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Gambogic acid induced oxidative stress dependent caspase activation regulates both apoptosis and autophagy by targeting various key molecules (NF-κB, Beclin-1, p62 and NBR1) in human bladder cancer cells

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ABSTRACT

Background: Gambogic acid is a potent anticancer agent and has been found effective against various types of cancer cells. The present study was addressed to explore the cytotoxic potential of Gambogic acid and the modulation of autophagy and apoptosis in bladder cancer cells T24 and UMUC3.
Methods: Bladder cancer cell lines T24 and UMUC3 were treated with Gambogic acid, apoptosis was checked by flow-cytometry and expression of various autophagy and apoptosis related proteins was monitored by Western blotting. Confocal microscope was used for colocalization of p62 and Beclin-1.
Results: Gambogic acid induces reactive oxygen species, and elicits a strong autophagic response by activating JNK at earlier time points, which is inhibited at later time points with the activation of caspases. Reactive oxygen species mediated caspase activation causes degradation of autophagic proteins, cleavage of molecular chaperones (Hsp90 and GRP-78) and adaptor proteins (p62 and NBR1). Gambogic acid treatment results in mitochondrial hyperpolarization and cytochrome c release and activates caspases involved in both extrinsic and intrinsic apoptotic pathways. Gambogic acid abrogates NF-κB activation by ROS mediated inhibition of IκB-α phosphorylation. Functionally Gambogic acid induced autophagy acts as a strong cell survival response and delays caspase activation.
Conclusion: Our study provides the new insights about the mechanism of Gambogic acid induced modulation of autophagy and apoptosis in bladder cancer cells. All the molecular events responsible for Gambogic acid induced autophagy and apoptosis are mediated by reactive oxygen species.
General significance: Since Gambogic acid targets various cell survival molecules therefore, it may be considered as a potential anticancer agent against bladder cancer.

1. Introduction

Plant derived compounds are beneficial for the treatment of several types of cancers [1] and over 60% of the anticancer drugs are derived from natural sources. Gambogic acid (GA, $C_{38}H_{44}O_8$, Fig. 1A) is one such recently discovered compound which specifically targets rapidly dividing cancerous cells but is not very toxic for normal cells [2,3]. GA has multiple targets in different types of cancer cells which include Bcl-2 family proteins [4], Hsp90 [5], and redox regulatory proteins [6] and is also known to act as a tissue specific proteasomal inhibitor [7]. GA has been also reported to inhibit kinase activity of IKK-β by covalent modification thereby blocking NF-κB activation [8]. Involvement in both intrinsic and extrinsic pathways of apoptosis has been documented in GA mediated cancer cell death. GA has showed promising results in *in vivo* system [2] and has been approved for clinical trials by the Chinese Food and Drug Administration [9].

GA has been also reported to exert its anticancer effect via generation of reactive oxygen species (ROS). ROS are the by-products of normal metabolism and are found to be elevated in cancer cells. Alterations in the ROS levels have been shown to have catastrophic effects on cancerous cells. ROS have been shown to induce apoptosis by activating caspase-3 [10], induce mitochondrial outer membrane permeabilization [11] and modulate various signaling molecules like NF-κB, HIF-1α, AP-1 and p53 [12].

Recently, ROS have been shown as one of the strongest triggers of autophagy associated with most of the cancers particularly solid tumors [13,14]. Autophagy is a general term for the degradation of cytoplasmic components within lysosomes. Autophagy has three main steps:

Abbreviations: ROS, reactive oxygen species; GA, gambogic acid; LC3, microtubule-associated protein (MAP) light chain 3; NAC, N-acetyl-L-cysteine

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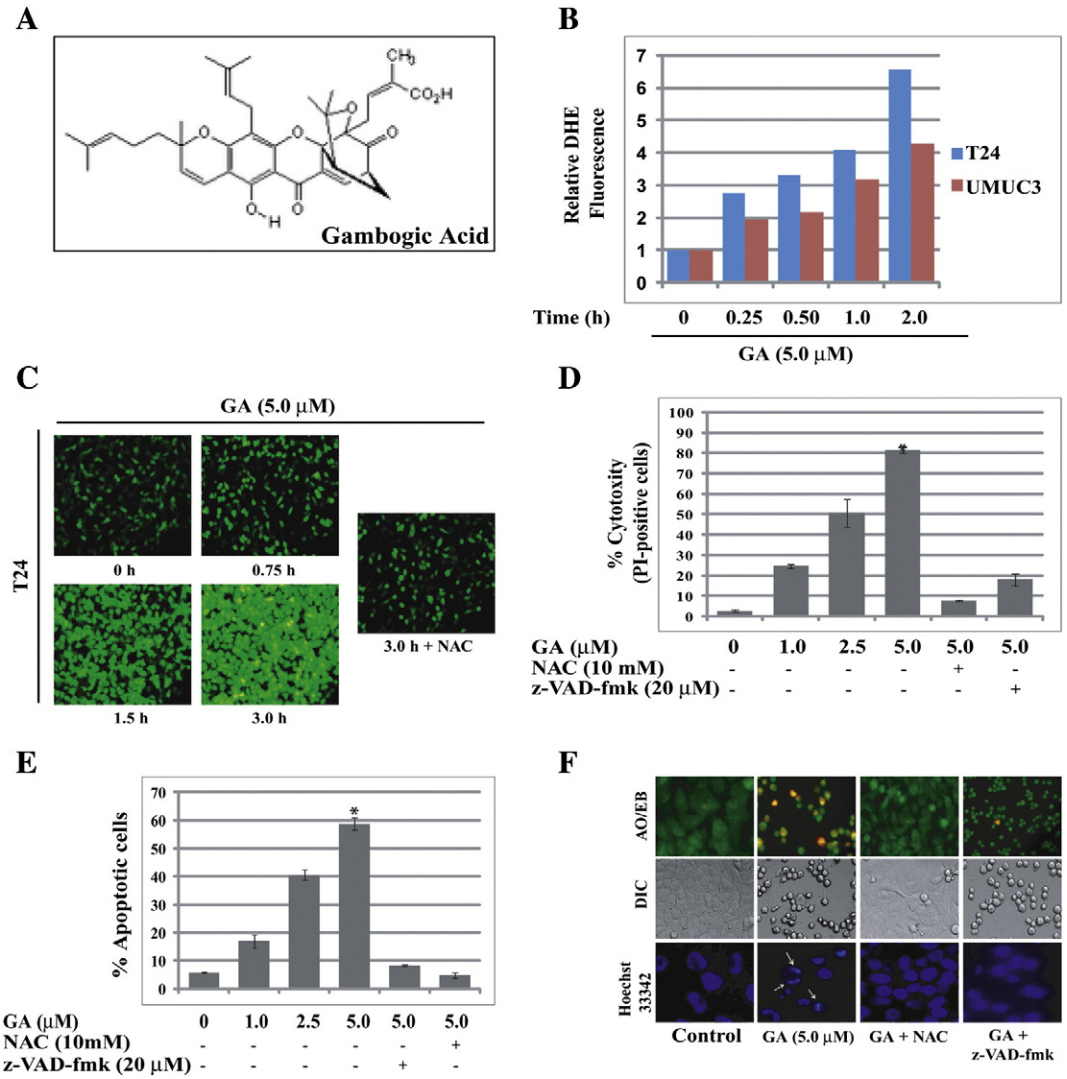

Fig. 1. GA induces apoptosis in bladder cancer cells. (A) Chemical structure of GA. (B) T24 and UMUC3 cells were treated with GA for indicated times, and the level of superoxide was assayed by dihydroethidium staining by flow-cytometry. Bar graph represents change in superoxide level relative to fluorescence intensity of control. Data represent the results from one of the three similar experiments. (C) ROS level was visualized by CM-H_2DCFDA staining under fluorescence microscope. (D, E) T24 cells were treated with GA in the presence and absence of NAC or z-VAD-fmk for 24 h and examined for cell viability by live PI and sub-G_1 analysis. Values are expressed as mean ± SD of three similar experiments. $^*P < 0.05$ vs. control. (F) AO/EB staining, DIC, Hoechst 33342 staining after 24 h treatment with GA, either alone or in the presence of NAC or z-VAD-fmk. Arrow indicates apoptotic cells.

initiation, elongation and maturation. Each step is regulated by specific autophagy related proteins (Atgs) like Beclin-1, LC3, Atg5 and Atg7 [15]. From the studies of the past few years, it has become increasingly apparent that autophagy and apoptosis are intricately linked with each other to ensure the tight regulation of cellular homeostasis. The crosstalk between autophagy and apoptosis is mediated by various proteins like Bcl-2 family members [16]. Caspases have been also reported to be important modulators of autophagy by cleaving various Atg proteins [17]. Autophagy was generally thought to be a nonselective degradation system, but p62 and NBR1 (neighbor of BRCA1 gene), the two adaptor proteins, have been shown to be involved in selective autophagy [18,19]. These scaffolding proteins selectively target some ubiquitinated proteins to autophagosomes for their degradation. p62 has been also shown to regulate apoptosis by activating caspase-8 adding another link for crosstalk between these two processes [20]. Apart from apoptosis and autophagy, p62 is also involved in the regulation of NF-κB and other signaling pathways [21,22].

Recent speculation that ROS generating compounds may show promising results against bladder cancer [23] and the fact that role of drug induced autophagy in bladder cancer cells has not been explored in detail. Since, GA can generate ROS which can modulate both apoptosis and autophagy and these properties of GA motivated us to explore this compound against bladder cancer. Bladder cancer is one of the most common urogenital cancers with very high recurrence rate and is the most expensive type of cancer to manage [24]. Bladder cancer cells constitutively express STAT-3 and some anti-apoptotic proteins such as Bcl-2 and c-FLIP, which confer resistance to various anticancer therapies [25,26]. Here, we report for the first time that GA induces apoptosis in bladder cancer cells by generating ROS. ROS leads to the induction of autophagy at earlier time points which is a strong cell survival response and delays caspase activation. Once caspases are activated which results in degradation of various autophagy and several other important cell survival proteins. Inhibition of GA induced autophagy results in early activation of caspases and increased apoptosis in bladder cancer cells.

2. Materials and methods

2.1. Cell culture and reagents

Human bladder cell lines, T24 and UMUC3 were procured from and authenticated by the European Collection of Animal Cell Cultures (ECACC). Cell line was passaged for less than three months before use

in this study. Both cell lines were cultured in RPMI-1640 medium supplemented with 10% heat inactivated Fetal Bovine Serum, GIBCO. GA was purchased from Calbiochem (La Jolla, CA). N-acetyl-L-cysteine (NAC), E64D, paraformaldehyde, pepstatin-A, rapamycin, wortmannin, bafilomycin A_1, chloroquine, propidium iodide(PI), Hoechst 33342, MG132, anti-ubiquitin antibody, anti-β actin antibody, anti-mouse HRP, anti-rabbit HRP, anti-goat HRP antibody, caspase-8 inhibitor, z-IETD-fmk, caspase-6 inhibitor, z-VEID-fmk, caspase-3 inhibitor, Ac-DEVD-CHO, pan-caspase inhibitor, z-VAD-fmk, caspase-8 colorimetric assay kit, caspase-3 colorimetric assay kit and 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) were purchased from Sigma-Aldrich (St. Louis, MO). Dihydroethidium and chloromethyl derivative of H_2DCFDA, CM-H_2DCFDA, Alexa-Fluor 488 anti-rabbit and anti-mouse 633 were purchased from Invitrogen (Carlsbad, CA, USA). PI/RNase staining buffer, Annexin V/PI kit and antibodies against cytochrome c, PARP, Bcl-xL, IKK-α, IKK-β, Hsp90, Beclin-1, p62, caspase-8, caspase-2, caspase-9, c-Jun, pc-Jun, pJNK, JNK and GRP-78 were purchased from BD (Becton Dickinson, San Jose, CA). LC3, Atg5, Atg7, Atg4c, and NBR1 antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Bid antibody was purchased from Abcam (Cambridge, MA). Antibodies against caspase-3, Bax, p50, p65, p-IκB-α and IκB-α and control siRNA, Beclin-1 siRNA and siRNA transfection reagent were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). NF-κB oligo was purchased from Promega (Madison, WI, USA).

All the experiments were performed in T24 cells and the experiments carried out in UMUC3 cells have been marked in the figures. NAC (10 mM), z-VAD-fmk (20 μM), z-IETD-fmk (20 μM), z-VIED-fmk (20 μM), Ac-DEVD-CHO (20 μM), MG132 (1.0 μM), CQ (50 μM), Wm (200 nM) and E64D and pepstatin (10 μg/ml each) treatment were given 1 h before the addition of GA for indicated time points and combinations as found in the figures.

2.2. Measurement of ROS

Dihydroethidium fluorescent probe was used to measure the intracellular generation of superoxide anion radical, $O_2^{-}\bullet$ as described earlier [27]. For fluorescence microscopy, cells were incubated with GA for different time points and then incubated with CM-H_2DCFDA (10 μM) at 37 °C for 5 min and subsequently washed twice in cold PBS and viewed using Zeiss fluorescence microscope [28].

2.3. Immunostaining

Cells were grown treated and fixed with 4% paraformaldehyde for 5 min, permeabilized with 0.1% Triton X-100 for 5 min, and then blocked with 2% BSA for 1 h at room temperature. Double immunostaining was performed using rabbit anti-Beclin-1 (1:50) and mouse anti-p62 (1:500) followed by staining with secondary antibodies (goat anti-rabbit Alexa Fluor 633 (1:500) and rabbit anti-mouse Alexa Fluor 488 (1:500)) [29]. Cover slips were visualized under confocal microscope (Nikon A1R).

2.4. Western blot analysis

Western blot analysis was performed as described previously [27]. Cytosolic extracts were prepared as described by Miyoshi et al. [30]. Densitometry of individual bands was determined using Scion Image software (Scion Corporation).

2.5. Live PI, sub-G_1 analysis and Annexin V/PI staining

Cell viability was determined by PI exclusion assay [27] and checked by sub-G_1 analysis as described earlier [31]. The percentage of apoptotic cells was determined using Becton Dickinson FACSCalibur with Cell Quest software. Annexin V/PI staining was performed as per the manufacturer's protocol. The percentage of PI positive and Annexin V positive cells was analyzed using FACSDiva software.

2.6. Fluorescence/morphological examination

Cell morphology was investigated by staining cells with a combination of fluorescent DNA binding dyes AO/EB (Acridine Orange/Ethidium Bromide). Briefly, cells were treated with different concentrations of GA for 24 h and cell viability was determined under Zeiss fluorescence microscope [31]. For analysis of nuclear morphology, Hoechst staining was carried out.

2.7. Electrophoretic mobility shift assay (EMSA)

NF-κB DNA-binding activity and supershift assay was determined by the method as described [27,28].

2.8. siRNA transfection

2×10^5 T24 cells were seeded overnight and transfected with Beclin-1 and control siRNA according to the manufacturer's protocol (Santa Cruz, CA). After 36 h of transfection cells were treated with GA (5.0 μM) for 3 h. Cell lysates were prepared and analyzed for LC3-I to LC-II conversion by Western blotting.

2.9. Caspase activity assay

Caspase-3 and caspase-8 activities were measured after GA treatment (0–5.0 μM) according to the manufacturer's protocol (Sigma).

2.10. Statistical analysis

Statistical significance of the differences was determined by the paired two-tailed Student's *t* test. *P* values < 0.05 were considered as statistically significant.

3. Results

3.1. GA induces apoptosis in bladder cancer cells in a ROS dependent manner

ROS generation by GA was measured in two different bladder cancer cell lines, T24 and UMUC3. Superoxide anion level was measured by dihydroethidium staining and was found to increase with time (Fig. 1B). Intracellular staining using CM-H_2DCFDA further confirmed our results and N-acetyl-L-cysteine (NAC, ROS scavenger) effectively reduces GA induced ROS generation (Fig. 1C). Cell viability was assessed by live PI and sub-G_1 analysis using flow-cytometry. Both cell lines showed a dose dependent increase in the percentage of apoptotic cells. Pretreatment with NAC or broad range caspase inhibitor, z-VAD-fmk significantly reduced GA induced apoptosis (Fig. 1D–E). GA induced cell death was further confirmed by Annexin V/PI staining. Our results showed that GA induces apoptosis (Annexin V positive) in a dose dependent manner which was inhibited by NAC and z-VAD-fmk (Sup Fig. 1A).

AO/EB staining was performed for cell death analysis in which live cells were found uniformly in green while the dead cells (orange/red) were observed after GA treatment (Fig. 1F). Morphological observation with light microscopy showed that cells become round and are detached from the surface after GA treatment. Normal morphology was regained when cells were pretreated with NAC but, on pretreatment of z-VAD-fmk cells were viable (green) and appeared round. GA treated T24 cells showed enhanced chromatin condensation when stained with Hoechst 33342. GA mediated nuclear condensation was blocked by NAC or z-VAD-fmk (Fig. 1F).

3.2. GA activates caspases in a ROS dependent manner in T24 cells

Oxidative stress is known to activate different caspases which in turn initiates and executes the process of apoptosis [27]. Activation of caspase-8 in GA treated T24 cells was measured by colorimetric method. Dose dependent increase in caspase-8 activity was observed which was blocked by caspase-8 specific inhibitor and NAC (Fig. 2A). Caspase-2 was also found to activate in a dose dependent manner by immunoblotting and NAC completely inhibited activation of caspase-2 (Fig. 2B). Time kinetic studies showed that GA leads to the activation of caspase-8, caspase-2 (Fig. 2C), caspase-9 and caspase-3 (Fig. 2D) in a time dependent manner. Dose dependent activation of caspase-3 was also studied and caspase-3 activation was inhibited by NAC or z-VAD-fmk (Fig. 2E). To further confirm our results, caspase-3 activity

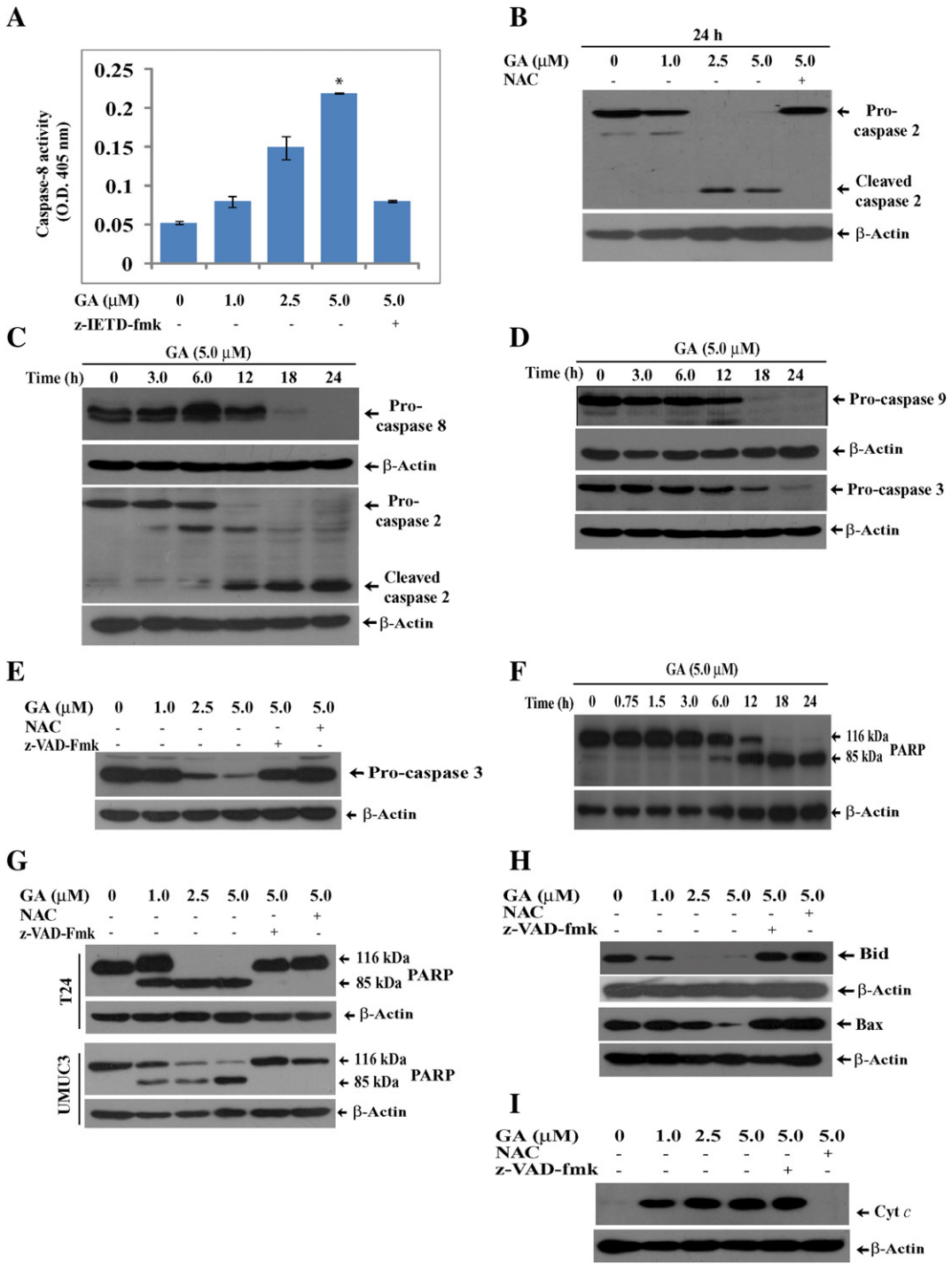

Fig. 2. GA induces caspase activation. (A) T24 cells were treated with GA for 24 h, and caspase-8 activation was measured by colorimetric assay. OD, optical density, $^{*}P < 0.05$ vs. control. T24 cells were treated as indicated; activation of caspase-2 (B), caspase-8, and caspase-2 (C), caspase-9 and-3 (D) was determined by immunoblotting. Antibodies of caspase-8, caspase-3 and caspase-9 recognize pro-forms of these caspases. (E) T24 cells were treated with GA (0–5 μM) for 24 h either alone or in the presence of NAC or z-VAD-fmk and caspase-3 activation was checked by immunoblotting. (F) PARP cleavage was checked in T24 cells for indicated time points. (G) PARP cleavage in both T24 and UMUC was checked as in E. (H) T24 cells were treated as indicated and analyzed for Bid and Bax expression in cytosolic fraction by immunoblotting. (I) Under similar conditions as in H analysis of cytochrome c by Western blotting was performed.

was measured by colorimetric assay (Sup Fig. 1B) which also showed that GA induces caspase-3 activation. Addition of caspase-3 specific inhibitor inhibited GA induced caspase-3 activation. PARP is a substrate for caspase-3 and its cleavage is an indicator of apoptosis. PARP cleavage was found to start after 6 h of GA treatment (Fig. 2F). We also observed a dose dependent increase in PARP cleavage in both cell lines that was inhibited by NAC or z-VAD-fmk (Fig. 2G).

GA treatment causes concentration dependent cleavage of Bid and decrease in the level of Bax in cytosolic fraction of T24 cells. Both the cleavage of Bid and level of Bax were reverted back in the presence of z-VAD-fmk or NAC (Fig. 2H). Changes in mitochondrial membrane potential (MMP) were studied by $DiOC_6$ staining. GA treatment leads to increase in MMP, which indicates mitochondrial hyper-polarization. Mitochondrial hyper-polarization was inhibited by NAC but not by z-VAD-fmk (Sup Fig. 1C). Alterations in MMP leads to release of cytochrome c into cytosol and GA was found to induce dose dependent release of cytochrome c. NAC completely inhibited the cytochrome c release but interestingly z-VAD-fmk was not able to do so (Fig. 2I).

3.3. Oxidative stress induced by GA induces autophagy through JNK pathway

ROS are well known inducers of autophagy; therefore, we next examined the effect of GA on autophagy. At 12 h time point, concentration dependent increase in the conversion of LC3-I/II was observed whereas at 24 h time point decrease in the conversion of LC3-I/II was seen (Fig. 3A). Beclin-1, an important autophagy protein involved in autophagosome biogenesis was significantly degraded at 24 h (Fig. 3A). Since, GA induces ROS at very earlier time points, we next examined at what time GA induces autophagy. Time kinetics experiment revealed that GA treatment induced LC3-I to LC3-II conversion until 12 h, thereafter, accumulation of LC3-I was evident in T24 cells; similar results were obtained in UMUC3 (Fig. 3B). Acridine orange staining also showed that GA induces autophagy up to 12 h time point (Sup Fig. 1D). Autophagy flux was studied by measuring the conversion of LC3-I to LC3-II in T24 cells after treatment with GA in the presence of chloroquine (CQ) at 3 h time point. CQ treatment accumulates LC3-II level due to inhibition of lysosomal function (Fig. 3C). Further, to confirm the involvement of functional/classical autophagic pathway in GA induced autophagy, siRNA against Beclin-1, an essential factor for execution of autophagy, was used to knockdown Beclin-1 in T24 cells. siRNA based depletion of Beclin-1 effectively blocked the conversion of LC3-I to LC3-II (Fig. 3D). GA treatment did not significantly affect the expression of Atg5-12 and Atg7 but cleavage of Atg4c was visible after 12 h time points (Fig. 3E).

Another useful marker for induction of autophagy is p62 and its expression is inversely related with LC3-II. Interestingly, we observed that GA leads to cleavage of p62 after 6 h (Fig. 3F). However, we did not find any change in the expression of p62 at earlier time points when p62 was not cleaved (before 6 h). Similar results were also observed in UMUC3 cells (Fig. 3F). In order to find out whether p62 is located on autophagosomes or not, co-localization of p62 with Beclin-1 was performed by confocal microscopy. Our results showed that p62 co-localizes with Beclin-1 in a time dependent manner up to 3 h. The co-localization was enhanced in the presence of lysosomal inhibitors (E64D, PepA) or CQ while NAC inhibited the co-localization (Fig. 3G) which further proved that GA increases autophagy flux in T24 cells. NBR1 is another adaptor protein and like p62 it interacts with ubiquitinated proteins and cooperates with p62 in targeting cargo proteins to autophagosomes [18,32]. As p62 was cleaved by GA we thought that NBR1 may functionally compromise for the loss of p62. But interestingly we found that GA leads to the cleavage of not only p62 but also NBR1 (Fig. 3H). Rapamycin, a known inducer of autophagy was taken as positive control to study LC3 and p62 in T24 cells. As expected, rapamycin treatment induced conversion of LC3-I to LC3-II, and LC3-II form was accumulated in the presence of autophagy inhibitors, E64D, PepA and bafilomycin A1 (Fig. 3I). Rapamycin treatment did not induce the cleavage of p62.

GA induced autophagy was dependent on the activation of JNK as inhibition of JNK activity leads to the reduction in LC3-I to LC3-II conversion. Phosphorylation of c-Jun was inhibited in the presence of JNK inhibitor which confirmed the effectiveness of inhibitor used (Fig. 3J). Phosphorylation of JNK is ROS dependent as NAC completely inhibited the JNK activation (Sup Fig. 1E).

3.4. GA induced caspase activation inhibits autophagy by degrading various Atg proteins

In our previous results (Fig. 3A, E, F and H), we have shown that autophagy related proteins are degraded at later time points. Therefore, we aimed to study whether the activated caspases are responsible for the cleavage of Beclin-1, p62 and NBR1 at 24 h time point. Cells were treated with various caspase inhibitors (z-VAD-fmk, z-IETD-fmk, z-VIED-fmk and Ac-DEVD-CHO) for 1 h prior to the addition of GA. Caspase inhibitors significantly blocked the GA mediated degradation of Beclin-1 (Fig. 4A). Since addition of NAC almost completely abolished the degradation of Beclin-1, therefore, it is clear that the process is ROS dependent. Similarly, GA mediated cleavage of p62 was also reverted back by NAC and specific caspase inhibitors and interestingly autophagy was also restored back under these conditions as evident by LC3-I/II conversion (Fig. 4B). When cells were pre-treated with either proteasomal inhibitor or lysosomal inhibitor (E64D, PepA or bafilomycin A_1) for 1 h before addition of GA, degradation of Beclin-1 was not prevented (Fig. 4C). Cleavage of NBR1 and Atg4c was also found to be mediated by GA induced ROS generation and caspase activation (Fig. 4D). GA mediated cleavage of Atg4c and NBR1 was entirely mediated by caspases as inhibition of caspases by z-VAD-fmk prevented their cleavage even in the presence of CQ and MG132 (Fig. 4E).

3.5. GA inhibits constitutive NF-κB independent of p62 and Hsp90 cleavage

Recently, p62 was shown to be an important regulator of NF-κB via interaction with RIPI and TRAF6 and is said to regulate both phosphorylation and ubiquitination of IKK complex [20,33]. GA is also known to inhibit Hsp90 and covalently modify IKK complex and both of these proteins play important role in the regulation of NF-κB [8]. Therefore, we were also interested to study the effect of GA on NF-κB. Our result clearly suggested that GA inhibits NF-κB in a dose dependent manner (Fig. 5A). Also, time dependent inhibition of NF-κB was observed with a maximum inhibition at 3 h (Fig. 5B). Supershift assay confirmed the involvement of p50 and p65 subunits as both antibodies shifted the band to a higher molecular mass, thus suggesting that the major NF-κB band in T24 cells consists of p50 and p65 subunits (Fig. 5C). GA inhibited p65 translocation from cytosol to the nucleus which supported our EMSA result (Fig. 5D).

Next, we wanted to see the role of Hsp90 and IKK in inhibition of NF-κB. Hsp90 is a molecular chaperone involved in various stress responses. Hsp90 has several client proteins and one of the important client proteins is IKK-α/β which is involved in NF-κB signaling [34]. GA induces dose dependent cleavage of Hsp90 in T24 cells and this cleavage was blocked by NAC or z-VAD-fmk (Fig. 5E). Cleavage of Hsp90 is initiated after 6 h (Fig. 5F) while degradation of IKK-α/β starts after 12 h (Fig. 5G). Degradation of IKK-α/β was also reverted back by z-VAD-fmk and NAC in both T24 (Fig. 5H–I) and UMUC3 (Fig. 5J). GA mediated degradation of IKK-α/β was found solely dependent on caspases as z-VAD-fmk inhibited IKK-α/β degradation even in the presence of proteasomal and autophagy inhibitors (Fig. 5I). Under similar settings both activation of caspase-3 and PARP cleavage was also inhibited (Sup Fig. 1F). IKK-α/β phosphorylates IκB and mediates its degradation via proteasomal pathway, leading to NF-κB activation [35]. GA inhibits phosphorylation of IκB-α which is reverted back by NAC (Fig. 5K). NBR1 and p62 selectively target some ubiquitinated proteins to autophagosomes for their degradation [19]. Therefore, we next monitored accumulation of ubiquitinated proteins in a time dependent manner by Western blotting. Our results clearly indicated that GA leads

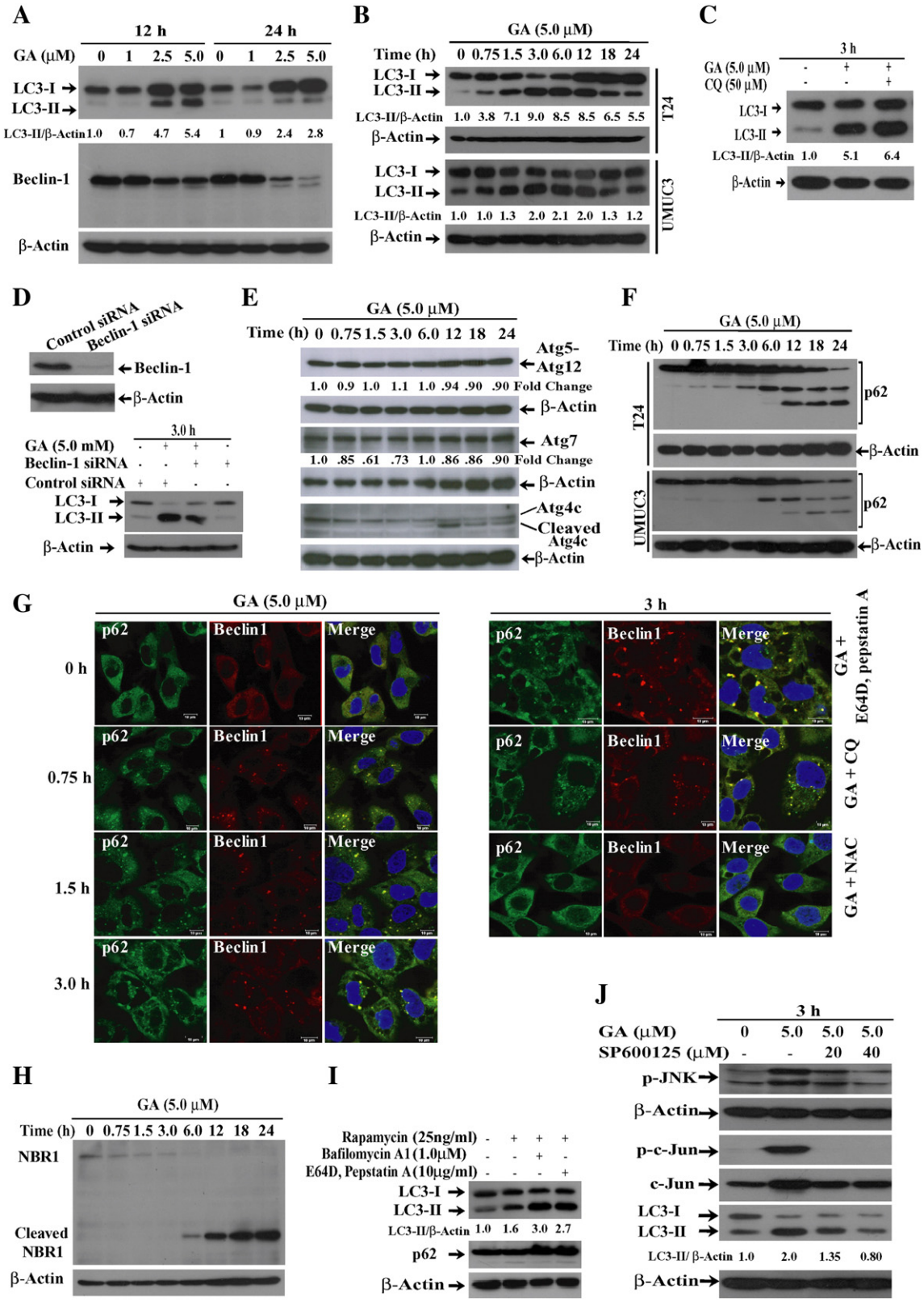

Fig. 3. GA induces autophagy. (A) Cells were treated with GA for 12 h or 24 h, and Western blotting was performed to check LC3 and Beclin-1 expression. (B) T24 and UMUC3 cells were treated with GA for different time points and LC3 was checked by immunoblotting. (C) T24 cells were treated with GA in presence and absence of CQ (50 μM) for 3 h and LC3 I/II conversion was studied by immunoblotting. (D) T24 cells were transfected with Beclin-1 siRNA or control siRNA and treated with GA (5.0 μM) for 3 h. LC3-I to LC3-II conversion was analyzed by Western blotting. Silencing of Beclin-1 was confirmed by Western blotting (D upper panel). (E–F) Cells were treated with GA for different time points, and expression of Atg5, Atg7 and Atg4C in T24 and p62 in both T24 and UMUC3 was checked by Western blotting. (G) T24 cells were treated with GA (5.0 μM) for 0 h–3 h alone or in the presence of NAC, E64D, PepA (10 μg/ml each) or CQ (50 μM) for 3 h. Co-localization was studied after double staining with p62 and Beclin-1 antibodies. Scale bars, 10 μm (H) T24 cells were treated as in E and expression of NBR1 was checked. (I) T24 cells were treated with rapamycin in the presence and absence of bafilomycin A1 or E64D/pepstatin-A and LC3 I/II conversion and p62 expression was checked by Western blotting. (J) Cells were cotreated with GA and JNK inhibitor for 3 h and phosphorylation of JNK and c-Jun was checked by immunoblotting. Under similar conditions LC3-I/II conversion was also monitored.

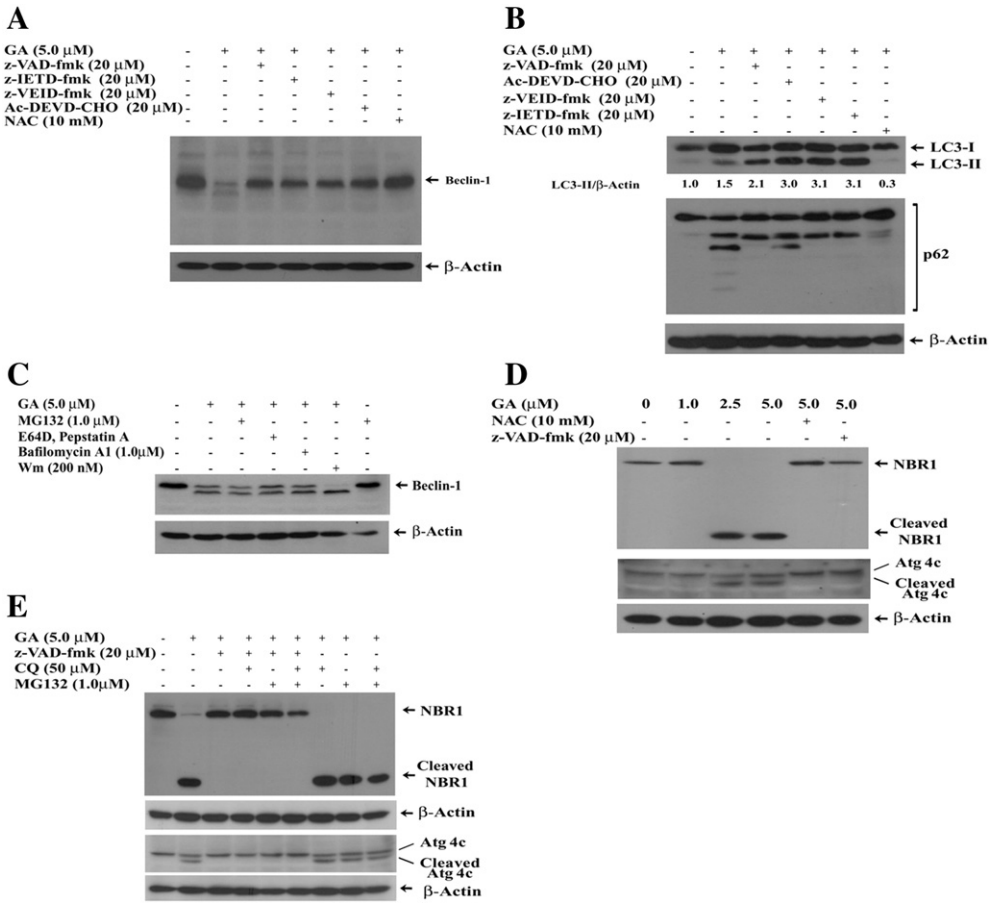

Fig. 4. Caspase activation degrades Atg proteins. (A and B) Cells were treated with GA after 1 h pretreatment of NAC, z-VAD-fmk and different caspase specific inhibitors for 24 h and expression of Beclin-1, LC3 and p62 was checked by immunoblotting. (C) Cells were pre-treated for 1 h either with MG132, E64D/PepA, bafilomycin A_1, or wortmannin (Wm) followed by GA treatment for 24 h and Beclin-1 expression was checked by immunoblotting. (D) T24 cells were treated with GA for 24 h in the presence or absence of NAC or z-VAD-fmk, NBR1 and Atg4c expression was analyzed by immunoblotting. (E) Cells were treated with z-VAD-fmk, CQ, or MG132 for 1 h prior to GA treatment as in the indicated combinations. Expression of NBR1 and Atg4c was examined by immunoblotting in whole cell lysates.

to accumulation of ubiquitinated proteins which is more pronounced after 6 h when p62 is cleaved (Sup Fig. 1G).

3.6. GA induced autophagy delays caspase activation

Autophagy has been reported to play pro-survival or pro-apoptotic role in drug treated cancer cells [36,37]. Therefore, we wanted to study the role of GA induced autophagy in bladder cancer cells. Inhibition of autophagy by CQ or Wm significantly increased cell death at earlier time point (12 h) with lower dose of GA (2.5 μM) as observed by live PI staining in both T24 (Fig. 6A) and UMUC3 (Fig. 6B) cells. Also, GA mediated caspase activation was enhanced when autophagy was inhibited by CQ (Fig. 6C) and Wm (Fig. 6D). Similar results were obtained in UMUC3 cells (Fig. 6E–F). Cleavage of PARP under similar condition further confirmed our results (Fig. 6G–H). Taken together these results suggest that GA induced autophagy is a cell survival response and delays activation of caspases.

4. Discussion

GA is a traditional medicine derived from *Garcinia hanburyi* and various recent studies have reported that GA is a potent anticancer agent with multiple cellular targets. One of the mechanisms by which GA has been shown to induce apoptosis is through generation of ROS. Our results also showed that GA through ROS activates both intrinsic and extrinsic arms of apoptosis by activating caspase-8, -2 -9 and -3. Truncation of Bid to t-Bid by caspase-8/2 links extrinsic to intrinsic pathway of apoptosis via alteration of mitochondrial membrane potential (MMP) [38]. We also observed truncation of Bid, mitochondrial hyperpolarization, and cytochrome c release in GA treated bladder cancer cells. All the molecular events leading to apoptosis induced by GA are regulated by ROS as NAC essentially blocks all these events. Interestingly, we observed that z-VAD-fmk is not able to prevent GA induced mitochondrial hyperpolarization and cytochrome c release. Alterations in MMP and cytochrome c are steps upstream of caspase activation; therefore z-VAD-fmk has no effect on these events. As reported by Madesh and Hajnoczky, ROS may directly induce cytochrome c release [39] and our results also indicate the same. Caspase mediated truncation of Bid facilitates migration of Bax to mitochondria [40]; therefore, both steps are effectively blocked by z-VAD-fmk. In conclusion ROS mediated activation of caspases seems to be the central mechanism of GA induced cell death, since z-VAD-fmk, effectively blocked GA mediated apoptosis of bladder cancer cells.

ROS have not only shown to be inducers of apoptosis but are also known as potent stimulators of autophagy. Functionally autophagy is a very complex process and presently no consensus can be found in the literature on the role of autophagy in cancerous cells [36,37]. However, autophagy has been proposed to be a cell stress response toward starvation, growth factor deprivation, hypoxia, radiation and exposure to various drugs [41]. The cytoprotective functions of autophagy are mediated by negative modulation of apoptosis and by clearing various aggregated proteins. Apoptosis and autophagy share several common factors and it is now well established that there is a strong crosstalk between these two processes. Some of the important points

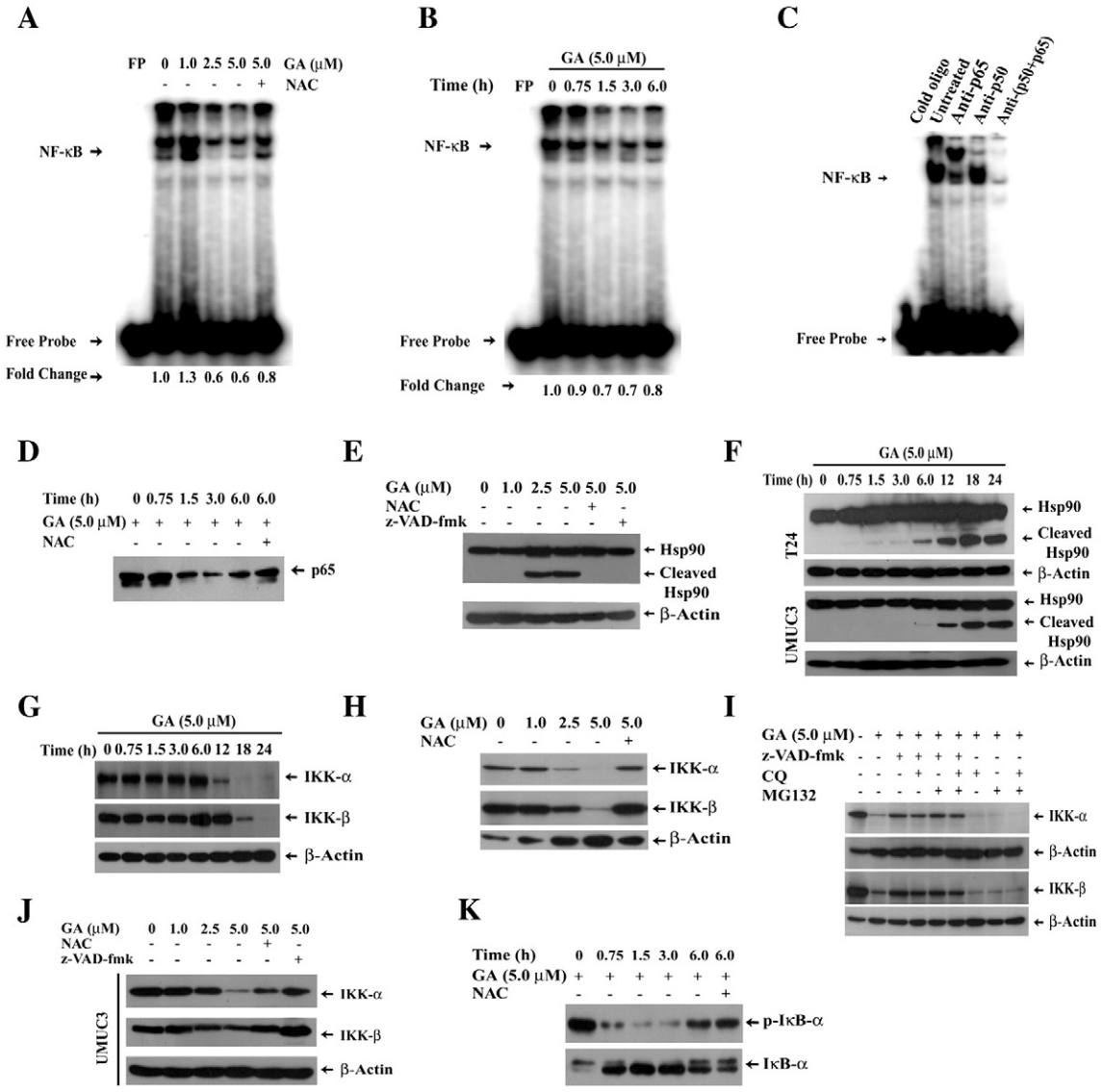

Fig. 5. GA inhibits NF-κB activation. (A) T24 cells were treated with different concentrations of GA as indicated for 6 h, (B) for different time points and NF-κB activation was checked by EMSA. FP represents free probe (no nuclear extracts). (C) Nuclear extracts were prepared from T24 cells, incubated for 60 min with different antibodies (p50 and p65) and then assayed for NF-κB by EMSA. (D) Cells were treated with GA alone or with GA in the presence of NAC as indicated, and expression of p65 in nuclear extract was monitored. (E) Expression of Hsp90 in the indicated combination was checked by immunoblotting. (F–G) Cells were treated with GA for indicated time points and expression of Hsp90 as well as that of IKK-α/β were examined. (H) Cells were treated with GA for 24 h either alone or in the presence of NAC, and expression of IKK-α/β was monitored by Western blotting. (I) Cells were pretreated for 1 h with z-VAD-fmk alone or in combination of CQ and MG132 prior to GA treatment for 24 h in the indicated combinations and expression of IKK-α/β was determined. (J) UMUC3 cells were treated with GA (5 μM) for 24 h in the presence or absence of NAC or z-VAD-fmk, and IKK-α/β expression was analyzed by immunoblotting. (K) Cytosolic fractions were subjected to Western blot analysis for the expression and phosphorylation of IκB-α.

of crosstalk comprise the interactions between Beclin-1 and Bcl-2 family members [42,43], interaction between FADD and Atg5 [44], and autophagy mediated degradation of caspases [45]. Caspases have been reported to play anti-autophagic functions by cleavage of various Atg proteins [17,42]. Recently, it has been shown that inhibition of GA induced autophagy in glioblastoma and colorectal cancer cells enhance apoptosis [46,47]. Zhang et al., have shown that GA induces autophagy by upregulating Atg12-Atg5, Beclin-1 and Atg7 and inhibition of autophagy by NAC leads to increased cell death [46]. However, our findings indicate that GA through generation of ROS actually, links autophagy and apoptosis via caspase mediated degradation of various Atg proteins. Inhibition of autophagy at later time points was well correlated with the degradation of Beclin-1. GA also leads to the cleavage of Atg4c a cysteine protease involved in the processing of LC3-I to LC3-II during autophagy [48]. The expression of Atg5 and Atg7 was not altered by GA which suggests that Beclin-1 and Atg4c are the main targets of GA for autophagy inhibition. Activation of caspases in GA treated cells not only induces apoptosis but also destroys several molecules associated with survival of cells. It is known that proteins like p62 and NBR1 are cytoprotective and help in scaffolding misfolded or mutated proteins to form aggregates and ultimately autophagic degradation. p62 plays an important role in cancer and recently it has been shown that p62 knockout mice fail to develop cancer [49]. For the first time we are reporting that GA, an anticancer agent cleaves p62 in a caspase dependent manner. GA also results in cleavage of ER stress chaperone protein GRP-78 which is involved in cell survival signaling pathways (Sup Fig. 1H, I). Only few anticancer agents are reported in the literature to degrade GRP-78, therefore, GA may be useful in targeting cancerous cells which have high p62 and GRP-78 expression [50].

p62 was originally identified as an interacting partner of atypical protein kinase Cs but subsequently it was noticed that this adaptor or scaffolding protein is the center of various signaling pathways and

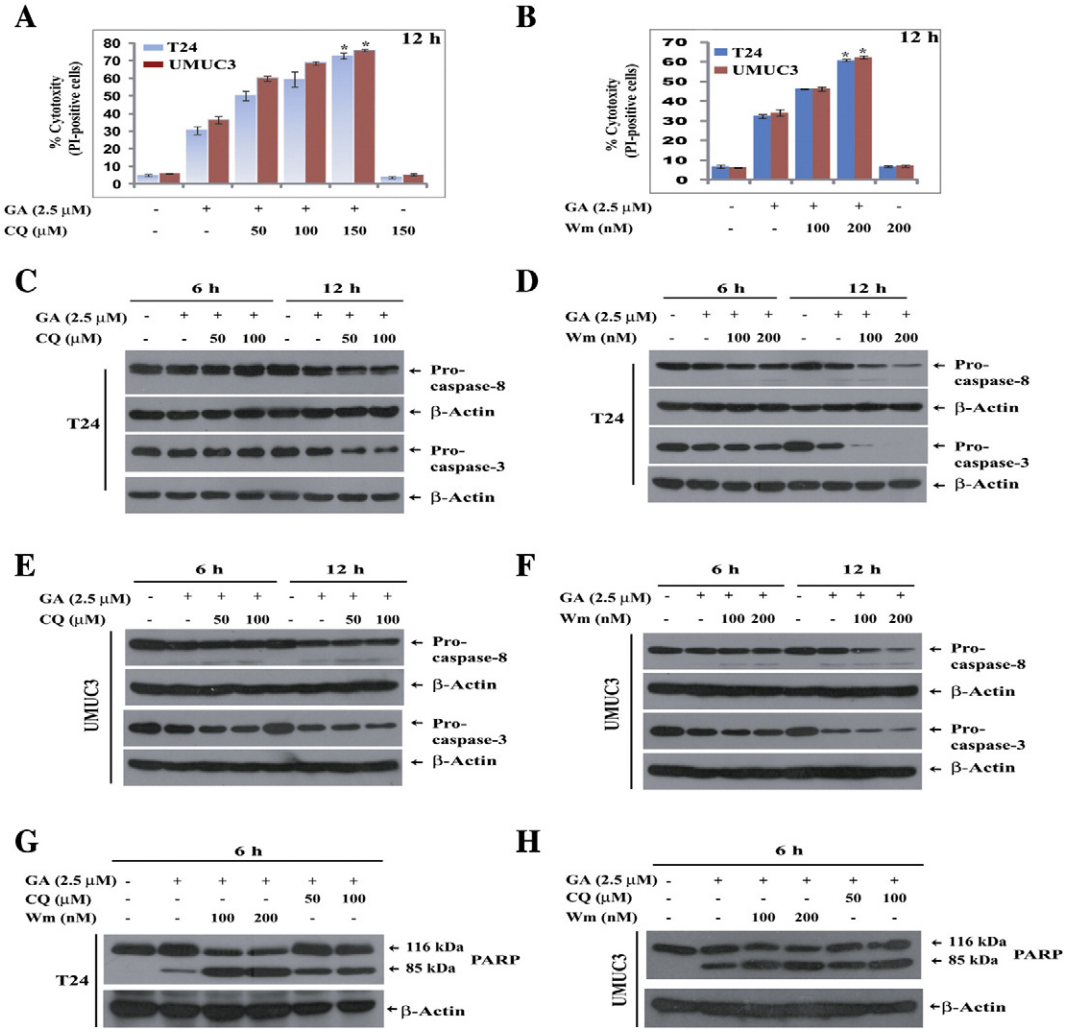

Fig. 6. GA induced autophagy delays caspase activation. (A–B) T24 and UMUC3 cells were treated with GA (2.5 μM) and different concentrations of CQ or Wm for 12 h and viability was checked by live PI exclusion method. Values are expressed as mean ± SD of three similar experiments. *$P < 0.05$ vs. control. (C–D) T24 and UMUC3 (E–F) cells were treated with GA (2.5 μM) for 6 h and 12 h either alone or in the presence of CQ (0–100 μM) and Wm (0–200 nM) and expression of caspase-8 and caspase-3 was checked by immunoblotting. (G–H) T24 and UMUC3 cells were treated with GA (2.5 μM) for 6 h in the presence and absence of CQ or Wm and PARP cleavage was monitored.

diverse cellular processes [22]. p62 on one hand was shown to be important for the full activation of caspase-8 but at the same time it was also reported to activate the NF-κB [22,51]. Apart from p62, Hsp90 is one of the important molecular chaperones required for the activation of NF-κB [34]. Our results showed that GA leads to the inhibition of NF-κB at very early time points with maximum inhibition at 3 h. NF-κB inhibition was independent of p62 and Hsp90 cleavage because p62 and Hsp90 degradation starts after 6 h while NF-κB is inhibited very early at 45 min. The two important regulatory proteins of NF-κB signaling axis (Hsp90 and IKK-α/β) are also degraded at later time points by caspases. The time points of NF-κB inhibition correlate well with the ROS generation and NAC was able to restore the NF-κB activation that leads us to propose that GA mediated NF-κB inhibition is regulated by ROS. Activation of NF-κB is inhibited by IκB-α by blocking its translocation to the nucleus. IKK-α/β phosphorylates IκB-α, which enhances its degradation via proteasomal pathway and makes NF-κB free to translocate to the nucleus. Palempalli et al., have reported that GA inhibits NF-κB by abolishing kinase activity of IKK-β via its covalent modification [8]. Our results clearly showed that NAC reverted back the phosphorylated form of IκB-α which would not have been possible if IKK-β was covalently modified by GA. Therefore, our results suggest that GA inhibits NF-κB by inhibiting phosphorylation of IκB-α and independently of Hsp90 and IKK degradation. However, ROS serve as central factors by which GA inhibits NF-κB. GA has been reported as a proteasomal inhibitor that results in accumulation of ubiquitinated proteins [7]. Interestingly, we observed more accumulation of ubiquitinated proteins after p62 was cleaved which may be due to impairment of proteasomal function as well as autophagic degradation of ubiquitinated proteins facilitated by p62.

GA is a unique compound as it first elicits a strong cell survival autophagic response by generating ROS but subsequently causes degradation of various cell survival proteins by activation of caspases. The molecular events induced by GA in bladder cancer cells can be divided into two distinct steps: before caspase activation and after caspase activation. The events before caspase activation include ROS generation, JNK activation, autophagy induction and NF-κB inhibition and the events after caspase activation include Hsp90 cleavage, degradation of various Atg proteins, GRP-78 cleavage, truncation of Bid, mitochondrial hyperpolarization and cell death (Fig. 7). Time dependent increase in oxidative stress induced by GA may be one possible element behind transition from one step to other. Cleavage of scaffolding proteins and molecular chaperones will essentially cause the cell to initiate the apoptotic program because in the absence of these proteins the normal signaling pathways and cellular homeostasis will be completely abolished. GA induced autophagy in bladder cancer cells acts as a cell survival mechanism, as inhibition of autophagy significantly enhanced cell

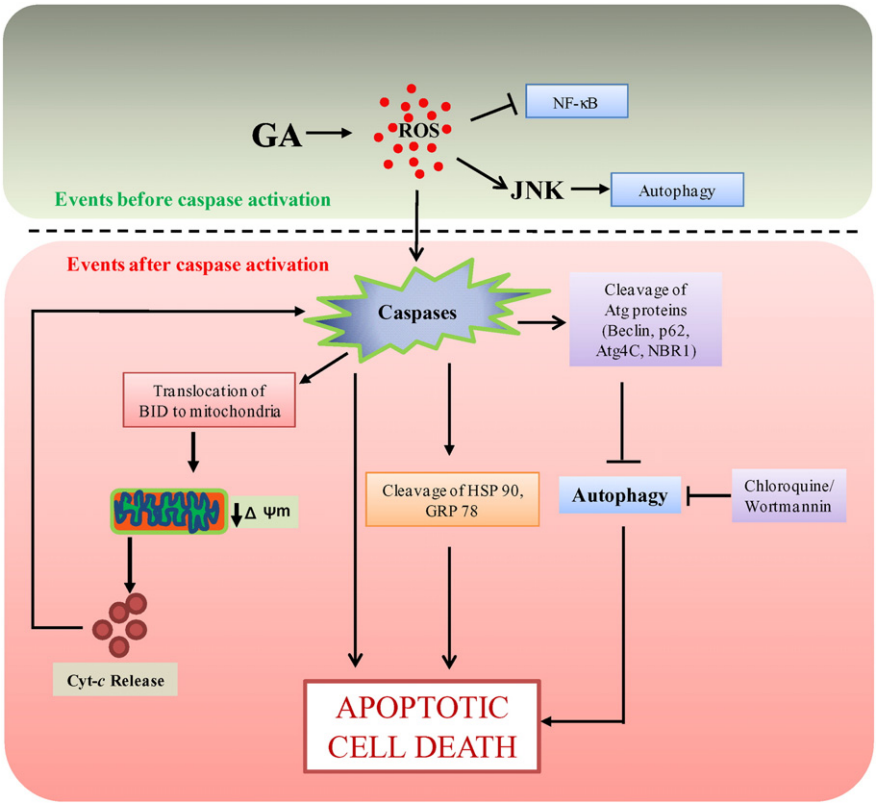

Fig. 7. GA induced molecular events. Model showing the modulation of autophagy and apoptosis by ROS mediated caspase activation. GA induced ROS activates JNK, inhibits NF-κB and induces autophagy at earlier time points before the activation of caspases. At later time points caspases are activated which leads to Hsp90 and GRP-78 cleavage, degradation of various Atg proteins, inhibition of autophagy and subsequent cell death via apoptosis.

death. Our results clearly showed that GA induced autophagy delays caspase activation as inhibition of autophagy leads to the activation of caspases at earlier time points.

In conclusion our findings clearly show that GA is a unique compound which generates ROS and first triggers ROS mediated autophagy and subsequent caspase activation which destroys cell survival autophagy machinery and initiates apoptotic pathway leading to cell death. Thus, GA may prove beneficial for the treatment of bladder cancer cells.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.08.019.

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