Ganoderic acid DM, a natural triterpenoid, induces DNA damage, G1 cell cycle arrest and apoptosis in human breast cancer cells

Guo-Sheng Wu a,b, Jin-Jian Lu a,b,⁎, Jia-Jie Guo a,b, Ying-Bo Li a,b, Wen Tan a,b, Yuan-Ye Dang a,b, Zhang-Feng Zhong a,b, Zeng-Tao Xu a,b, Xiu-Ping Chen a,b, Yi-Tao Wang a,b,⁎

a State Key Laboratory of Quality Research in Chinese Medicine (University of Macau), Macao, 999078, PR China
b Institute of Chinese Medical Sciences, University of Macau, Macao, 999078, PR China

Abstract

Ganoderic acid DM (GADM) is a triterpenoid isolated from Ganoderma lucidum, a well-known edible medicinal mushroom. In the present study, we found that GADM effectively inhibited cell proliferation and colony formation in MCF-7 human breast cancer cells, which was much stronger than that of MDA-MB-231 breast cancer cells. GADM both concentration- and time-dependently mediated G1 cell cycle arrest and significantly decreased the protein level of CDK2, CDK6, cycle D1, p-Rb and c-Myc in MCF-7 cells. Moreover, GADM obviously induced DNA fragmentation and cleavage of PARP which are the characteristics of apoptosis and decreased the mitochondrial membrane potential in MCF-7 cells. Besides, we also showed that GADM elicited DNA damage as measured by comet assay which is a sensitive method for DNA damage detection. γ-H2AX, a marker of DNA damage, was also slightly up-regulated after treated with GADM for 6 h, suggesting that the G1 cell cycle arrest and apoptosis induced by GADM may be partially resulted from GADM-induced DNA damage. These results have advanced our current understandings of the anti-cancer mechanisms of GADM.

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1. Introduction

Breast cancer is one of the most common cancers worldwide and the leading cause of cancer death in women [1]. The treatment of this disease includes surgery, radiation, chemotherapy and hormonal therapy. To most patients, chemotherapy drugs are vital to attenuate the malignant growth of tumor. However, the efficacy of the chemotherapy is limited by intrinsic and acquired therapeutic resistance. Therefore it is urgent to develop interesting chemotherapeutic or adjuvant drugs to combat this disease [2,3].

Ganoderma lucidum, also called Lingzhi in China, is an edible mushroom and a Traditional Chinese Medicine widely used for thousands of years to promote health and increase life expectancy [4]. It was reported that G. lucidum presented a wide spectrum of biological effects including prevention of chronic diseases, such as hepatitis, hepatopathy, hypertension and cancer [5,6]. Among the active compounds in G. lucidum, triterpenoids have been demonstrated as one of the main components responsible for the pharmacological activities including immunomodulation, anti-oxidative, anti-metastasis, and anti-tumor effects [7–13]. In vitro and in vivo assays have revealed that the mixtures of triterpenoids in G. lucidum exerted anti-proliferation effects by inducing apoptosis and cell cycle arrest [14,15]. Though the current interest for these triterpenoids and related compounds isolated from G. lucidum is in the realm of “alternative” cancer treatment, it is also necessary to exploit the acting mechanisms using purified compounds and recent
studies have focused on this area and many progresses have been received [8,16].

Ganoderic acid DM (GADM, Fig. 1), a lanostane-type triterpenoid extracted from the G. lucidum, inhibits osteoclastogenesis by regulation of c-Fos and nuclear factor of activated T cells c1 [17] and exerts anti-prostate cancer effects partially via inhibiting 5α-reductase activity [18]. The purpose of this study was to determine whether GADM affects the proliferation of breast cancer cells, and if so, the possible underlying mechanisms involved.

2. Materials and methods

2.1. Materials

GADM (93.3%) was purchased from ChromaDex Inc. (Santa Ana, CA) and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 50 mM, stored at −20 °C. Doxorubicin (DOX) was obtained from Sigam (St. Louis, MO). 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide (MTT) and 5,5′,6,6′-tetrachlоро-1,1′,3,3′-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR).

2.2. Cell culture

Estrogen receptor (ER)-positive human breast cancer MCF-7 cells and ER-negative MDA-MB-231 cells (ATCC, Rockville, MD) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (GIBCO BRL, Carlsbad, CA), 100 U/ml penicillin and 100 mg/ml streptomycin, and grown with 10% fetal bovine serum (GIBCO BRL, Carlsbad, CA).

2.3. Observation of morphologic changes

MCF-7 and MDA-MB-231 cells were seeded into 6-well plates and treated with the indicated concentration of GADM for 48 h. The cellular morphology was observed with AxioCam HRC CCD camera (Carl Zeiss).

2.4. MTT assay

Exponentially growing MCF-7 cells and MDA-MB-231 cells were planted into 96-well plates and after adhesion the cells were treated with serious concentration of GADM. The cell viability was determined after 48 h-incubation by adding 20 μl MTT (5 mg/ml). Then the MTT-containing medium was aspirated slightly after 4 h and 100 μl DMSO was added to solubilize the formazan followed by shaking 10 min under the dark. The absorbance at 570 nm was recorded using a Multilabel counter (Perkin Elmer, Singapore).

2.5. Colony formation assay

MCF-7 cells were treated with series concentration of GADM for 48 h. After treatment, the cells were suspended and re-seeded into 6-well plates at a density of 200 cells per well. After two weeks, cells were fixed using 4% paraformaldehyde (PFA) and stained with Crystal Violet Staining Solution (Beyotime Institute of Biotechnology, China). The visible colonies (≥50 cells) were counted and the typical images were photographed by a common NIKON camera.

2.6. Flow cytometry analysis of DNA content

MCF-7 cells seeded into 6-well plates were treated with series concentrations of GADM for the indicated time. Cells were harvested and fixed in 70% ethanol and then stored at 4 °C overnight. Cells were stained in PBS containing 5 μg/ml RNase and 20 μg/ml PI in the dark at room temperature for 30 min and analyzed using a flow cytometry (Becton Dickinson FACS Canto™, Franklin Lakes, NJ). At least 10,000 events were counted for each sample. The DNA content in the G0/ G1, S, and G2/M phases was analyzed using ModFit 161 LT version 3.0 software (Verity Software House, Topsham, USA).

2.7. DNA fragmentation assay

MCF-7 cells were seeded into 6-well plates and treated with 50 μM GADM for the indicated time. The DNAs were extracted and purified with the Apoptotic DNA Ladder Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer’s instructions. Equal amounts of purified apoptotic DNA were applied to electrophoresis on a 1% agarose gel at 90 V (1.5 h, RT). Then the DNA fragments were stained and visualized by a UV light and photographed.

2.8. Mitochondrial membrane potential (MMP) assay

The MMP of intact cells was measured by a fluorescent inverted microscope with the lipophilic probe JC-1 [19]. MCF-7 cells were planted in the 6-well plates and treated with various concentrations of GADM for 24 h. Following treatment described as above, JC-1 fluorescence was observed with fluorescent microscopy and pictures were taken with an Axiovert 200 fluorescent inverted microscope and AxioCam HRC CCD camera (Carl Zeiss).

2.9. Western blot analysis

Cells were lysed in the lysis buffer. The proteins of the lysates were quantified with BCA™ Protein Assay Kit (Pierce, Rockford, IL). Fifty micrograms of total proteins was subjected to 6–12% SDS-PAGE and transferred onto nitrocellulose membranes, blocked with 5% nonfat milk in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween-20) at room temperature for 2 h with rocking. The membranes were probed with specific primary antibodies against CDK2, CDK6, cyclin D, p-Rb, c-Myc, PARP and phospho-H2AX-S139 (γ-H2AX) (Cell Signaling

Fig. 1. The chemical structure of GADM.
Technology, Beverly, MA) overnight at 4 °C. After washing with TBST three times for 15 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology Inc.) in TBST at room temperature for 1 h. After washing three times in TBST for 15 min, the specific protein bands were visualized using an ECL advanced Western blot detection kit. Equal protein loading was verified by re-hybridization of membranes and re-probed with anti-β-actin antibody.

2.10. Comet assay

DNA damage was evaluated using the comet assay as described [20] with modifications. Briefly, treated cells were harvested after exposed to 50 μM GADM for the indicated time, mixed with 0.75% low melting point agarose, layered onto microscope slides pre-coated with 0.75% normal melting point agarose, then the slides were submerged in pre-chilled lysis solution (1% Triton X-100, 2.5 M NaCl, 1% lauroylsarcosinate and 10 mM EDTA, pH 10.5) for 1 h at 4 °C. After soaking with pre-chilled unwinding and electrophoresis buffer (0.3 M NaOH and 1 mM EDTA, pH 13) for 20 min, the slides were subjected to electrophoresis for 20 min at 0.5 V/cm (20 mA), then stained with PI. Individual cells were viewed using an Olympus IX81 fluorescence microscope.

2.11. Statistical analysis

Data were presented as mean ± SD and significance was analyzed with Student t-test. Difference was considered significant where \( P < 0.05 \).

3. Results

3.1. GADM decreases the cell viability in breast cancer cells

Previous study showed that GADM effectively inhibited the proliferation of prostate cancer LNCaP and PC3 cells in vitro possible via 5α-reductase inhibitory and androgen receptor (AR) binding activity [18]. Herein, the breast cancer cell lines MCF-7 (ER-positive) and MDA-MB-231 (ER-negative) were used to detect the anti-proliferative potential of GADM. Cells were treated with various concentrations of GADM for 48 h and the morphological changes were observed using phase contrast microscopy. As shown in Fig. 2A, GADM decreased the percentage of adherent cells in a concentration-dependent manner in MCF-7 cells. Consistently, GADM also notably decreased the viability of MCF-7 cells as detected by MTT assay (Fig. 2B). However, as shown in Fig. 2C,D, GADM had much less anti-proliferative potential to MDA-MB-231 cells compared with that of MCF-7 cells. To further investigate the anti-cancer effect of GADM in human MCF-7 cells, the colony formation assay was used. The number of colony foci was obviously decreased after treatment with 50 μM or 100 μM GADM which is consistent with the results of morphological changes and MTT assay, indicating that GADM effectively presents anti-proliferative activity in MCF-7 human breast cancer cells (Fig. 1E,F).

3.2. GADM mediates G1 cell cycle arrest in MCF-7 cancer cells

Generally, cell cycle arrest and apoptosis induction are two major causes of cell proliferative inhibition. We thus firstly detected the distribution of cell cycle after GADM treatment in MCF-7 cells. As shown in Fig. 3A, GADM effectively mediated G1 cell cycle arrest after 48-h treatment even at low concentration (25 μM) in MCF-7 cells. Consistently, the cells distributed in S phase were significantly reduced. GADM also obviously increased the cell numbers in G1 phase with a time-dependent manner (Fig. 3B). However, 50 μM GADM hardly induced G1 cell cycle arrest in MDA-MB-231 cells (data not shown). To further determine GADM-mediated G1 cell cycle arrest, some proteins which are related with G1 cell cycle proceeding were also examined by western blot analysis. Consistent with the result of FACS assay, the expression of CDK2, CDK6, cyclin D1 and p-Rb was down-regulated after GADM treatment (Fig. 3C). It’s notable that the oncoprotein c-Myc which is vital for cells progressing into S phase [21] was also reduced after GADM treatment (Fig. 3C). These results indicate that GADM-induced G1 arrest in MCF-7 cells may be partially due to modulating of CDKs, cyclins and c-Myc.

3.3. GADM induces apoptosis in MCF-7 cancer cells

Next, we performed apoptotic assay to determine whether GADM induced apoptosis in MCF-7 cells. We first examined the internucleosomal DNA fragmentation after 50 μM GADM treatment. Upon treatment with GADM for 24 h, GADM induced internucleosomal DNA fragmentation (Fig. 4A) and the fragmentation appeared more obvious accompanied with the increased incubate time (Fig. 4A). As MCF-7 cells do not express caspase-3 [22] we chose poly (ADP-ribose) polymerase (PARP) as a maker of apoptosis [23,24]. After GADM treatment, apoptosis was observed by detecting a cleavage fragment of PARP (Fig. 4B), indicating GADM indeed induces apoptosis in MCF-7 cells. Because the mitochondrion plays an essential role in death signal transduction, we subsequently detected the MMP after GADM treatment. JC-1 is a dual-emission fluorescent dye that is internalized and concentrated by respiring mitochondria and can reflect changes in MMP in live cells [19,25]. It forms aggregates in normal polarized mitochondria that result in a green red emission and upon loss of MMP, JC-1 forms monomers that emit only green fluorescence [19,25,26]. In 24 h-treated MCF-7 cells, mitochondrial membrane depolarization, was moderately induced by GADM in a concentration-dependent manner (Fig. 4C).

3.4. GADM induces DNA damage in MCF-7 cancer cells

It is believed that DNA damage triggers cell cycle arrest and apoptosis [27]. To elucidate the possible mechanism of GADM-induced G1 arrest and apoptosis, we further detected the DNA damage after GADM exposure by comet assay and examined the expression of γ-H2AX, which is a specific marker of DNA damage [28,29]. As shown in Fig. 5A, the comet assay indicated that GADM elicited comet tail in a time-dependent manner whereas the control cells had little tails. Slightly elevated levels of γ-H2AX protein in exposed cells were also revealed after treated with GADM for 6 h
These results suggested that GADM exposure induces DNA damage which may cause cell cycle arrest and apoptosis.

4. Discussion

Terpenoids constitute a rich resource of compounds for drug discovery. GADM belongs to tetracyclic triterpenoids, and others such as pachymic acid, curcurbitacins, etc. and pentacyclic triterpenoid (ursolic acid, celastrol, etc.) also presents anti-cancer activities via triggering cell cycle arrest, inducing apoptosis, retarding metastasis and so on [30-33]. It seems that the anti-cancer mechanisms are similar among these compounds though the doses to reach the indicated effects may be different. It should be noted that the molecular targets of these terpenoids might be different which deserves further study.

GADM has been shown to exert anti-proliferation effects on both androgen-dependent and independent prostate cancer cell lines in a concentration-dependent manner [18]. One of the underlying mechanisms is that GADM attenuates the conversion of testosterone to dihydrotestosterone (DHT) by inhibition of the 5α-reductase activity and blocks DHT binding to the androgen receptor (AR) by competitive inhibition in prostate cancer cells [18]. Human breast cancer is also a hormone-sensitive malignancy and can be treated by
hormonal therapies. Both cancers share many properties and new treatment strategies have been advanced benefiting from these common issues [34]. Considering that GADM blocks the androgen signaling pathway by competitive binding to AR, we investigate the anti-proliferation effects of GADM on ER-positive MCF-7 cells and ER-negative MDA-MB-231 cells. We found that MCF-7 cells were much more sensitive to GADM compared with that of MDA-MB-231 cells, indicating that affecting of ER may partially response the anti-cancer activity of GADM though direct evidence and more samples are needed for further study.

Cell cycle arrest and apoptosis are two important events involved in the anti-cancer drug treatment and GADM effectively induced G1 cell cycle arrest and apoptosis in MCF-7 cells. Both CDK2 and CDK6 are catalytic subunits of the cyclin-dependent kinase complex and essential for the G1/S transition. Cyclin D is one of the major cyclins and cyclin D-CDKs complex partially phosphorylates Rb which is an important regulator of genes responsible for progression through G1 phase. These protein are all important for G1 cell cycle progression [35,36], and might partially be responsible for GADM-induced G1 cell cycle arrest. It has been demonstrated that c-Myc promotes cell proliferation and many investigators have uncovered the target genes that regulate the cell cycle such as CDKs and cyclins [21]. Thus, GADM-mediated c-Myc downregulation may also contribute to the G1 cell cycle arrest. The results of DNA fragmentation assay, JC-1 staining and the fact of cleavage of PARP, indicating apoptosis was indeed induced by GADM. However, as shown in Fig. 4A,B, DNA fragmentation was detected at 24 h, while PARP cleavage at that time was not obvious. The possible reasons are as follows: First, caspase 3 which is the upstream of PARP is deficient in MCF-7 breast cancer cells [22,37,38]; Second, other DNase such as endonuclease G also induces DNA fragmentation [39,40]. Anyhow, our results suggest that G1 cell cycle arrest and apoptosis induction both contributed to the anti-cancer activity of GADM.

DNA damage is one of the molecular events associated with cell cycle arrest and apoptosis and many anti-cancer reagents induce DNA damage [41,42]. It has been known that all organisms have the ability to restore genomic integrity through DNA repair. If the repair is faulty or the cell is overwhelmed by damage, chances are that the cell will despair and be removed by apoptosis [27,43]. In our study, we found that GADM elicited DNA damage after 6-h treatment measured by the comet assay and the up-regulated γ-H2AX protein levels, we thus suppose that the cell cycle arrest and apoptosis may be attributed to GADM-induced DNA damage while the detail mechanisms remain to be clearly elucidated and deserve further study.

In conclusion, the pure compound GADM isolated from G. lucidum exerts an anti-proliferative effect by inducing G1 arrest and apoptosis in MCF7 cells. DNA damage induced by GADM may be associated with apoptosis and cell cycle arrest. Our results confirm the anti-tumor effects of GADM and the potential of GADM as an agent of chemotherapeutic activity in human breast cancer cells.

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![Fig. 3. GADM mediates G1 cell cycle arrest in MCF-7 cancer cells.](image-url)
References

[14] Lin SB, Li CH, Lee SS, Kan LS. Triterpene-enriched extracts from Ganoderma lucidum inhibit growth of hepatoma cells via suppressing

Fig. 4. GADM induces apoptosis in MCF-7 cancer cells. (A) MCF-7 cells were treated with 50 μM GADM for the indicated time and the DNA fragments were separated on 1% agarose gel by electrophoresis. (B) MCF-7 cells were treated with 50 μM GADM for indicated time and the level of PARP was detected by western blot analysis. (C) MCF-7 cells in 6-well plates were treated with various concentrations of GADM for 24 h, and JC-1 mitochondrial probe was employed to test the MMP.

Fig. 5. GADM induces DNA damage in MCF-7 cancer cells. (A) Cells were treated with 50 μM GADM for the indicated time and the DNA damage was detected by comet assay. Nuclei with damaged DNA have a comet feature with a bright head and a tail, whereas nuclei with undamaged DNA appear round with no tail. Typical micrographs of comet assays were shown. (B) Cells were treated with 50 μM GADM or 2 μM DOX for 6 h and the level of γ-H2AX was detected using western blot analysis.


