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Bullatacin, a potent antitumor Annonaceous acetogenin, induces apoptosis through a reduction of intracellular cAMP and cGMP levels in human hepatoma 2.2.15 cells

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Abstract

Bullatacin, a potential antitumor Annonaceous acetogenin (AA), is isolated from the seed of the Formosa *Annona atemoya*. We reported previously that bullatacin inhibits the secretion of hepatitis B surface antigen from 2.2.15 cells (human hepatoma HepG2 cells transfected with hepatitis B virus DNA plasmid). In the present study, we determined cell apoptosis by using double-dye staining with fluorescein-isothiocyanate-labeled annexin V and propidium iodide. We found that bullatacin induced apoptosis in 2.2.15 cells in a time-dependent manner; the most significant apoptotic change appeared at 16 hr. Moreover, different concentrations (10^{-3} to 1.0 μM) of bullatacin induced apoptosis in a concentration-dependent manner at 16 hr. The determination of intracellular cyclic AMP (cAMP) and cyclic GMP (cGMP) levels in 2.2.15 cells after exposure to bullatacin demonstrated that bullatacin caused both to decrease in a time- and concentration-dependent manner. A time course (0.33, 1, 6, 16, 24 hr) study indicated that while both cAMP and cGMP levels decreased early (at 0.33 hr), the most dramatic decline appeared at 6 hr. Meanwhile, the inhibitory effect on cAMP and cGMP levels reached a maximum at 16 hr (90.5 ± 3.2 and 47.3 ± 12.8%, respectively). The concentration-dependent decrease of both cAMP and cGMP induced by bullatacin was parallel with the magnitude of apoptosis induced by various concentrations (10^{-3} to 1.0 μM) of bullatacin. Additionally, the bullatacin-induced apoptosis was inhibited by the addition of cAMP and cGMP elevating agents (forskolin and *S*-nitrosoglutathione). Our results suggest that a decrease of both cAMP and cGMP levels may play a crucial role in bullatacin-induced apoptosis in 2.2.15 cells.
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Keywords: Annonaceous acetogenin; Bullatacin; cAMP; cGMP; Apoptosis; Hepatoma 2.2.15 cells

1. Introduction

The AAs are a new and rapidly growing class of waxy natural compounds found, thus far, only in the Annonaceae family [1]. They have attracted considerable attention in recent years because of their special structures and wide range of biological activities [2]. In general, the AAs are comprised of 35 to 37 carbon atoms encompassing two long hydrocarbon chains, a terminal 2,4-disubstituted-γ-lactone, and a variable number of THF rings.

Since the initial discovery in 1982 of uvaricin by activity-directed fractionation using the P388 murine leukemia antitumor system [3], over 350 natural AAs have been isolated [2–4]. We have reported 10 more new AAs and their related pharmacological activities [5–14]. Currently, AAs have been demonstrated to possess pesticidal, antimalarial, T-cell suppressant, antiparasitic, antimicrobial, cytotoxic, and *in vivo* antitumor effects [2,12–17].

We have reported that bullatacin induces cytotoxicity in various cancer cell lines [7,11,12]. Bullatacin was isolated from the seed of the Formosa *Annona atemoya*, which is a potent antitumor AA with an adjacent bis-THF ring structure (MW: 622) (Fig. 1) [11,12,18,19].

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Abbreviations: AAs, Annonaceous acetogenins; cAMP, cyclic AMP; cGMP, cyclic GMP; DMEM, Dulbecco's minimal essential medium; FBS, fetal bovine serum; FITC, fluorescein-isothiocyanate; GSNO, *S*-nitrosoglutathione; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NO, nitric oxide; PI, propidium iodide; PS, phosphatidylserine; and THF, tetrahydrofuran.

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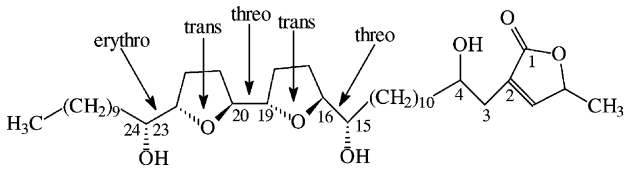

Fig. 1. Chemical structure of bullatacin.

HBV causes acute and chronic hepatitis, which affects nearly 300 million people worldwide [20,21]. Chronic HBV infection is recognized as a major risk factor for the development of primary hepatocellular carcinoma. An effective antiviral therapy against HBV infection has not been fully developed, and studies have been hampered by the extremely narrow host range and limited access to experimental culture systems [22].

The 2.2.15 cells (human hepatoma HepG2 cells transfected with HBV DNA plasmid) secreting hepatitis B virions make it possible to examine the effectiveness of potential anti-HBV-associated hepatoma drugs [22,23].

In one of our previous studies, we reported the inhibitory effects of bullatacin on HBsAg secretion from 2.2.15 cells [12]. In the present study, we investigated the ability of bullatacin to induce apoptosis by double staining 2.2.15 cells with FITC-labeled annexin V and PI.

Thus far, there have been no reports of any AAs regulating intracellular second messengers or apoptosis signal transduction. Since little is known about the nature of the intracellular signal transduction pathways involved in bullatacin-induced apoptosis, we examined the roles of cAMP and cGMP as possible candidates for modulating apoptosis in 2.2.15 cells.

cAMP [24–26] and cGMP [27–29] are important mediators in apoptosis. Here, we report that bullatacin decreased the levels of intracellular cAMP and cGMP in a time- and concentration-dependent manner and that the decreased concentrations of these messengers correlated with the magnitude of bullatacin-induced apoptosis.

We also evaluated the effects of forskolin, a potent activator of adenylate cyclase leading to increased cAMP levels, and of GSNO, an NO donor leading to increased cGMP levels, on bullatacin-induced apoptosis. We have determined a relationship between apoptosis and the bullatacin-mediated alteration of cAMP and cGMP levels. Our data indicated that apoptosis induced by bullatacin in 2.2.15 cells may be through a mechanism that reduces intracellular cAMP and cGMP levels.

2. Materials and methods

2.1. Materials

Bullatacin was isolated from the seed of the Formosa *Annona atemoya* by Dr. Yang-Chang Wu (Graduate Institute of Natural Products, Kaohsiung Medical University). DMSO, forskolin, GSNO, and PI were purchased from the Sigma Chemical Co.; annexin V-FITC was purchased from Boehringer Mannheim.

cAMP and cGMP kits were obtained from Amersham. Tissue culture plates (24-well) and 100-mm diameter plastic petri dishes were purchased from the Falcon Co. DMEM, PBS, FBS, and other tissue culture reagents were purchased from Gibco.

2.2. Cell culture

The 2.2.15 cells [22,23] were provided by G. Acs (Mount Sinai Medical Center) and Dr. Y.C. Cheng (Department of Pharmacology, School of Medicine, Yale University). They were cultured in 24-well plates with mycoplasma-free medium, maintained at 1.0×10^5 cells/mL in DMEM supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/mL), and streptomycin (100 μg/mL), and incubated at 37° in a humidified atmosphere containing 5% CO_2 and 95% air.

2.3. Annexin V-FITC labeling of apoptotic cell membranes

Bullatacin-induced apoptosis in 2.2.15 cells was quantified using annexin V-FITC/PI flow cytometry. The use of annexin V as a marker for apoptosis is based on the translocation of PS from the inner face of the plasma membrane to the cell surface during the early stages of apoptosis [30,31]. Cell surface PS can be detected by staining with annexin V-FITC. Apoptotic cells exclude PI, while necrotic cells do not. Thus, PI was used as a marker to detect necrotic cells. Apoptotic cells were identified as those intensely stained with annexin V-FITC at the cell membrane in the absence of nuclear staining by PI.

Double staining for annexin V-FITC binding and for cellular DNA using PI was performed as follows: After washing twice with PBS, 1×10^6 cells were resuspended in the dark in HEPES buffer (10 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) containing annexin V-FITC (1:50, Boehringer Mannheim) and 1 μg/mL of PI for 15 min. Following labeling, cells were fixed with 4% paraformaldehyde for 10 min. The quantitative analysis of apoptotic cells was performed immediately after staining, using an EPICS flow cytometer (Coulter Electronics) and estimated with CytoLogic software.

2.4. cAMP quantitation

After 2.2.15 cells were trypsinized, cell density was adjusted to 1×10^5 cells/mL. Cells (1.0 mL) were distributed into 24-well plates and incubated at 37° for 24 hr before treatment. Then they were preincubated with bullatacin for different times (0.33, 1, 6, 16, 24 hr) before the addition of 20 μM forskolin for 20 min. After terminating the reaction, cAMP was extracted with 65% ice-cold alcohol.

The cAMP alcohol extracts were centrifuged (2000 *g* for 15 min at 4°) and evaporated at 60°. The dried residue was dissolved in 1 mL of kit assay buffer, and 100-μL aliquots were analyzed using a cAMP enzyme immunoassay (EIA) kit purchased from the Amersham Pharmacia Biotech Co. [26].

The assay is based on competition between unlabeled cAMP in the sample or standard and a fixed quantity of peroxidase-labeled cAMP (cAMP–peroxidase conjugate) for a limited number of binding sites on a cAMP-specific antibody. The cAMP–peroxidase conjugate-bound antibody was immobilized on polystyrene microtiter wells precoated with a second antibody. Unbound ligands were removed from the wells by washing. The amount of bound cAMP–peroxidase conjugate is determined by the addition of a tetramethylbenzidine/hydrogen peroxide substrate. With fixed amounts of antibody and cAMP–peroxidase conjugate, the amount of bound cAMP–peroxidase conjugate will be inversely proportional to the concentration of added unlabeled cAMP.

The assay was performed as follows: A 100-μL sample or standard and 100 μL antibody were added into the wells of a microtiter plate and gently mixed for 2 hr at 3–5°. After the incubation, 50 μL of the cAMP–peroxidase conjugate was added, and the mixture was incubated for an additional 1 hr. Then the wells were washed four times with 400 μL of wash buffer. Following washing, 150 μL substrate was added, and the plates were shaken for 1 hr at 15–30°. One hundred microliters of 1.0 M sulfuric acid was added to each well to stop the reaction, and the optical densities were determined at 450 nm within 30 min. The concentration of cAMP was calculated according to the cAMP standard curve generated in each assay.

2.5. cGMP quantitation

After 2.2.15 cells were trypsinized, the cell density was adjusted to 1×10^5 cells/mL. Cells (1.0 mL) were distributed into 24-well plates and incubated at 37° for 24 hr before treatment. They were also preincubated with bullatacin for different times (0.33, 1, 6, 16, 24 hr) before treatment with 100 μM GSNO for 10 sec. Then the reaction was terminated, and cGMP was extracted with 65% ice-cold alcohol.

The cGMP alcohol extracts were centrifuged (2000 *g* for 15 min at 4°) and evaporated at 60°. The dried residue was dissolved in 1 mL of kit assay buffer, and 100-μL aliquots of cGMP were analyzed using an Amersham cGMP EIA kit [27].

The cGMP assay, performed according to the instructions of the manufacturer, was similar to the assay used to determine cAMP. Briefly, a 100-μL sample or standard and an equal volume of antibody were added to the wells of a microtiter plate and gently mixed for 15–18 hr at 3–5°. After the incubation, 50 μL of cGMP–peroxidase conjugate was added, and the mixture was incubated for an additional 3 hr. Then the wells were washed four times with 400 μL of wash buffer. Following washing, 200 μL of substrate was added, and the plates were shaken for 30 min at room temperature. One hundred microliters of 1.0 M sulfuric acid was added to each well to halt the reaction, and the optical densities were estimated at 450 nm within 30 min. The concentration of cGMP was calculated according to the cGMP standard curve generated in each determination.

2.6. Statistical analysis

Results were expressed as means ± SD and analyzed by Student's *t*-test. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Bullatacin-induced apoptosis in 2.2.15 cells assessed by annexin V-FITC/PI double staining

Annexin V-FITC binding identifies membrane asymmetry due to transposition of PS from the inner to the outer leaflet of the surface membrane [30,31]. Development of this asymmetry may be an early marker for apoptosis, which allows selective recognition and clearance of the apoptotic cells by macrophages [32,33].

It has been reported that annexin V-FITC binding is a sensitive marker for apoptosis and could clearly define cAMP-mediated apoptosis [34]. Thus, we measured this quantitative marker to demonstrate the early apoptotic reaction.

To shed light on the time course of bullatacin-induced apoptosis in 2.2.15 cells, a kinetics study was performed. Apoptosis in 2.2.15 cells, determined by double staining with annexin V-FITC/PI, is exemplified by the representative flow cytometric analysis depicted in Fig. 2A. The lower left quadrant of each panel shows the viable cells, which exclude PI and are negative for annexin V-FITC binding. The upper right quadrants contain the non-viable, necrotic cells, positive for annexin V-FITC binding and for PI uptake. The lower right quadrants represent the apoptotic cells, annexin V-FITC positive and PI negative, demonstrating cytoplasmic membrane integrity.

As shown in Fig. 2B, the FITC positive and PI negative apoptotic cell population was dramatically increased by 16 hr (72 ± 5.5%). Treatment with different concentrations (10^{-3} to 1.0 μM) of bullatacin for 16 hr demonstrated that the effect of bullatacin on inducing apoptosis was concentration-dependent (Fig. 3).

3.2. Effects of bullatacin on the intracellular concentration of cAMP

cAMP has been shown to play an important role in the induction of apoptosis in various cell types [24–26,34].

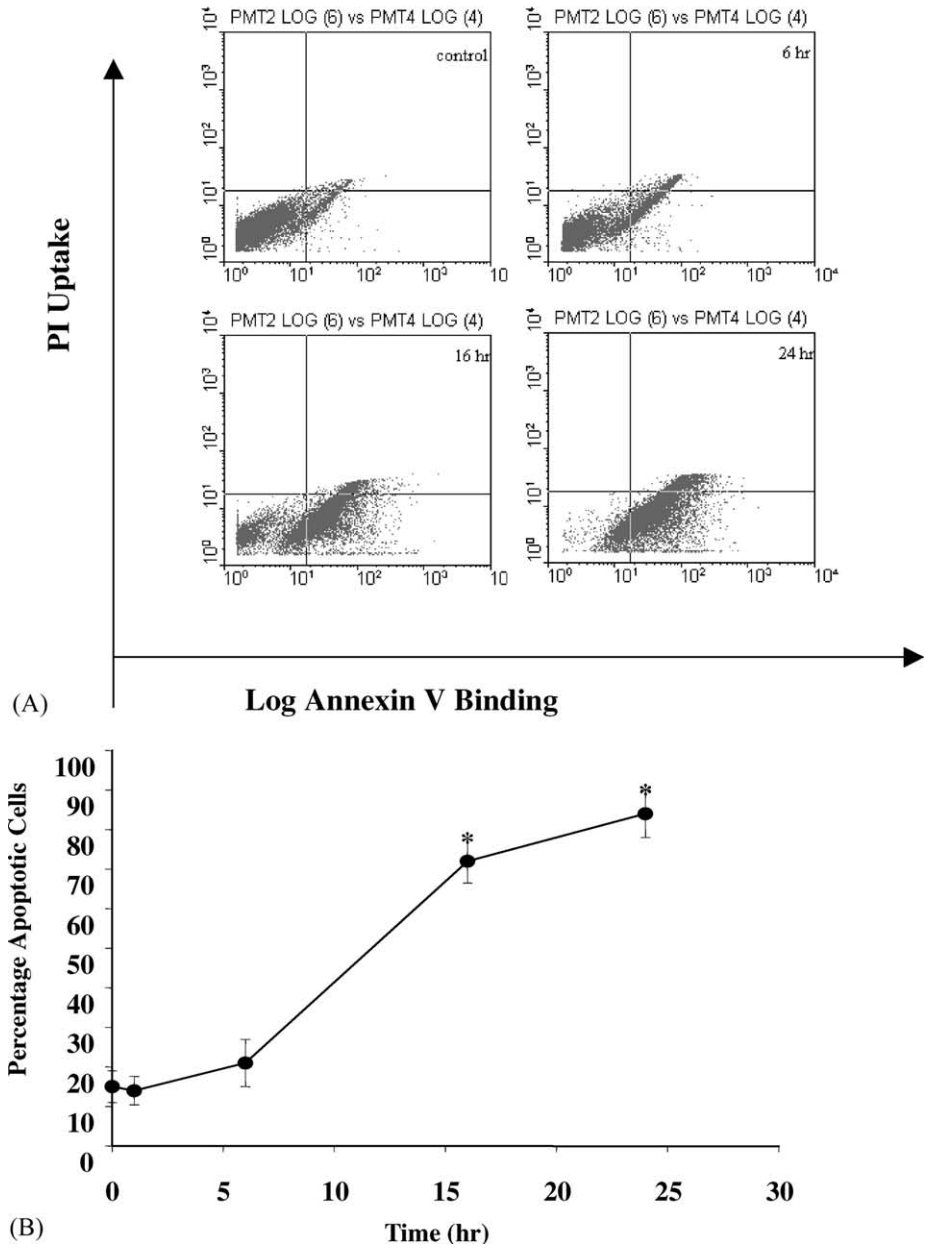

Fig. 2. Kinetic study of bullatacin-induced changes in the distribution of PS in the cell membrane. (A) Contour diagram of annexin V-FITC/PI flow cytometry of 2.2.15 cells treated for the indicated time intervals with and without (control) 0.1 μM bullatacin. The lower left quadrant of each panel shows the viable cells, which exclude PI and are negative for annexin V-FITC binding. The upper right quadrants contain the non-viable, necrotic cells, positive for both annexin V-FITC binding and PI uptake. The lower right quadrants represent the apoptotic cells, annexin V-FITC positive and PI negative, demonstrating cytoplasmic membrane integrity. Ten thousand cells were analyzed at each time point. One representative experiment out of three is shown. (B) Time course of bullatacin-induced increases in the percentage of apoptotic cells. Results are expressed as the means ± SD of three separate experiments. Key: (*) statistically significant ($P < 0.05$) vs control.

However, the role of this intracellular transducer in bullatacin-induced apoptosis has not been characterized. To determine the relationship between the regulation of cAMP by bullatacin and bullatacin-induced apoptosis, we determined the time course of the modulation of cAMP levels by measuring the level of cAMP in 2.2.15 cells with the ELISA method.

2.2.1.5 cells were pretreated with 0.1 μM bullatacin for increasing intervals before treatment with 20 μM forskolin for an additional 20 min. Forskolin, a potent activator of adenylate cyclase, was efficient in raising cAMP in 2.2.15 cells, which reached a peak by 20–30 min. As shown in Fig. 4, the intracellular level of cAMP dropped sharply just after the start of the treatment with 0.1 μM bullatacin and decreased to 9.5 ± 3.2% of the control value at 6 hr.

To observe the correlation between changes in intracellular cAMP levels and the percentage of apoptosis, we measured the intracellular cAMP levels of 2.2.15 cells that were pretreated with various concentrations (10^{-3} to 1.0 μM) of bullatacin for 6 hr and then stimulated with

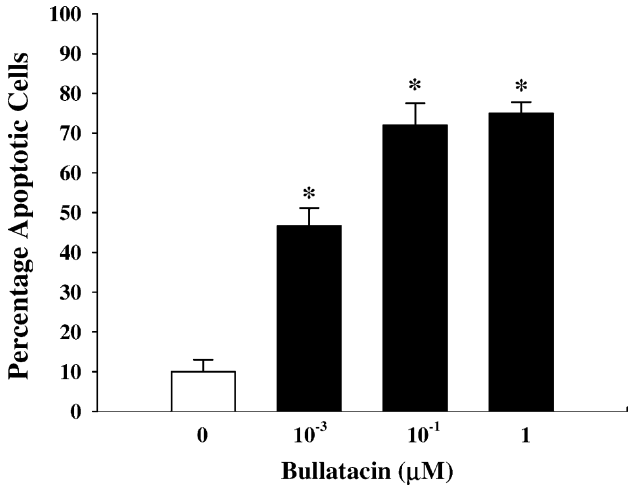

Fig. 3. Concentration–response effect of bullatacin on 2.2.15 cell apoptosis. Apoptosis was assessed by flow cytometric analysis of the annexin V-FITC/PI double staining method. Cells were cultured in the absence or presence of bullatacin (10^{-3} to 1.0 μM) for 16 hr prior to being stained. Apoptosis was determined at 16 hr. Ten thousand cells were analyzed in each sample. Results are expressed as the means ± SD of six experiments. Key: (*) statistically significant ($P < 0.05$) vs control.

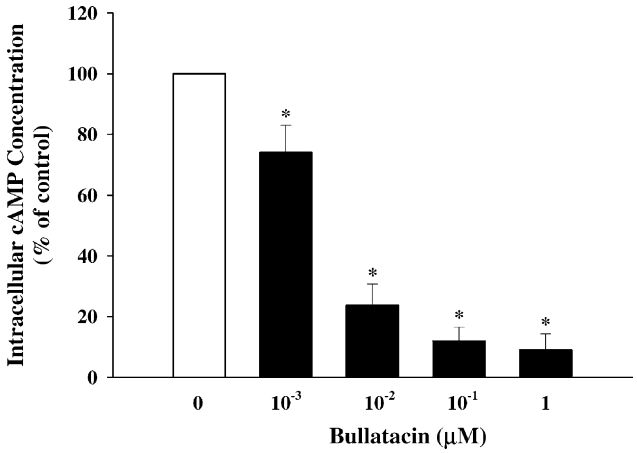

Fig. 5. Concentration–response effect of bullatacin on forskolin-stimulated intracellular cAMP levels. Cells were incubated in the absence (control) or presence of bullatacin (10^{-3} to 1.0 μM) for 6 hr. cAMP concentration was determined after a 20-min stimulation with 20 μM forskolin. Control value: 100% = 17 pmol/1.0 × 10^5 cells. Data represent the means ± SD of three separate experiments. Key: (*) statistically significant ($P < 0.05$) vs control.

20 μM forskolin for an additional 20 min. As shown in Fig. 5, bullatacin decreased the level of intracellular cAMP in a concentration-dependent manner, and this effect was not reversed by forskolin treatment. There was a close correlation between the decrease of intracellular cAMP levels and the high incidence of cellular apoptosis.

3.3. Effects of bullatacin on the intracellular concentration of cGMP

The cGMP pathway is known to be of paramount importance in the modulation of apoptosis [27–29]. However, how cGMP is involved in bullatacin-induced apoptosis has not been characterized. To examine whether bullatacin altered the intracellular level of cGMP in 2.2.15 cells, we measured the cGMP concentration by EIA.

2.2.15 cells were pretreated with 0.1 μM bullatacin for increasing intervals before treatment with 100 μM GSNO for 10 sec. GSNO, a stable NO donor, was efficient in raising cGMP in 2.2.15 cells, which reached a peak around 10 sec. As shown in Fig. 6, the intracellular level of cGMP in cells dropped sharply just after the start of the treatment with 0.1 μM bullatacin and decreased to 52.7 ± 12.8% of the control value at 6 hr.

To investigate the correlation between intracellular cGMP levels and the percentage of apoptosis, we measured

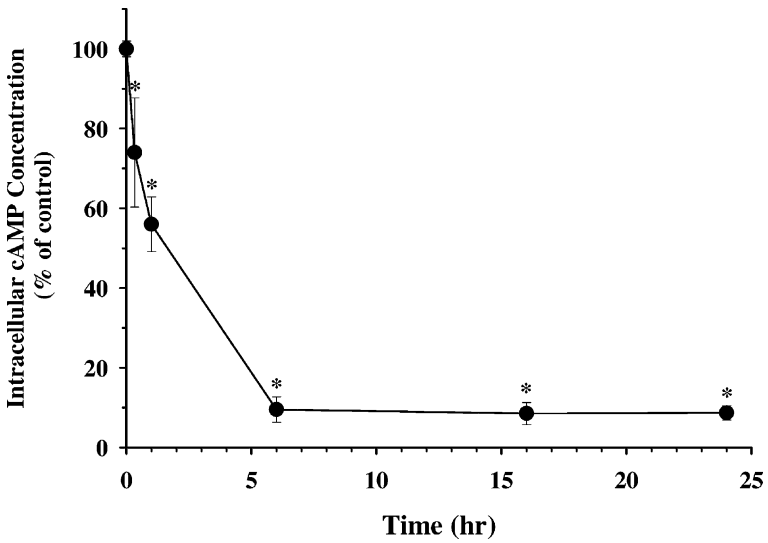

Fig. 4. Time course of the inhibitory effects of bullatacin on forskolin-induced intracellular cAMP elevation in 2.2.15 cells. Cells were treated in the absence (control) or presence of 0.1 μM bullatacin for the time periods indicated. cAMP concentration was determined after a 20-min stimulation with 20 μM forskolin. Control value: 100% = 13.32 pmol/1.0 × 10^5 cells. Data represent the means ± SD of six experiments. Key: (*) statistically significant ($P < 0.05$) vs control.

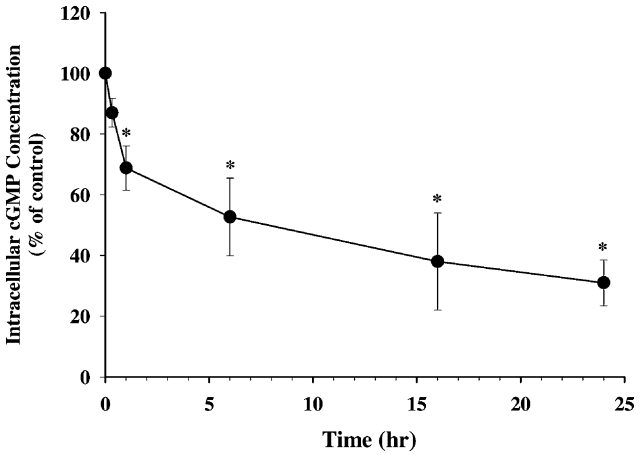

Fig. 6. Time course of the inhibitory effects of bullatacin on GSNO-stimulated intracellular cGMP levels. Cells were treated in the absence (control) or presence of 0.1 μM bullatacin for the time periods indicated. cGMP concentration was determined after a 10-sec stimulation with 100 μM GSNO. Control value: 100% = 2870 fmol/1.0 × 10^5 cells. Data represent the means ± SD of three separate experiments. Key: (*) statistically significant ($P < 0.05$) vs control.

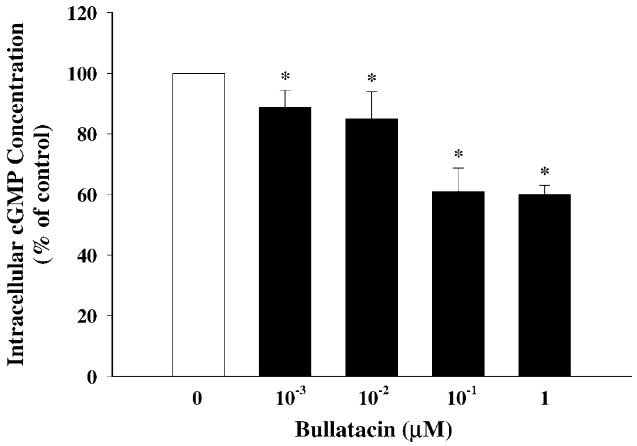

Fig. 7. Concentration–response effects of bullatacin on GSNO-stimulated intracellular cGMP levels. Cells were incubated in the absence (control) or presence of bullatacin (10^{-3} to 1.0 μM) for 6 hr. cGMP concentration was determined after a 10-sec stimulation with 100 μM GSNO. Control value: 100% = 3470 fmol/1.0 × 10^5 cells. Data represent the means ± SD of three separate experiments. Key: (*) statistically significant ($P < 0.05$) vs control.

intracellular cGMP levels of 2.2.15 cells pretreated with various concentrations of bullatacin for 6 hr followed by treatment with 100 μM GSNO for a further 10 sec. As shown in Fig. 7, bullatacin decreased intracellular cGMP levels in a concentration-dependent fashion, and this effect was not reversed by GSNO treatment. There was a close correlation between the decrease of intracellular cGMP levels and the high incidence of cell apoptosis.

3.4. Effects of forskolin and S-nitrosoglutathione on bullatacin-induced apoptosis of 2.2.15 cells

Bullatacin treatment caused a marked decrease of intracellular cAMP and cGMP concentrations in 2.2.15 cells, suggesting that a decrease of cAMP and cGMP may be a primary component of the signaling pathway leading to the activation of apoptosis in 2.2.15 cells. To further examine

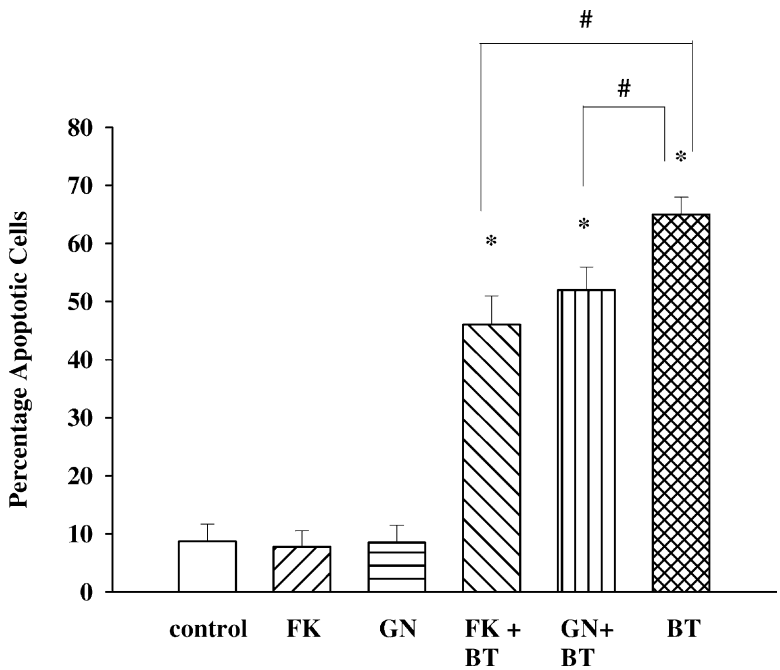

Fig. 8. Protective effects of forskolin and *S*-nitrosoglutathione on bullatacin-induced cell apoptosis. Cells were co-treated with bullatacin (BT) and 5 μM forskolin (FK) or with 25 μM *S*-nitrosoglutathione (GN) for 16 hr. Apoptosis was assessed by flow cytometric analysis of annexin V-FITC and PI double staining. Ten thousand cells were analyzed. Data represent the means ± SD of three separate experiments. Key: (*) indicates $P < 0.05$ compared with the control, untreated cells; and (#) indicates $P < 0.05$ compared with bullatacin-treated cells.

this possibility, cells were simultaneously administered 0.1 μM bullatacin and 5.0 μM forskolin, a potent activator of adenylate cyclase that increases cAMP levels, or 25 μM GSNO, a stable NO donor that increases cGMP levels. After a 16-hr incubation, both forskolin and GSNO decreased the percentage of bullatacin-induced apoptosis by approximately 25 ± 5 and $20 \pm 5\%$, respectively (Fig. 8). These data confirmed the involvement of cAMP and cGMP in bullatacin-induced cell apoptosis.

4. Discussion

AAs are a new class of natural products that have attracted increasing interest as new potential anticancer agents [2,16,17]. The compounds have been categorized into four structural subclasses according to the tetrahydrofuran (THF) ring(s): (a) adjacent bis-THF ring, (b) non-adjacent bis-THF ring, (c) mono-THF ring, and (d) non-THF ring [2–4]. Bullatacin is a potent antitumor Annonaceous acetogenin with an adjacent bis-THF ring structure (Fig. 1) [11,12,16–19].

In the present study, we used annexin V-FITC/PI double staining as a quantitative marker to estimate cell apoptosis induced by bullatacin. Detection of apoptosis by annexin V is based on the transposition of PS from the inner to the outer leaflet of the cell surface membrane during the early stages of apoptosis [30,31]. Our data showed that bullatacin induced the exposure of PS on the surface of 2.2.15 cells in a time- and concentration-dependent manner. Development of this asymmetry is a critical event that allows recognition and removal of the apoptotic cells by macrophages before they are lysed [32,33]. Thus, the leakage of cell constituents with secondary inflammation is prevented.

The 2.2.15 cells are human hepatocarcinoma HepG2 cells that are transfected with a plasmid containing HBV DNA. We have reported previously that bullatacin exhibited inhibitory effects on hepatitis B virion secretion, [^{3}H]thymidine incorporation, and cell proliferation through the induction of apoptosis [12]. It has been widely reported that apoptosis may be an important goal of cancer therapy [35,36]. The induction of apoptosis by bullatacin in 2.2.15 cells indicates that bullatacin may be a potential anti-HBV-associated hepatoma drug.

Martin *et al*. [30] reported that activation of an inside–outside PS translocase is an early and widespread event during cellular apoptosis.

In our time course study, bullatacin-induced changes of PS asymmetry were detected at 16 hr, prior to significant apoptotic change detected by terminal deoxynucleotide transferase-mediated dUPT nick-end labeling (TUNEL) at 24 hr (data to be published elsewhere).

Many studies have reported that cAMP has an important role in the regulation of apoptosis [24–26,34]. However, the signals involved in bullatacin-induced apoptosis remain unknown. We found that treatment with bullatacin dramatically inhibited the intracellular cAMP levels of 2.2.15 cells (Figs. 4 and 5). Therefore, we suggest that cAMP may play a crucial role in bullatacin-induced cell apoptosis.

There is some evidence that confirms our hypothesis. The time course studies indicated that bullatacin caused a persistently time-dependent decrease of cAMP; a significant inhibitory effect appeared at 6 hr, which was prior to the marked incidence of PS transposition (early apoptosis) at 16 hr. Meanwhile, the fact that forskolin, a cAMP-elevating agent, decreased the percentage of cell apoptosis caused by bullatacin indicated a correlation between the decrease of intracellular cAMP levels and the high incidence of apoptosis induced by bullatacin.

Wolvetang *et al*. [37] reported that specific mitochondrial respiratory chain inhibitors rotenone and antimycin A and a highly specific mitochondrial ATP-synthase inhibitor, oligomycin, have the ability to induce apoptosis within 12–18 hr. Bullatacin was reported previously to be a potent enzyme inhibitor of mitochondrial electron transport [1,19] and NADH oxidase activity [38,39], which may lead to a decrease in ATP, the precursor of cAMP, thereby causing a decrease in the levels of intracellular cAMP. Further studies must define whether a global decrease in ATP precedes changes in cAMP and cGMP, thereby providing a basis for bullatacin-induced 2.2.15 cell apoptosis.

The regulation of apoptosis by cAMP has been investigated in several different cell types [24–26,34,40]. Parvathenani *et al*. [34] found that the delay in caspase-3 activity was parallel to the cAMP-induced delay of human neutrophil apoptosis. Garcia-Bermejo *et al*. [41] reported that cAMP-elevating agents produce an inhibitory effect on retinoblastoma (Rb) type I phosphatase and ICE/CED-3-like protease activities, as well as the abrogation of c-*myc* expression in myeloid cells. Their results indicated that the increase of cAMP inhibited the apoptosis process. Our results are consistent with all these reports.

It is possible that bullatacin via decreased cAMP levels mediates the expression of key regulatory genes, transcription factors, and proteins reportedly associated with the regulation of apoptosis. However, the exact mechanisms by which cAMP is involved in the induction of apoptosis need to be studied further.

cGMP has been proposed as an important mediator of apoptosis [27–29]. Our study showed that the administration of bullatacin dramatically inhibited cGMP in a time- and concentration-dependent manner (Figs. 6 and 7). Therefore, we also hypothesize that bullatacin-induced apoptosis may be associated with alteration of the cGMP level.

In our experiments, we have demonstrated a marked inhibitory effect of bullatacin on the intracellular concentration of cGMP at 6 hr, prior to the translocation of PS to the outer surface of the cell membrane at 16 hr. The high incidence of apoptosis is parallel with the decline of the intracellular cGMP level. These results are similar to the finding in our cAMP experiment. Moreover, the addition of GSNO, a stable NO donor that increases cGMP levels, has

the ability to prevent the apoptosis induced by bullatacin. Based on these results, we suggest that the decrease in the cGMP level may be strongly correlated with 2.2.15 cell apoptosis.

It was reported that NO inhibits apoptosis in splenic B lymphocytes [29], human eosinophils [42], and hepatocytes [43]. NO acts, in part, by stimulating soluble guanylate cyclase to increase levels of intracellular cGMP [44–46]. The ability of cGMP to activate cGMP-dependent protein kinase (PKG) leads to many physiological effects, such as vascular smooth muscle relaxation, neutrophil activation, and hepatoprotection [47].

NO, via the cGMP-dependent mechanism, directly or indirectly inhibits caspase-3-like activity, resulting in the prevention of apoptosis in hepatocytes [43]. In the present investigation, we found the decrease of intracellular cGMP level to be coincident with bullatacin-induced 2.2.15 cell apoptosis. Reducing intracellular cGMP levels may promote caspase-3-like activity, resulting in bullatacin-induced 2.2.15 apoptosis. However, the exact mechanisms by which cGMP mediates bullatacin-induced 2.2.15 apoptosis remain to be established.

In summary, our data demonstrate for the first time that decreased intracellular cAMP and cGMP levels may play important roles in the bullatacin-induced apoptosis pathway in human hepatoma 2.2.15 cells. Our study also indicated that the pharmacological manipulation of cAMP and cGMP levels may influence the cytotoxic and antitumor activity of bullatacin.

However, the inhibitory bioactivities of bullatacin on cAMP and cGMP are different. The inhibitory effect on cAMP ($90.5 \pm 3.2\%$ at 6 hr) was more potent than that on cGMP ($47.3 \pm 12.8\%$ at 6 hr). The details of the pharmacological mechanisms of bullatacin on cellular apoptosis will be investigated in our future studies.

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