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Gambogic acid enhances proteasome inhibitor-induced anticancer activity

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ABSTRACT

Proteasome inhibition has emerged as a novel approach to anticancer therapy. Numerous natural compounds, such as gambogic acid, have been tested *in vitro* and *in vivo* as anticancer agents for cancer prevention and therapy. However, whether gambogic acid has chemosensitizing properties when combined with proteasome inhibitors in the treatment of malignant cells is still unknown. In an effort to investigate this effect, human leukemia K562 cells, mouse hepatocarcinoma H22 cells and H22 cell allografts were treated with gambogic acid, a proteasome inhibitor (MG132 or MG262) or the combination of both, followed by measurement of cellular viability, apoptosis induction and tumor growth inhibition. We report, for the first time, that: (i) the combination of natural product gambogic acid and the proteasome inhibitor MG132 or MG262 results in a synergistic inhibitory effect on growth of malignant cells and tumors in allograft animal models and (ii) there was no apparent systemic toxicity observed in the animals treated with the combination. Therefore, the findings presented in this study demonstrate that natural product gambogic acid is a valuable candidate to be used in combination with proteasome inhibitors, thus representing a compelling anticancer strategy.

1. Introduction

The ubiquitin–proteasome system controls the turnover of regulatory proteins involved in critical cellular processes including cell cycle progression, cell development and differentiation, apoptosis, angiogenesis and cell signaling pathways [1–4]. Since aberrant proteasome-dependent proteolysis appears to be associated with the pathophysiology of some malignancies, it was suggested that pharmacological inhibition of proteasome function may prove useful as a novel anticancer strategy [5–7]. Indeed, the first proteasome inhibitor bortezomib was approved by the US Food and Drug Administration (FDA) in 2003 for the treatment of multiple myeloma which provided "proof of concept" that targeting the ubiquitin–proteasome pathway is a viable route for the treatment of human cancer [8,9]. Although bortezomib has achieved significant clinical benefit for multiple myeloma in clinical trials, its effectiveness and administration have been

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limited by toxic side effects, including asthenic conditions (such as fatigue, generalized weakness), gastrointestinal events (nausea, diarrhea, vomiting, poor appetite, etc.), hematological toxicity (low platelet and erythrocytes counts), peripheral neuropathy and high rate of shingles [10,11]. Therefore, efforts are ongoing to discover adjuvant agents, especially from natural resources, to chemosensitize malignant cells to proteasome inhibitors. The ideal adjuvant should augment the effect of proteasome inhibitors to achieve optimal therapeutic effects at lower doses, while resulting in minimal toxicity.

Gambogic acid (GA, Fig. 1A) is a natural product isolated from gamboge, which is a dry resin secreted from the *Garcinia hurburyi* tree. The gamboge resin has been used as a coloring agent and in traditional Chinese medicine for the treatment of human diseases including indigestion, inflammation, and ulcers [12]. Early studies revealed that GA acts as a potent inducer of apoptosis in cancer cells [13,14]. Recent studies have demonstrated that GA has anticancer effects and inhibits the growth of several types of human cancer cells, including prostate, breast, gastric carcinoma, hepatocarcinoma, epithelial cervical cancer, lung cancer and leukemia *in vitro* and *in vivo* [13–21]. GA has been approved by the Chinese Food and Drug Administration for the treatment of various cancers in clinical trials [22]. However, whether GA possesses synergistic anticancer effects when combined with proteasome inhibitors in malignant cells has yet to be reported.

In the present study, we report for the first time that the combination of natural product GA with two proteasome inhibitors (MG132, MG262) produces a significant synergistic effect in both malignant cells and tumors, resulting in reduced cell viability/proliferation and apoptotic cell death.

2. Materials and methods

2.1. Materials, reagents, and antibodies

MG132, MG262, z-VAD-fmk and Gambogic acid were purchased from BIOMOL International LP (Plymouth Meeting, PA, USA). MG132, MG262 and GA were dissolved in DMSO (Sigma; St. Louis, MO) at a stock concentration of 10 mM, aliquoted and stored at −80 °C. Cremophor EL was purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS), RPMI 1640, penicillin and streptomycin were purchased from Invitrogen by Life Technology (Carlsbad, CA, USA). Rabbit polyclonal antibody against GAPDH (FL-335) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibody against nuclear poly(ADP-ribose) polymerase (PARP), rabbit monoclonal antibody against caspase-3 (8G10), and mouse monoclonal antibodies against caspase-8 (1C12) and caspase-9 (C9) were purchased from Cell Signaling (Beverly, MA, USA). Enhanced chemiluminescence (ECL) reagents were purchased from Amersham Biosciences (Piscataway, NJ, USA). Propidium idodide (PI) and Annexin V-FITC Apoptosis Detection Kit were purchased from Keygen Company (Nanjing, China).

2.2. Cell lines and cell culture

Murine hepatoma H22 and human leukemia K562 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and grown in RPMI 1640 supplemented with 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin. Cell cultures were maintained at 37 °C and 5% CO_2.

2.3. MTS assay

The effects of compounds on cell viability were determined by the MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation assay, Promega Corporation, Madison, WI, USA). The MTS tetrazolium compound is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium. This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells. Exponentially growing cells were harvested and seeded at 2500 cells/well in a 96-well plate. After 6 h incubation, compounds or DMSO, as the untreated control, were added, followed by continuous incubation for 48 h. Twenty microliter of MTS were added to each well and the incubation was continued for an additional 3 h. The absorbance was measured with a microplate reader (Sunrise, Tecan) at 490 nm. The percent inhibition was calculated as follows: Inhibition rate (IR) (%) = [1 − (absorbance of experimental well − absorbance of blank)/(absorbance of untreated control well − absorbance of blank)] × 100%.

2.4. Cell death detection assay via flow cytometry

Apoptotic cell death was measured by Annexin V-FITC and propidium iodide (PI) double staining followed by flow cytometry as previously described [23]. Briefly, cultured K562 and H22 cells were harvested and washed with cold PBS and resuspended with the binding buffer, followed by Annexin V- FITC incubation for 15 min and PI staining for another 15 min at 4 °C in dark. The stained cells were analyzed with flow cytometry within 30 min.

2.5. Morphological characterization of cell death

K562 or H22 cells were treated as described. To monitor temporal changes in the incidence of cell death in the live culture condition, propidium idodide (PI) was added to the cell culture medium and at the desired sequential time points, the cells in the culture dish were imaged with an inverted fluorescence microscope equipped with a digital camera (Axio Obsever Z1, Zeiss). PI is not able to enter the normal live cells but the dying or dead cells lose their membrane integrity and PI can enter their nucleus, bind to double-stranded DNA, and thereby positively stain the dying and dead cells.

2.6. Establishment and treatment of H22 allografts

H22 allograft model was established as previously described [24]. Briefly, murine hepatoma H22 cells (10×10^6) suspended in 0.2 ml of serum-free RPMI 1640