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# Calcium channel blocker verapamil accelerates gambogic acid-induced cytotoxicity *via* enhancing proteasome inhibition and ROS generation

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ABSTRACT

Verapamil (Ver), an inhibitor of the multidrug resistance gene product, has been proved to be a promising combination partner with other anti-cancer agents including proteasome inhibitor bortezomib. Gambogic acid (GA) has been approved for Phase II clinical trials in cancer therapy in China. We have most recently reported that GA is a potent proteasome inhibitor, with anticancer efficiency comparable to bortezomib but much less toxicity. In the current study we investigated whether Ver can enhance the cytotoxicity of GA. We report that (i) the combination of Ver and GA results in synergistic cytotoxic effect and cell death induction in HepG2 and K562 cancer cell lines; (ii) a combinational treatment with Ver and GA induces caspase activation, endoplasmic reticulum (ER) stress and reactive oxygen species (ROS) production; (iii) caspase inhibitor z-VAD blocks GA + Ver-induced apoptosis but not proteasome inhibition; (iv) cysteine-containing compound N-acetylcysteine (NAC) prevents GA + Ver-induced poly(ADP-ribose) polymerase cleavage and proteasome inhibition. These results demonstrate that Ver accelerates GA-induced cytotoxicity *via* enhancing proteasome inhibition and ROS production. These findings indicate that the natural product GA is a valuable candidate that can be used in combination with Ver, thus representing a compelling anticancer strategy.



## 1. Introduction

Proteasome inhibition has been demonstrated as a novel therapeutic strategy in cancer therapy. Bortezomib (Velcade), a specific proteasome inhibitor, has been approved by the United States Food and Drug Administration (FDA) to treat multiple myeloma patients (Adams, 2004; Adams et al., 1999; Kane et al., 2006; Orlowski and Dees, 2003). Several second generation proteasome inhibitors are now under clinical trials for cancer therapy (Yang et al., 2009). Although bortezomib has achieved significant clinical benefit for multiple myeloma in clinical trials, its effectiveness and administration have been limited by toxic side effect. To improve the efficacy of proteasome inhibition-based treatments, drugs augmenting the antitumor properties of bortezomib or other proteasome inhibitors and/or reducing their dose-dependent toxicities are required.

Gambogic acid (GA) is a natural product isolated from gamboge, which has been used as a coloring agent and in traditional Chinese medicine for the treatment of various human conditions for hundreds of years (Auterhoff et al., 1962; Panthong et al., 2007). Recent studies have demonstrated that GA has a spectrum anticancer effect both *in vitro* and *in vivo* with low toxic side effects (Huang et al., 2011a; Liu et al., 2005; Yang et al., 2007; Yi et al., 2008). GA has been approved by the Chinese FDA for the treatment of solid cancers in Phase II clinical trials (Zhou and Wang, 2007). Even though several molecular targets of GA have been suggested, most of them might not be responsible for GA-induced cytotoxicity (Kasibhatla et al., 2005; Pandey et al., 2007; Rong et al., 2009; Wu et al., 2004; Xu et al., 2009; Yu et al., 2007). We have demonstrated that GA is able to selectively inhibit tumor proteasome activity, with potency comparable to bortezomib but lower toxicity. Compared with bortezomib, GA could produce tissue-specific

*Abbreviations:* GA, gambogic acid; Ver, verapamil; Ab, antibody; ER, endoplasmic reticulum; ROS, reactive oxygen species; z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone; CT-like, chymotrypsin-like; DCFH-DA, 2′,7′-dichlorfluorescein-diacetate; PARP, poly ADP-ribose polymerase; MDR, multidrug resistance; UPR, unfolded protein response; PI, propidium iodide; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; NAC, N-acetylcysteine.

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proteasome inhibition and tumor-specific toxicity (Li et al., 2013; Shi et al., 2013). We have also demonstrated that GA could overcome hypoxia-induced myocardial hypertrophy *via* targeting the proteasome (Zhao et al., 2013).

As an L-type calcium channel blocker, verapamil (Ver) has been used in clinics for many years. It was approved by the United States FDA in 1981 and has been used for the treatment of cardiac arrhythmias, hypertension, and, most recently, as a promising combination partner with bortezomib (Chen et al., 2012; Meister et al., 2010). Ver has also been reported as an inhibitor of the multidrug resistance (MDR) gene product in cancer therapy (Endicott and Ling, 1989). In the current study, we report that the combination of GA and Ver synergistically enhanced cytotoxicity and cell death in tumor cell cultures, which was associated with enhanced proteasome inhibition, caspase activation, generation of reactive oxygen species (ROS) and induction of endoplasmic reticulum (ER) stress.

## 2. Materials and methods

### 2.1. Materials

GA and z-VAD-FMK [Carbobenzoxy-valyl-alanyl-aspartyl-(O-methyl)-fluoromethylketone] were purchased from BIOMOL International LP (Plymouth Meeting, PA). Ver was from National Institute for the Control Pharmaceutical and Biological Products (Beijing, China). Propidium iodide (PI)/Annexin V-FITC Apoptosis Detection Kit was from Keygen Company (Nanjing, China). Rabbit polyclonal antibodies (Abs) against nuclear PARP (poly (ADP-ribose) polymerase); rabbit monoclonal Abs against BIP (C50B12), caspase-3 (8G10) and Bcl-2 (50E3); mouse monoclonal Abs against C/EBP homologous protein (CHOP, L63F7), caspase-8 (1C12) and caspase-9 (C9) were from Cell Signaling (Beverly, MA). Rabbit polyclonal Abs against cleaved caspase-8 (Cleaved Asp384) was from Assay biotechnology Company, Inc. Rabbit polyclonal antibodies against cleaved caspase-9 p35 (D315) and cleaved caspase-3 p17 were from Bioworld Technology, Inc. Mouse monoclonal Abs against ubiquitin (P4D1) and Bax (B-9), rabbit polyclonal Ab against GAPDH (FL-335) and horseradish peroxidase (HRP)-labeled secondary Abs were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences (Piscataway, NJ). DCFH-DA (2′,7′-dichlorfluorescein-diacetate) was from Beyotime Institute of Biotechnology (Jiangsu, China).

### 2.2. Cell viability assay

Cell viability was detected by trypan blue exclusion assay. Briefly, HepG2 or K562 cancer cells ($5 \times 10^3$ cells/well) were seeded in 96-well plate and treated with GA, Ver and the combination for 72 h, then the cells were detached by trypsinization and viable cells were determined by counting the viable cells with trypan blue staining. All the results are from at least three independent experiments.

### 2.3. Cell death assay

Apoptosis assay was performed as we previously described (Huang et al., 2010). In brief, cultured HepG2 and K562 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.

The morphological changes of cell death were performed as we reported (Huang et al., 2012). To monitor temporal changes in the incidence of cell death in the live culture condition, HepG2 and K562 cells were seeded into 12-well plates and PI was added directly to the cell culture medium, then the cells in the culture dish were kinetically imaged with an inverted fluorescence microscope equipped with a digital camera (Axio Obsever Z1, Zeiss).

### 2.4. Western blot analysis

Western blot was performed as we described previously (Li et al., 2013). Briefly, an equal amount of total protein extracted from cultured cells were separated by 12% SDS–PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were blocked with 5% milk for 1 h. Primary Abs and horseradish peroxidase (HRP)-conjugated secondary Abs were each incubated for 1 h. The bounded secondary antibodies were reacted to the ECL detection reagents and exposed to X-ray films (Kodak, Japan).

### 2.5. ROS production

HepG2 or K562 cells were treated with GA and/or Ver for 24 h, and then the cells were incubated with the free serum medium with addition of 10 μM of DCFH-DA for 20 min at 37 °C. Then cells were collected for flow cytometry analysis. DCFH penetrates the cells and is in turn oxidized to DCF in the presence of ROS, and the DCF fluorescence intensity was determined by flow cytometric analysis. The fold changes of mean fluorescence intensities are shown in the diagram. Mean values and standard deviations were calculated from triplicates.

### 2.6. Cell-based chymotrypsin-like (CT-like) activity assay

This was performed as we previously reported (Huang et al., 2011b). Cancer cells (4000 cells) were treated with either vehicle or GA, Ver and the combination for 6 h. The treated cells were incubated with the Promega Proteasome-Glo Cell-Based Assay Reagent (Promega Bioscience, Madison, WI) for 10 min. The CT-like proteasome activity was detected. Luminescence generated from each reaction was detected with luminescence microplate reader (Varioskan Flash 3001, Thermo Scientific, USA). Means and standard deviations are calculated from three independent experiments.

### 2.7. Statistical methods

Mean ± SD are presented where applicable. Unpaired Student's *t*-test or one way ANOVA is used where appropriate for determining statistic probabilities. *P* value less than 0.05 is considered significant.

## 3. Results

### 3.1. The combination of Ver and GA induced apoptosis in cancer cells in a synergistic manner

Human hepatoma HepG2 and leukemia K562 cells were grown in RPMI 1640 supplemented with 10% FBS. The effect of GA or Ver alone and the combination on the cell viability was assessed by trypan blue exclusion assay. Treatment with increasing doses of either GA (0.3, 0.4, 0.5 μM) or Ver (20, 30, 40 μM) only slightly decreased cell viability in HepG2 cells after 72 h, while the combination of GA and Ver dramatically decreased the HepG2 cell viability (Fig. 1A). In K562 cells, GA plus Ver also yielded the similar synergistic effects, compared to each treatment alone (Fig. 1B).

Next the induction of cell death by GA and Ver combination treatment was analyzed. As displayed in Fig. 1C, the combination of GA and Ver markedly increased Annexin V/propidium iodide