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Endoplasmic reticulum stress, autophagic and apoptotic cell death, and immune activation by a natural triterpenoid in human prostate cancer cells

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Abstract

Though the current therapies are effective at clearing an early stage prostate cancer, they often fail to treat late-stage metastatic disease. We aimed to investigate the molecular mechanisms underlying the anticancer effects of a natural triterpenoid, ganoderic acid DM (GA-DM), on two human prostate cancer cell lines: the androgen-independent prostate carcinoma (PC-3), and androgen-sensitive prostate adenocarcinoma (LNCaP). Cell viability assay showed that GA-DM was relatively more toxic to LNCaP cells than to PC-3 cells (IC₅₀s ranged 45–55 μ M for PC-3, and 20–25 μ M for LNCaP), which may have occurred due to differential expression of p53. Hoechst DNA staining confirmed detectable nuclear fragmentation in both cell lines irrespective of the p53 status. GA-DM treatment decreased Bcl-2 proteins while it upregulated apoptotic Bax and autophagic Beclin-1, Atg5, and LC-3 molecules, and caused an induction of both early and late events of apoptotic cell death. Biochemical analyses of GA-DM-treated prostate cancer cells demonstrated that caspase-3 cleavage was notable in GA-DM-treated PC-3 cells. Interestingly, GA-DM treatment altered cell cycle progression in the S phase with a significant growth arrest in the G2 checkpoint and enhanced CD4⁺ T cell recognition of prostate tumor cells. Mechanistic study of GA-DM-treated prostate cancer cells further demonstrated that calpain activation and endoplasmic reticulum stress contributed to cell death. These findings suggest that GA-DM is a candidate for future drug design for prostate cancer as it activates multiple pathways of cell death and immune recognition.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

Keywords

apoptosis; calpain; endoplasmic reticulum stress; ganoderic acid DM; immune activation; prostate cancer

1 | INTRODUCTION

Prostate cancer is the most common form of cancer affecting men worldwide and the second leading cause of cancer-related deaths in men of the western world.^{1–3} Many cases of prostate cancer require only active surveillance including regular digital rectal examination and monitoring rather than treatment, as the cancer does not often progress beyond the initial site.⁴ Nonetheless, a treatment regimen is initiated if the cancer displays a more aggressive phenotype.^{5,6} Early treatment options for prostate cancer include surgery, radiation, and androgen ablation therapy.^{7–9} Surgical removal of the prostate is the most common form of treatment in early-stage prostate cancer but carries the risk of serious side effects including impairing urinary and sexual functions through damage to the prostate nerve plexus.^{10–12} Androgen ablation is used in cases of high-risk metastasis but leads to side effects including muscle atrophy, hair loss, anemia, and insulin resistance.^{13–15} As prostate cancer progresses, androgen dependence for survival and growth becomes minimal.^{16,17} Once androgen ablation therapy fails and the tumor progresses to castrate-resistant prostate cancer (CRPC), no curative therapies exist.^{17–19}

Androgens act on the androgen receptor (AR) in the process of regulation and maintenance of the prostate and also plays a role in prostate cancer development. Specifically, the conversion of androgen precursors to testosterone and dihydrotestosterone (DHT) is an important process in prostate cancer.^{20,21} The enzyme 5- α -reductase reduces testosterone to its more active form, DHT, which displays higher binding affinity and increased ligand-receptor binding time with the AR than testosterone.²² This enzyme has been the target of several anticancer drugs, most notably the steroidal inhibitor finasteride.²³ In CRPC, AR becomes hypersensitive to small amounts of circulating androgens and broadens its specificity to allow binding by androgen antagonists and corticosteroids.^{24,25} AR also becomes upregulated in the absence of androgens, and the enzymes involved in the conversion of steroid precursors to testosterone are also upregulated.²⁶ Another major problem associated with prostate cancer is its ability to metastasize, particularly to the bone,^{27,28} which is less responsive to existing therapeutics and cannot be cured. The cellular and molecular mechanisms by which prostate cancer cells metastasize to bone are likely to involve cellular proliferation, adhesion, invasion, and the release of soluble mediators from tumor cells. Thus, an effective therapy that targets prostate tumor growth and metastasis is desired for alleviating the disease and improving patient outcomes.

Tumor cells adapt to endoplasmic reticulum (ER) stress through a set of conserved intracellular pathways, as part of a process termed the unfolded protein response (UPR). Prostate cancer cells often gain resistance to early therapeutic interventions, and relapse is common. Recently, the ability to mount the UPR has been linked to prostate tumorigenesis and cancer progression. Human prostate cancer cells also express significantly more GRP78

than their benign counterparts, and increasing GRP78 expression correlates with recurrence and poor survival.^{29–31} The classical ER stress–inducing agents may activate the autophagic pathway in mammalian cells. While autophagy is a cellular response to adverse environment and stress, its significance in cell survival/death is not always clear. The ER is highly sensitive to any disturbance to its internal environment. Moderate disruption of ER function may lead to the accumulation of misfolded or unfolded proteins, triggering the ER stress or UPR. Recently, ER stress-inducing substances, such as thapsigargin have shown some promise in prostate cancer therapy, suggesting the need for a better candidate that induces ER stress with less toxicity.

Natural therapeutics with toxicity to cancer cells but limited residual toxicity, have been the subject of recent studies, such as those from the *Ganoderma lucidum* mushroom. Extracts from the *G. lucidum* mushroom have been used for centuries to maintain vitality and prolong life expectancy.^{22,32,33} Recent scientific reports have shown wide-ranging medicinal benefits of *Ganoderma* extracts, most notably targeting cancer cells with a limited toxicity to surrounding healthy cells.³⁴ Triterpenoid extracts including various biologically active ganoderic acid (GA) subtypes have shown some degree of antitumor activities.^{35,36} While GA-DM was found to be cytotoxic to androgen-dependent (LNCaP) and androgen-independent prostate cancer (PC-3),²² the mechanisms of drug-induced cytotoxicity remain unknown. GA-DM has also been shown to inhibit 5- α -reductase activity and the binding of DHT to the AR, thus preventing the downstream AR-mediated prosurvival signaling pathway. However, little is known about the molecular mechanisms underlying the killing of prostate cancer cells by GA-DM. Therefore, we aimed to investigate the possible downstream cell death pathways induced by GA-DM including ER stress, apoptosis, autophagy, and cell cycle progression. The unique effects of GA-DM to stimulate HLA class II–induced CD4⁺ T cell antitumor immune responses were also investigated.

2 | MATERIALS AND METHODS

2.1 | Cell lines and reagents

Human prostate cancer cell lines were obtained from Dr James Norris (Department of Microbiology and Immunology at MUSC). The PC-3 cell line was originally purchased from the American Type Culture Collection (Rockville, MD). LNCaP cells were obtained from Dr Voelkel-Johnson (Department of Microbiology and Immunology at MUSC). LNCaP cells were originally obtained from Urocor (Oklahoma City, OK). Cell lines PC-3 and LNCaP were cultured in complete RPMI-1640 (Invitrogen, Grand Island, NY) medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 50 U/mL penicillin, and 50 μ g/mL streptomycin (Mediatech Inc, Manassas, VA), and 1% L-glutamine (Mediatech).³⁷ The primary antibodies used in this study were human caspase-3 (31A1067) (Alexis Biochemicals, Plymouth Meeting, PA); Bcl-2 (C-2), Bax (B-9), Beclin-1 (G-11), light chain 3 β (LC3 β) (N-20), Atg5 (Santa Cruz Biotechnology Inc, Santa Cruz, CA); and β -actin (clone AC-15) (Sigma-Aldrich, St Louis, MO). HLA class II DR β -specific mAb (XD5) and DR α -specific mAb (DA6) were obtained from Dr Janice Blum (Indiana University). GRP78 was a gift from Dr Bei Liu (MUSC). The secondary antibodies used were anti-mouse and antirabbit immunoglobulin (IgG) (Santa Cruz Biotechnology Inc),

horseradish peroxidase conjugated anti-mouse (Pierce, Rockford, IL), anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology Inc). Bafilomycin A1 was purchased from Sigma-Aldrich. GA-DM, originally isolated from the *G. lucidum* mushroom, was purchased from Planta Analytica, LLC (Brookfield, CT).³⁸ The purity of GA-DM was determined by the vendor as 97.9% using Liquid chromatography-mass spectrometry analysis. GA-DM was dissolved in dimethyl sulfoxide (DMSO; Fisher Scientific, Hampton, NH) for use in all treatments, in which DMSO final concentration was adjusted to 1%.

2.2 | MTS cell viability assay

Prostate cancer cells (PC-3 and LNCaP) reaching 80% confluence were harvested using 0.05% trypsin/EDTA (CellGro) for a few minutes at room temperature, washed, and seeded at 5×10^4 cells per well in 100 μ L of appropriate culture medium in a flat-bottom 96-well plate (Corning Inc, Corning, NY). GA-DM was then added to appropriate wells for making a series of concentrations ranging from 20 to 80 μ M. After incubation for 1, 6, 12, and 24 hours at 37°C, cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI).^{38,39} Then, 20 μ L of MTS reagent was added to all wells, and the plate was incubated for 2 hours at 37°C. Following incubation, absorbance was read at 490 nm. Cells treated with vehicle alone were used as controls. The percent cell death induced by GA-DM was calculated using the equation: $[(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{treated}}) / \text{Absorbance}_{\text{control}}] \times 100$. Experiments were repeated at least three times and the data were expressed as percent cell death $\pm$ SD of triplicate wells.

2.3 | Hoechst staining

Cells were adjusted to 4×10^5 /mL of appropriate culture medium in a six-well tissue culture plate (Corning Inc; cat #3506), and were treated with 40 μ M of GA-DM or vehicle alone for 24 hours at 37°C. Cells were then incubated with Hoechst stain ($\times 1$, Sigma-Aldrich) for 10 minutes, and examined under a fluorescence microscope with an Axiovert 200 ultraviolet filter (Carl Zeiss, Jena, Germany) at $\times 200$ magnification.

2.4 | Western blot analysis

Cells were cultured for 24 hours in the presence of vehicle alone or GA-DM (20 or 40 μ M) for 24 hours. Thereafter, cells were washed in phosphate-buffered saline (PBS), and whole cell lysate was obtained using a standard lysis buffer (10 mM Trizma base, 150-mM NaCl, 1% Triton-X 100).⁴⁰ Equal protein concentrations from designated cell lysates were separated on a 4% to 12% Bis/Tris NuPage gel (Invitrogen).^{41–43} Proteins were transferred onto a nitrocellulose membrane (Pierce), and probed with specific antibodies for the expression of apoptosis (caspase-3, Bcl-2, and Bax; Santa Cruz Biotechnology Inc), ER-stress (GRP78, CHOP, and HSP70; Santa Cruz Biotechnology Inc), and autophagy-related proteins (Beclin-1, LC3, Atg5; Santa Cruz Biotechnology Inc), and HLA class II molecules (HLA-DR). The secondary antibodies used were horseradish peroxidase conjugated anti-mouse (Pierce), anti-rabbit or anti-goat IgG (Santa Cruz Biotechnology Inc). Monoclonal antibody for β -actin (Santa Cruz Biotechnology Inc) was used as a protein loading control.

2.5 | Flow cytometry

Cells were cultured for 24 hours in the presence of vehicle alone or 40 μ M of GA-DM. Following treatment, cells were washed with staining buffer (PBS + 1% heat-inactivated BGS) (HyClone), and resuspended in a binding buffer containing 0.1M HEPES (pH 7.4), 1.4M NaCl₂ and 25 mM CaCl₂ (BD Bioscience, Mountain View, CA; cat #556454). Cells were stained with annexin V-FITC (BD Bioscience; cat #556420), followed by the addition of propidium iodide (PI) (BD Bioscience; cat #556463). Samples were then analyzed for cell apoptosis using FACSscan and CellQuest software (BD Bioscience).^{39,40,43}

2.6 | Confocal microscopy

PC-3 cells were cultured on glass coverslips (cat #12–545-80; Fisher Scientific Co) in the presence of 40 μ M of GA-DM or vehicle alone for 24 hours. Cells were washed, fixed with 1% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 15 minutes, and blocked with 5% BSA for 10 minutes. Primary antibodies were then added to each slide for 1 hour at room temperature. Cells were washed twice with 1% BSA/PBS and then coated with 5% normal serum (50 μ L donkey serum in 1 mL of 1% BSA/PBS) for 10 minutes. Specific secondary antibodies were added to cells for 1 hour in the dark room. The slides were then washed twice in PBS before staining with DAPI (1:5000 v/v) for 10 minutes. The slides were mounted in fluorescent mounting medium G (SouthernBiotech, Birmingham, AL), and analyzed by a Leica TCS SP5 confocal laser scanning microscope using Las-AF software (Buffalo Grove, IL).^{41,44}

2.7 | Cell cycle analysis

To determine cell cycle distribution, PC-3 cells were treated with 40 μ M GA-DM or vehicle alone for 12 and 24 hours, respectively. Cells were pelleted in FACS tubes and then resuspended in 100 μ L of PBS, and 2 ml of 70% ethanol was added slowly to each tube while vortexing. Cells were fixed overnight at 4°C. Cells were pelleted again in microcentrifuge tubes and resuspended in 100 μ L PBS, followed by the addition of 100 μ L RNase (1 mg/mL), and 200 μ L of propidium iodide (100 μ g/mL). Tubes were wrapped in foil and stored at 4°C for 30 minutes before reading by flow cytometry.³⁸ Data acquisition was performed in the flow cytometer with the accompanying software (CellQuest; BD Bioscience). Appropriate gating was used to select the easily distinguished single population. Twenty thousand events per sample were counted, and determinations were made at least in triplicate to assure the distribution of each cell cycle.

2.8 | Antigen presentation assay

PC-3 cells were cultured in complete RPMI medium and treated with GA-DM (40 μ M) or vehicle alone for 24 hours. Thereafter, cells were washed and coincubated with human primary CD4⁺ T cells in a 96-well flatbottom plate for another 72 hours. Human T cells were obtained from DR⁺ healthy individuals with written consent. The plate was stored at –80°C until the supernatants were clarified and tested for human interferon- γ (IFN- γ) production by enzyme-linked immunosorbent assay protocol as mentioned previously.^{38,43,44}

2.9 | Statistical analysis

The data are expressed as the mean $\pm$ SD and analyzed using the Student *t* test or one-way ANOVA, with *P* = 0.05 considered statistically significant.

3 | RESULTS

3.1 | Triterpenoid GA-DM attenuates prostate cancer cell growth

Initial screening of GA-DM anticancer effects was carried out on two human prostate cancer cell lines; androgen-independent human prostate carcinoma (PC-3) and androgen-sensitive adenocarcinoma (LNCaP). Results obtained indicated that low concentrations of GA-DM markedly reduced the viability of PC-3 and LNCaP cells in a dose- and time-dependent manner, with a notably greater influence on androgen-sensitive LNCaP cells (Figure 1A and 1B). The IC₅₀s ranged from 45 to 55 μ M for PC-3 and 20 to 25 μ M for LNCaP cells. Microscopic observation in Figure 1C illustrates typical morphology of apoptotic nuclei stained with Hoechst (Figure 1C), in which chromatin was condensed and aggregated at the nuclear membrane as indicated by a bright fluorescence at the periphery. PC-3 cells are p53-null⁴⁵ and LNCaP cells express p53.⁴⁶ Thus, our data indicate that GA-DM induce nuclear fragmentation and cell death regardless of p53 status in prostate cancer cells.

3.2 | Alteration of Bcl-2/Bax proteins and apoptotic events in prostate cancer cells by GA-DM

To understand the mechanism(s) of cytotoxicity triggered by GA-DM in prostate cancer cells, we next investigated its effects on the expression of proteins regulating cell death and survival. We analyzed the cellular expression of two Bcl-2 family proteins, Bcl-2 and Bax, in PC-3 and LNCaP cells treated with GA-DM. Western blot analysis of protein lysates showed a profound reduction in protein expression of Bcl-2 that followed a dose-dependent manner in both PC-3 and LNCaP prostate cancer cell lines (Figure 2A and 2B). Meanwhile, the GA-DM treatment caused a significant increase in Bax protein expression in both PC-3 and LNCaP cells, but was most notable in LNCaP cells (Figure 3B). Although Bax, a Bcl-2 antagonist,^{47–49} was upregulated in GA-DM-treated prostate cancer cells, GA-DM induced a greater level of the active caspase-3 protein in PC-3 cells (Figure 2C). These data suggested that GA-DM treatment induced a change in Bax/Bcl-2 ratios in both PC-3 and LNCaP cells that may favor apoptotic cell death. LNCaP cells are p53-positive,⁴⁶ and showed an increased sensitivity to GA-DM. On the contrary, PC-3 cells are p53-null and showed less sensitivity to low concentrations of GA-DM; thus, we focused on relatively resistant PC-3 cells for the rest of the mechanistic studies. We first looked at apoptotic events by GA-DM in PC-3 cells using annexin V staining (Figure 2D). Flow cytometric analysis of annexin V/PI-stained PC-3 cells confirmed that apoptotic cell death plays a role in prostate cancer cells treated with 40 μ M of GA-DM for 24 hours. The results shown in Figure 2D represent both qualitative and quantitative state of early and late stages of apoptosis induced by GA-DM, where apoptosis (10.3%) was observed in PC-3 cells when compared with those treated with vehicle alone. These data suggest that GA-DM induced apoptosis in PC-3 cells by altering the ratio of cell survival Bcl-2 to apoptotic Bax proteins, and the process could be executed by the generation of effector caspase-3 molecules. These data also suggest that

other cell death pathway(s) could be involved in the GA-DM-mediated killing of prostate cancer cells as only 10% of the cells were annexin V positive.

3.3 | Regulation of autophagy and ER stress by GA-DM in prostate cancer cells

Since our data suggest the involvement of other mechanisms in GA-DM-induced cell death, we examined the role of autophagy in determining the fate of prostate cancer cells. While autophagy functioned as a mechanism of cell survival under certain conditions, some stimuli can lead to autophagic cell death.⁵⁰ To evaluate the role of GA-DM in the induction of autophagy, we performed confocal microscopy and Western blot analysis for the detection of autophagic markers Beclin-1 and LC3. Confocal microscopic analysis showed an upregulation of Beclin-1 and LC3 proteins in PC-3 cells (Figure 3A). We then conducted a posttreatment timeline using Western blot analysis to evaluate the expression of autophagic marker proteins Beclin-1 and Atg5 that are known as Bcl-2-interacting partners in the interplay between apoptosis and autophagy.^{51,52} Results showed that GA-DM-treated PC-3 cells expressed higher levels of Beclin-1 proteins (Figure 3B). It was also notable that Atg5 expression was increased first and then drastically reduced at GA-DM treatments exceeding 20 μ M. These data indicate a possible binding of Atg5 to the survival protein Bcl-2, triggering further apoptotic events characterized by the activation of caspase-3 observed in GA-DM-treated PC-3 cells (Figure 2A). Further cleavage of Atg5 has been reported previously to compromise the Bcl-2 protective ability.⁴⁶ We then analyzed the cellular expression of lipidated LC3 (LC3-II) as another prominent marker of the autophagic activity because the amount of LC3-II is often correlated with the extent of autophagosome formation.^{46,50} Data showed that LC3 cleavage to active LC3-II was strictly dose- and time-dependent (Figure 3C). Taken together, these results demonstrate the potential of autophagy as a possible player in triggering subsequent cell death in GA-DM-treated prostate cancer cells.

During autophagy, entire organelles or part of the cytoplasm are sequestered into autophagosomes, which ultimately fuse with lysosomes, forming autophagolysosomes and degrading their cellular contents.^{53,54} This autophagic process leads to an induction of type II cell death. Under conditions in which the fusion between lysosomes and autophagosomes is inhibited, the formation of autophagic vacuoles especially LC3-II could be enhanced at a preapoptotic stage. Thus, we treated PC-3 cells with bafilomycin A1, which prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes. While blocking phagolysosomal fusion could prevent autophagic cell death, our results showed that a combination of GA-DM and bafilomycin A1 enhanced prostate cancer cell death (Figure 4A) suggesting that blocking autophagy may promote GA-DM-initiated apoptotic cell death. We then measured the mitochondrial membrane potential of GA-DM-treated (20–40 μ M) PC-3 cells by staining cells with tetramethylrhodamine, ethyl ester (TMRE) (Figure 4B). TMRE is a cell-permeant, positively charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE. Flow cytometric analysis showed that GA-DM treatment reduced the membrane potential of mitochondria in PC-3 cells (76.03 vs 55.48 by 20 μ M GA-DM). These data suggest that GA-

DM induces mitochondrial cell death initiated by activation of autophagy and apoptosis in prostate cancer cells.

To further investigate the mechanisms of GA-DM-induced cell death, we also monitored the level of ER stress protein GRP78 because it acts as a molecular chaperone and is required for ER integrity and stress-induced autophagy. ER stress may also lead to the activation of multiple pathways, triggering the transcription of GRP78 and regulation of the cell growth. GRP78 is also called BiP, and is thought to be a central regulator for ER stress in the cells.^{55–57} The HSP70 family of heat shock proteins played a critical role in protein homeostasis and cellular stress.⁵⁸ CHOP is also strongly upregulated during periods of ER stress.⁵⁹ Interestingly, GA-DM treatment significantly increased GRP78, HSP70, and CHOP expression in PC-3 cells as determined by Western blot analysis (Figure 4C and 4D). Taken together, these data suggest that GA-DM activates multiple cellular pathways leading to cellular stress and prostate cancer cell death.

3.4 | Alteration of cell cycle progression by GA-DM

Cell cycle progression in treated PC-3 was monitored by the flow cytometric analysis of propidium iodide-stained cells. Data showed that GA-DM treatment resulted in a significant reduction of the S-phase population and the accumulation of G2-arrested cells compared with the untreated control (Figure 5). Flow cytometric analysis also showed an apparent delay in G1 phase following 12 or 24 hours incubation with 40 μ M of GA-DM (Figure 5C and 5D). The percentages of treated cells in the G2 phase were increased by 36.5% and 51.2% at 12 and 24 hours, respectively. These data suggested that GA-DM treatment delayed PC-3 cell cycle progression in the G1/S phase resulting in an accumulation of arrested cells in G2 phase. While activation of p53 can modulate the progression of cell cycle and genes involved in the regulation of apoptosis,⁵¹ PC-3 cells are p53 null,^{52,60} thus, the GA-DM-induced cell cycle arrest in G2 phase appears to be independent of p53.

3.5 | Calpain-mediated cell death and immune activation by GA-DM in PC-3 cells

ER stress and calpain activation have been implicated in cell death by cleaving either proapoptotic or antiapoptotic molecules, such as Bax and Bcl-2, depending on the nature of the stimuli and type of cells involved.^{61–63} Calpain expression may also influence cell death pathway (s) initiated by ER stress because ER is one of the major sources of calcium storage.^{29,31,64} As stated in Figure 4C, induction of ER stress was confirmed by the significant upregulation of ER stress protein GRP78 and CHOP following exposure to GA-DM. In addition, calpain may influence mitochondrial pathway of cell death as observed in Figure 4B. Thus, we measured if GA-DM treatment altered calpain levels in prostate cancer cells. Results showed that GA-DM markedly elevated calpains 2 and 8 in PC-3 cells (Figure 6), which may contribute to prostate cancer cell death. Inhibition of calpain by calpain inhibitor calpeptin confirmed that indeed calpain was responsible for cell death because calpeptin significantly reduced the killing of PC-3 cells by GA-DM.

Studies suggest that autophagic activity facilitates antigen processing and presentation via HLA molecules, and is critical for the deployment of the immune response against malignant tumors.^{65,66} We investigated whether GA-DM-induced autophagy in PC-3 cells

influenced the intracellular expression of HLA class II proteins that may promote an immune response against tumor cells. Data showed a remarkable elevation of cellular expression of HLA class II molecules in GA-DM-treated PC-3 cells (Figure 6C), which correlated with the autophagic events observed in those cells (Figure 3). CD4 + T cells that were cocultured with GA-DM-treated PC-3 cells produced a significant amount of IFN- γ when compared with those treated with the vehicle alone (Figure 6D). These data suggests that GA-DM may activate HLA class II antigen processing machinery of PC-3 cells, a crucial step in antigen presentation, immune recognition, and execution of prostate cancer cells.

4 | DISCUSSION

Prostate cancer is among the leading causes of cancer-related death in men in the western world, and the current therapies often fail to treat late stage, metastatic forms of the disease. Malignant prostate tumors develop a resistance to natural apoptotic activity through alterations of normal tumor suppressor p53, overexpression of prosurvival Bcl-2 family members, and other cellular signaling pathways.^{46,60,67,68} Despite their deterring side effects, chemotherapeutic drugs have become a mainstay of treatment, destroying cancer cells by inducing apoptosis,^{69,70} which effectively reduces the size of the tumor and prevents further tumor growth. Unfortunately, it often fails to prevent disease recurrence particularly following androgen ablation therapy. Another less well-defined pathway of drug-induced cell death is autophagy, a process within the cell utilizing lysosomal machinery to sequester its own molecules.^{70–72} Although autophagy is crucial to maintaining cellular homeostasis and preventing the accumulation of defective cellular structures, in some settings, autophagy and apoptosis seem to be interconnected introducing the concept of ‘molecular switches’ between them which lead to cell death following the hallmarks typical to apoptotic events.^{72,73} Nevertheless, the link between autophagy and apoptosis is not well elucidated, although understanding the interplay between these two processes may help investigators design new anticancer therapeutics. Also, in view of the fact that a contributing factor in tumor development is the disorder and poor performance of the immune system, it has become important to search for natural therapeutics that are known to augment the immune defense, and specifically target tumor cells with limited toxicity to normal cells. One of the attractive sources of antitumor products is the *G. lucidum* mushroom, which has been used for centuries as an herbal medicine for the prevention and treatment of a variety of inflammatory and malignant diseases.^{74–76} In this study, we have found that a triterpenoid extract of the *G. lucidum* mushroom, GA-DM, induces orchestrated apoptotic and autophagic events, as well as enhanced immunogenicity in human prostate cancer cells. Our data suggest that GA-DM causes significant cytotoxicity in both androgen-dependent and androgen-independent prostate tumor cells, with higher levels of cytotoxicity in the androgen-sensitive LNCaP cells. These findings are supported by previous reports showing the antagonistic effects of GA-DM on the conversion of testosterone to DHT, a process essential in regulating and maintenance of the prostate cells.^{20,22,23} We also found for the first time that GA-DM treatment induces ER stress and activates calpain processing and mediators of cell death. Thus, this study finds a connection between calpain activation and disruption of the ER following exposure of prostate cancer cells to GA-DM.

Our study showed that GA-DM caused remarkable cell death in prostate cancer through multiple mechanisms including p53-dependent and p53-independent apoptotic events. We found that GA-DM-treated cells featured a typical morphology of apoptotic nuclei stained with Hoechst, in which chromatin was condensed and aggregated at the nuclear membrane. GA-DM treatment can also induce a time-dependent surge of active caspase 3, which was pronounced in PC-3 cells but relatively lower in LNCaP cells. Quantitative figures of apoptosis observed in flow cytometric analysis of annexin V/PI-stained preparations confirms that apoptosis is a minor contributor to cell execution by GA-DM. To investigate the potential mechanism by which GA-DM induced apoptosis of human prostate cancer cells, the effect of GA-DM on the expression of apoptosis-related proteins was examined. GA-DM treatment upregulated the expression of Bax and downregulated the expression of Bcl-2, and also increased the active forms of caspase-3 in PC-3 cells. These data suggest that the upregulation of proapoptotic protein Bax may break mitochondrial potential and cause eventual activation of effector caspases and induction of apoptosis of prostate cancer cells.

Analysis of Bcl-2 and Bax proteins following GA-DM treatment suggests that alteration of Bax/Bcl-2 ratios may in part contribute to mitochondrial dysfunction and cell death as reported in other models previously.^{47–49} Interestingly, inhibition of autophagy promoted cell death by GA-DM, but calpain inhibition significantly block the cytotoxic effect of GA-DM in PC-3 cells, suggesting that GA-DM-induced cell death may be largely dependent on ER stress and calpain activation. In this scenario, the induction of ER stress by GA-DM is much more severe in that multiple cell death pathways such as autophagy and apoptosis are activated. However, GA-DM-mediated cell death may also occur following the loss of calcium homeostasis if ER calcium regulation was disrupted.

We investigated the induction of toxic autophagy as a cellular mechanism independent of caspases that may contribute to GA-DM-induced cell death. Autophagy can function as a cell survival or a cell death mechanism depending on the type of the stimulus.^{50,71} We found that treatment with GA-DM upregulated autophagic markers Beclin-1 and Atg5 that are known as Bcl-2-interacting partners in the interplay between apoptosis and autophagy. A possible binding of Beclin-1 to the survival protein Bcl-2 could possibly trigger further apoptotic events similar to the activation of caspase-3 observed in GA-DM-treated PC-3 cells. It has been previously shown that Atg5 activation compromises the Bcl-2's protective ability.^{52,77} Further cell cycle progression monitored at 12 and 24 hours posttreatment showed that the cycle was altered by the S phase, with a significant cell cycle arrest at the G2 checkpoint. Induction of autophagy events presumed to drive up antigen processing and presentation via HLA molecules, which was observed in the further initiation of the immune response against malignant tumors.^{78–80} We found that GA-DM treatment caused an upregulation of HLA class II molecules in PC-3, which correlated with enhanced antigen presentation to T cells and immune activation. Treatment of prostate cancer cells with GA-DM markedly induced the formation of autophagosomes, which were documented at the cellular and structural level.

A number of studies have shown that the induction of GRP78/BiP is a marker for ER stress.^{55,56} The HSP70 family of HSPs plays important roles in cellular stress, protein homeostasis, cell survival, and death.⁸¹ CHOP is involved in the process of cell death

associated with ER stress.⁵⁹ Our study found that GRP78, HSP70, and CHOP proteins are elevated in prostate cancer cells following GA-DM treatment.^{58,59} In the ER, accumulation of misfolded proteins causes cellular stress and activates the UPR to induce the expression of chaperones and proteins involved in the recovery process. HLA class II molecules are also abundant in the ER, especially in ER vesicular structures. We showed that higher levels of HLA-DR molecules along with HSP70 chaperone were detected in prostate cancer cells in response to GA-DM. While ER stress proteins can bind to HLA class II molecules and retain them in the ER,⁸² GA-DM-induced autophagic events may promote HLA-mediated Antigen presentation and immune activation.⁸³ Disruption of autophagy rendered cells sensitive to apoptotic death, suggesting that autophagy may play dual roles in cell survival and death after ER stress. It is important that while gaining the advantage of tumor control with cytotoxic chemotherapy, restoring the immunogenicity of cancer cells could reinstate much more effective treatment, preventing further disease recurrence. Given that GA-DM prevents prosurvival signaling pathways and activates immune recognition, it could be considered as an adjunct chemoimmunotherapy for further testing in vivo in a mouse model of advanced prostate cancer.

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FIGURE 1.

Antiproliferative effects of GA-DM on human prostate cancer cell lines. A, PC-3 and LNCaP cells were treated with vehicle (DMSO, final conc., < 0.01%) alone or GA-DM (20, 40, and 80 μM) for 24 hours at 37°C, followed by the MTS viability assay as described in Section 2. B, Cells treated with 40 μM of GA-DM were assayed for cytotoxicity after 3, 6, 12, and 24 hours posttreatment. Cells treated with vehicle alone for 3, 6, 12, and 24 hours were utilized to calculate percent cell death induced by GA-DM. Data are representative of at least three separate experiments performed in triplicate wells. C, Hoechst staining of PC-3 and LNCaP cells after 24 hours of GA-DM treatment, illustrating a typical morphology of the apoptotic nuclei with chromatin condensation and aggregation. DMSO, dimethyl sulfoxide; GA-DM, ganoderic acid DM; PC-3, prostate carcinoma-3

FIGURE 2.

GA-DM treatment altered cell death and survival regulatory proteins in prostate cancer cell lines. A, Western blot analysis of whole cell lysates showed the expression of Bcl-2 and Bax proteins in PC-3 and LNCaP cells. Cells were treated with the vehicle alone, 10, 20, or 40 μM of GA-DM for 24 hours at 37°C, followed by Western blot analysis for Bcl-2 and Bax proteins as described earlier. β-Actin was used as a loading control. B, Densitometric analysis of protein bands detected in A. Data was represented as relative density, mean ± SD. Significant differences between control and test groups were calculated by the Student *t* test; **P* < 0.05, ***P* < 0.01. C, PC-3 and LNCaP cells were treated with GA-DM for 3 to 24 hours, followed by Western blot analysis for pro- and active caspase-3. β-Actin was used as a loading control. D, Cells were treated with 20 μM of GA-DM and stained with annexin V, followed by the addition of PI as described earlier. Flow cytometric analysis of annexin V/PI-stained cells was performed to determine apoptotic cells (R2). Experiments are repeated at least three times and the data are representative of three separate experiments. GA-DM, ganoderic acid DM; PC-3, prostate carcinoma-3; PI, propidium iodide

FIGURE 3.

GA-DM treatment induces cytotoxic autophagy in PC-3 cells. A, Representative confocal microscopy images of PC-3 cells showing increased levels of Beclin-1 (image, $\times 20$) and LC3 (image, $\times 40$) proteins, following treatment with 40 μ M of GA-DM. B, C, Western blot analysis of whole cell lysates showing dose-dependent upregulation of Beclin-1, Atg5, and LC3-3 proteins, as well as a time-dependent upregulation/cleavage of LC3 proteins (arrow) in GA-DM-treated (40 μ M) PC-3 cells. β -Actin was used as a loading control. Data are representatives of at least three independent experiments with similar patterns. GA-DM, ganoderic acid DM; LC3, light chain 3; PC-3, prostate carcinoma-3

FIGURE 4.

Autophagic and apoptotic cell death may be initiated via GA-DM-induced ER stress. A, Blocking autophagy enhances cell death. PC-3 cells were incubated with autophagy inhibitor bafilomycin A1 (20 nM) or triterpenoid GA-DM (20 μ M) for 24 hours. Cells were also pretreated with bafilomycin A1 for 3 hours and GA-DM was added for an additional 21 hours, followed by MTS assay as described. B, PC-3 cells were treated with vehicle alone or GA-DM (20–40 μ M) and stained with TMRE, followed by flow cytometric analysis to determine mitochondrial potential. C, PC-3 cells were treated with vehicle alone or GA-DM (20–40 μ M) for 24 hours and subjected to Western blot analysis for various stress proteins GRP78, CHOP, and HSP70. β -Actin was used as loading controls. D, Densitometric analysis of protein bands detected in C showing significant upregulation of ER stress protein GRP78, HSP70, and CHOP. Data represent relative density $\pm$ SD. Significant differences between control and test groups were calculated by the Student *t* test; * P < 0.01, ** P < 0.05 (compared with vehicle treatment). ER, endoplasmic reticulum; GA-DM, ganoderic acid DM; PC-3, prostate carcinoma-3; TMRE, tetramethylrhodamine, ethyl ester

FIGURE 5.

GA-DM treatment induces cell cycle arrest and apoptosis in prostate cancer cells. A, PC-3 cells were treated with 40 μ M of GA-DM for 18 hours. Cells were harvested and stained with propidium iodide (PI) as described in Section 2. Data shows that GA-DM treatment decreased the number of cells in the S phase. Data represent average $\pm$ SD. Significant differences from the controls were calculated by the Student *t* test; $**P < 0.01$. B, Representative DNA histograms showed the distribution of PC-3 cells in distinct phases of cell cycle progression when treated with the vehicle alone. C, D, Cells were treated with 40 μ M of GA-DM for 12 or 24 hours. Data suggest that GA-DM treatment of PC-3 cells induces cell cycle arrest in G2 phase. GA-DM, ganoderic acid DM; PC-3, prostate carcinoma-3

FIGURE 6.

GA-DM treatment regulates calpain processing and cell death and enhances HLA class II-mediated antigen presentation in human prostate cancer cells. A, Western blot analysis of various calpain subunits. PC-3 cells treated with 40 μ M of GA-DM were analyzed by Western blot analysis for processed calpains. The blot was reprobed with β -actin and was used as a loading control. B, PC-3 cells were pretreated with calpain inhibitor calpeptin (0–2 μ M) for 3 hours, and incubated with 40 μ M of GA-DM for 24 hours at 37°C, followed by the MTS viability assay as described in Section 2. C, GA-DM treatment increased the expression of HLA-DR proteins in PC-3 cells. Western blot analysis of GA-DM-treated PC-3 cells for detecting HLA-DR molecules. The blot was reprobed with β -actin antibody and served as loading controls. D, PC-3 cells were treated with 20 μ M of GA-DM for 24 hours. Cells were washed and cocultured with human CD4 + T cells for 72 hours. The production of IFN- γ in the culture supernatant was measured by ELISA and expressed as mean pg/mL $\pm$ SD of triplicate wells. Improved antigen presentation was featured by the concurrent increase in IFN- γ production, which also corresponds to T cell proliferation. Significant differences to controls were calculated by the Student *t* test; ***P* < 0.01. ELISA, enzyme-linked immunosorbent assay; GA-DM, ganoderic acid DM; HLA, human leukocyte antigen; IFN- γ , interferon- γ ; PC-3, prostate carcinoma-3