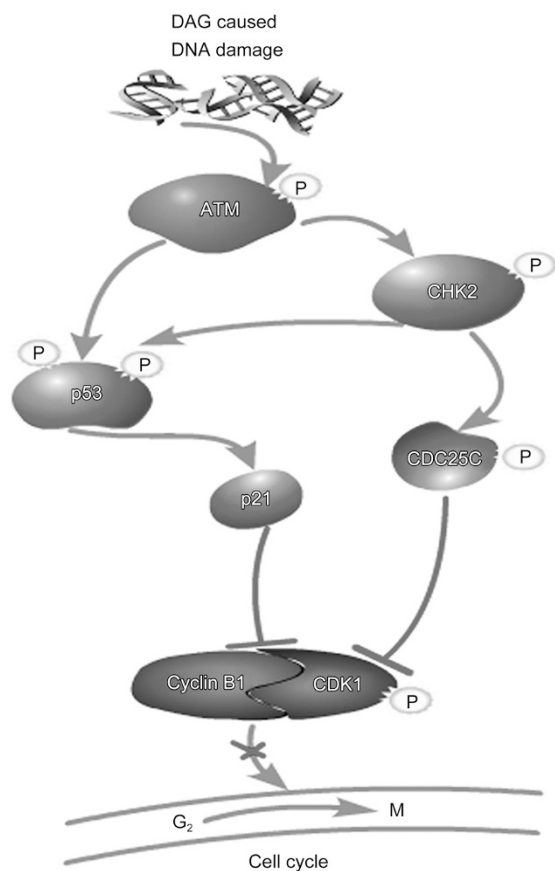


Figure 7. Model of the mechanism of DAG induced G₂/M phase cell cycle arrest in glioma cells.

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Author contribution

Jin REN and Ling-ling MIAO participated in research design; Chun PENG, Ling-ling MIAO, and Xin-ming QI conducted experiments; Chun PENG performed data analysis; Ling-ling MIAO, Chun PENG and Jin REN wrote or contributed to the writing of the manuscript.

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