BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2009), 158, 1405–1412

0007-1188/09
www.brjpharmacol.org

RESEARCH PAPER

Inhibition of the JNK signalling pathway enhances proteasome inhibitor-induced apoptosis of kidney cancer cells by suppression of BAG3 expression

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Background and purpose: Proteasome inhibitors represent a novel class of anti-tumour agents that have clinical efficacy against haematological and solid cancers. The anti-apoptotic protein BAG3 is a member of the Bcl-2-associated athanogene family. We have previously shown that BAG3 is up-regulated after exposure to proteasome inhibitors and that inhibition of BAG3 sensitized cells to apoptosis induced by proteasome inhibition. However, the mechanisms by which proteasome inhibition induced BAG3 expression remained unclear and the present experiments were designed to elucidate these mechanisms.
Experimental approach: Effects of the proteasome inhibitor MG132 on activation of mitogenic signalling pathways were evaluated in kidney cancer cells (A498, Caki1, Caki2), with Western blotting. Specific inhibitors against individual mitogenic signalling pathways, real-time reverse transcription-polymerase chain reaction and luciferase reporter assays were used to investigate the roles of mitogenic signalling pathways in BAG3 induction after proteasome inhibition. Cell death was evaluated using Annexin V/propidium iodide staining and subsequent FACS.
Key results: MG132 activated several key mitogenic signalling pathways including extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) activities. Induction of BAG3 by MG132 was inhibited by blocking JNK, but not ERK1/2 and p38 MAPK signalling pathways. In addition, SP600125 and dominant-negative JNK1 suppressed BAG3 promoter-driven reporter gene expression. Furthermore, activation of the JNK pathway induced BAG in kidney cancer cells after treatment with MG132.
Conclusions and implications: Our results suggested that the JNK pathway was associated with the protective response against proteasome inhibition, by mediating induction of BAG3.
British Journal of Pharmacology (2009) **158,** 1405–1412; doi:10.1111/j.1476-5381.2009.00455.x; published online 13 August 2009

Keywords: BAG3; MAPK; MG132; JNK; proteasome; cancer; apoptosis

Abbreviations: AMC, 7-amino-4-methylcoumarin; bp, base pair; DN, dominant-negative; ERK, extracellular signal-regulated kinase; IP, immunoprecipitation; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; PCR, polymerase chain reaction; RNAi, RNA interference; RT-PCR, reverse transcription-polymerase chain reaction; WT, wild type

Introduction

Proteasome inhibitors represent a new class of drugs that have anti-tumour activity through a number of mechanisms, including interfering with cell cycle progression, inducing apoptosis and inhibiting angiogenesis (Voorhees *et al.*, 2003; Rajkumar *et al.*, 2005). However, it is increasingly recognized that, as is the case for other drugs, they may also activate some anti-apoptotic survival pathways that limit their own anti-tumour efficacy. The anti-apoptotic protein BAG3 is such an example and this protein was induced by proteasome inhibition and impaired tumour response to proteasome inhibitor treatment (Wang *et al.*, 2008). BAG3, also known as Bis or CAIR-1, binds to the ATPase domain of heat shock protein (Hsp)70 with high affinity and inhibits its chaperone

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Received 9 April 2009; revised 30 May 2009; accepted 1 July 2009

activity (Takayama *et al*., 1999; Rosati *et al*., 2007a). BAG3 has also been reported to bind with Bcl-2 and have a synergistic effect on the anti-apoptotic activity of Bcl-2 (Lee *et al*., 1999). With regard to cancer, expression of BAG3 is elevated in leukaemia and some solid tumours (Liao *et al*., 2001; Romano *et al*., 2003; Chiappetta *et al*., 2007). Notably, BAG3 expression can be induced by some stressful agents such as high temperature and heavy metal exposure (Liao *et al*., 2001; Pagliuca *et al*., 2003), HIV-1 infection (Rosati *et al*., 2007b), and proteasome inhibition (Wang *et al*., 2008), suggesting that it is a protein inducible by stress stimuli.

The mitogen-activated protein kinase (MAPK) family of proteins includes p38 MAPK, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK). MAPKs are proline-directed serine/threonine kinases that are activated by dual phosphorylation on threonine and tyrosine residues in response to a wide variety of stress stimuli (Pearson *et al*., 2001). They mediate signal transduction from the cell surface to the nucleus and are involved in the expression of a variety of genes (Karin, 1995). Pharmacological inhibitors of p38 enhanced the ability of proteasome inhibitors to induce apoptosis and seemed also to help overcome resistance to proteasome inhibitors in models of lymphoma (Meriin *et al*., 1998; Chauhan *et al*., 2003; Hideshima *et al*., 2004), indicating that manipulation of these signalling pathways might influence the anti-tumour effectiveness of proteasome inhibitors.

In the present study, we characterized the role of MAPK signalling pathways in the induction of BAG3, following exposure to proteasome inhibitor. Proteasome inhibition activated several sequential protein kinase pathways, leading to increased activity of ERK, JNK and p38 MAPK in kidney cancer cells. We demonstrated that the JNK inhibitor SP600125 significantly suppressed the induction of BAG3 by MG132, whereas the ERK inhibitor PD98059 and the p38 MAPK inhibitor SB203580 had little effect on BAG3 induction. We also demonstrated that the JNK inhibitor SP600125 or dominant-negative JNK1 (DN-JNK1) significantly suppressed the induction of BAG3 by MG132. We further observed that inhibition of the JNK pathway significantly increased levels of apoptosis induced by MG132. In addition, ectopic expression of BAG3 reversed the enhancing effects of the JNK inhibitor, suggesting that, in kidney cancer cells, the JNK pathway plays anti-apoptotic roles after proteasome inhibition, mediated, at least in part, by induction of BAG3.

Methods

Cell culture

The A498, Caki-1 and Caki-2 kidney cancer cell lines were maintained in DMEM supplemented with 10% FBS (Sigma-Aldrich, Saint Louis, MO).

20S proteasome activity assay

Cytosolic extracts (without protease inhibitors) were used to measure proteasome activity using a 20S proteasome assay kit (Chemicon International, Temecula, CA) following the manufacturer's instructions. The assay is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labelled substrate LLVY-AMC. Levels of released AMC were measured using an excitation wavelength of 380 nm and an emission wavelength of 460 nm with an automatic multi-well plate reader. The relative activity was standardized by protein concentration, determined using Coomassie Protein Assay Reagent (Pierce, Rockford, IL).

Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-X100) containing a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO). Cell extract protein amounts were quantified using the BCA protein assay kit. Equivalent amounts of protein (20 μg) were separated using 12% SDS-PAGE and transferred to PVDF membranes (Millipore Corporation, Billerica, MA).

Luciferase assay

The 5′-flanking region of human BAG3 genomic DNA between –1556 and +5 (+1 represents the translation start site) was amplified by polymerase chain reaction (PCR) from HeLa genomic DNA and subcloned into the reporter plasmid pGL4 (Promega, Madison, WI). The luciferase activity was determined using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI), according to the manufacturer's instructions. All transfection experiments were repeated for three times, each in triplicate. Firefly (*Photinus pyralis*) luciferase activities normalized by *Renilla* (*Renilla reniformis*) activities are presented as fold induction relative to the normalized firefly luciferase activity in cells transfected with the pGL4 empty vector only, which was taken as 1.0.

Construction of plasmids and transfection

A cDNA encoding human BAG3 or JNK1 was generated by PCR from human brain cDNA library (Invitrogen, Carlsbad, CA) and subcloned into the eukaryotic expression plasmid pcDNA3 tagged with Flag epitope. The DN-JNK1 cDNA was produced by amino acid substitution of threonine (ACG) 183 for alanine (GCG) and tyrosine (TAT) 185 for phenylalanine (TTT) in the dual-activating phosphorylation sites of wild-type JNK1 and subcloned into pcDNA3 tagged with Flag epitope. A498 cells were transfected with indicated vector using Lipofectamine 2000 reagent according to the protocol of the manufacturer.

RNA isolation and real-time reverse transcription PCR

RNA isolation and real-time reverse transcription PCR was performed as previously reported (Wang *et al*., 2007). For BAG3, the forward primer was 5′-ATGCAGCGATTCCGAA CTGAG-3′ and the reverse was 5′-AGGATGAGCAGTCAG AGGCAG-3′, the amplicon size is 191 base pair (bp). For β-actin, the forward primer was 5′-GAGACCTTCAAC ACCCCAGCC-3′ and the reverse was 5′-GGATCTTCAT GAGGTAGTCAG-3′, the amplicon size is 205 bp. Results were normalized against those of β-actin.

Chromosomal immunoprecipitation assay

Chromosomal immunoprecipitation (IP) assays were performed using a kit from Upstate Biotechonology Inc. (Lake

Placid, NY) according to the supplied protocol. In brief, cells were exposed to 2 μM MG132 for 8 h and fixed with 1% formaldehyde in phosphate-buffered saline to cross-link chromatin. Cell lysates were prepared and sonicated on ice to break chromatin DNA to an average length of 400 bp. After a preclearing step, IP was carried out at 4°C overnight with anti-HSF1 antibody or normal goat IgG (negative control antibody). Immune complexes were collected with salmon sperm DNA saturated protein A-agarose beads. After extensive washing the immunoprecipitated complexes were eluted with 0.1 M $NaHCO_3$ and 1% SDS, and then protein-DNA cross-links were reversed by incubating at 65°C for 5 h. DNA was purified using proteinase K digestion, phenol: chloroform extraction and ethanol precipitation. Real-time PCR was performed using primers specific for the BAG3 promoter sequence between –327 and –174 (forward: 5′-GAT TATAGCCGATGACTCAGGGCG-3′ and reverse: 5′-AGTGT CTGGAAATAGCCTCC-3′) to generate a 154 bp amplification product containing the HSE sequence. A standard curve was prepared using serial dilutions of the pBAG3/-825 promoter construct. The amount of BAG3 promoter that was present in the IP and input fractions was calculated from the standard curve. The input represents 1% of the material used in the IP assay. The results were expressed as the IP/input ratios of the PCR products,which were used for comparison.

Detection of cell death

For cell death assays, cells were washed twice in phosphate-buffered saline and then stained with Annexin V-FITC (Biovision, Mountainview, CA) and propidium iodide (Sigma-Aldrich) according to the manufacturer's instructions. After staining with annexin V-FITC and propidium iodide, samples were analysed by fluorescence-activated cell scanner (FACScan) flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Statistics

The statistical significance of the difference was analysed by ANOVA and *post hoc* Dunnett's test. Statistical significance was defined as $P < 0.05$. All experiments were repeated three times, and data were expressed as the mean ± SD (standard deviation) from a representative experiment.

Materials

MG132, PD98059, SB203580 and SP600125 were purchased from Calbiochem (La Jolla, CA). The following antibodies were used in this study: mouse anti-p44/42 MAPK (ERK1/2) monoclonal antibody (Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) monoclonal antibody (Cell Signaling Technology, Danvers, MA), rabbit anti-c-Jun polyclonal antibody (Abcam, Cambridge, MA), rabbit anti-JNK monoclonal antibody (Cell Signaling Technology, Danvers, MA),mouse anti-phospho-JNK (Thr183/Tyr185) monoclonal antibody (Cell Signaling Technology, Danvers, MA), rabbit anti-p38 MAPK monoclonal antibody (Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-p38 (Thr180/Tyr182) monoclonal antibody (Cell Signaling Technology, Danvers, MA) and rabbit anti-BAG3 polyclonal antibody (Abcam, Cambridge, MA).

Results

Sequential activation of protein kinase pathways by MG132

MG132 quickly suppressed proteasome activity in A498, Caki1 and Caki2 renal cancer cells, and more than 40% suppression was observed upon exposure to 2 μM MG132 for 30 min (Figure 1A). The suppression peaked at 2 h and was maintained for 24 h (Figure 1A), suggesting that the dose of MG132 used in this study effectively suppressed proteasome activity in kidney cancer cells. A498 cells were then treated with MG132 and the activation state of mitogenic signalling pathways (ERK, p38 MAPK and JNK) was measured over 24 h using antibodies against the phosphorylated active forms of the proteins and Western blot analysis (Figure 1B). ERK, JNK and p38 activities are all activated by MG132 exposure. However, the regulation of each pathway by MG132 is different as characterized below. ERK activity increased rapidly to a maximal level within 1 h of treatment. Levels of phospho-p38

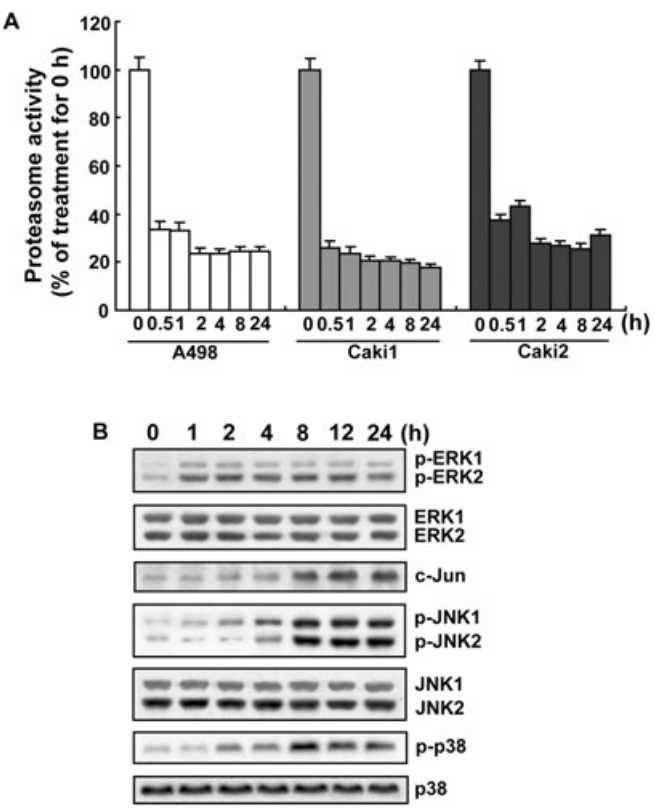

Figure 1 Proteasome inhibition activates several mitogenic signalling pathways. (A) A498, Caki1 and Caki2 kidney cancer cells were treated with 2 μM MG132 for the indicated times and 20S proteasome activity was analysed. (B) A498 cells were incubated in the presence of vehicle or 2 μM MG132 for the indicated time. Total cytosolic proteins were isolated and subjected to Western blotting to assess levels of three mitogen-activated protein kinases, extracellular signal-regulated kinase (ERK)1/2, c-Jun N-terminal kinase (JNK)1/2 and p-38. We used antibodies for the active, phosphorylated protein and for the total level of protein. Blots are representative of three individual experiments.

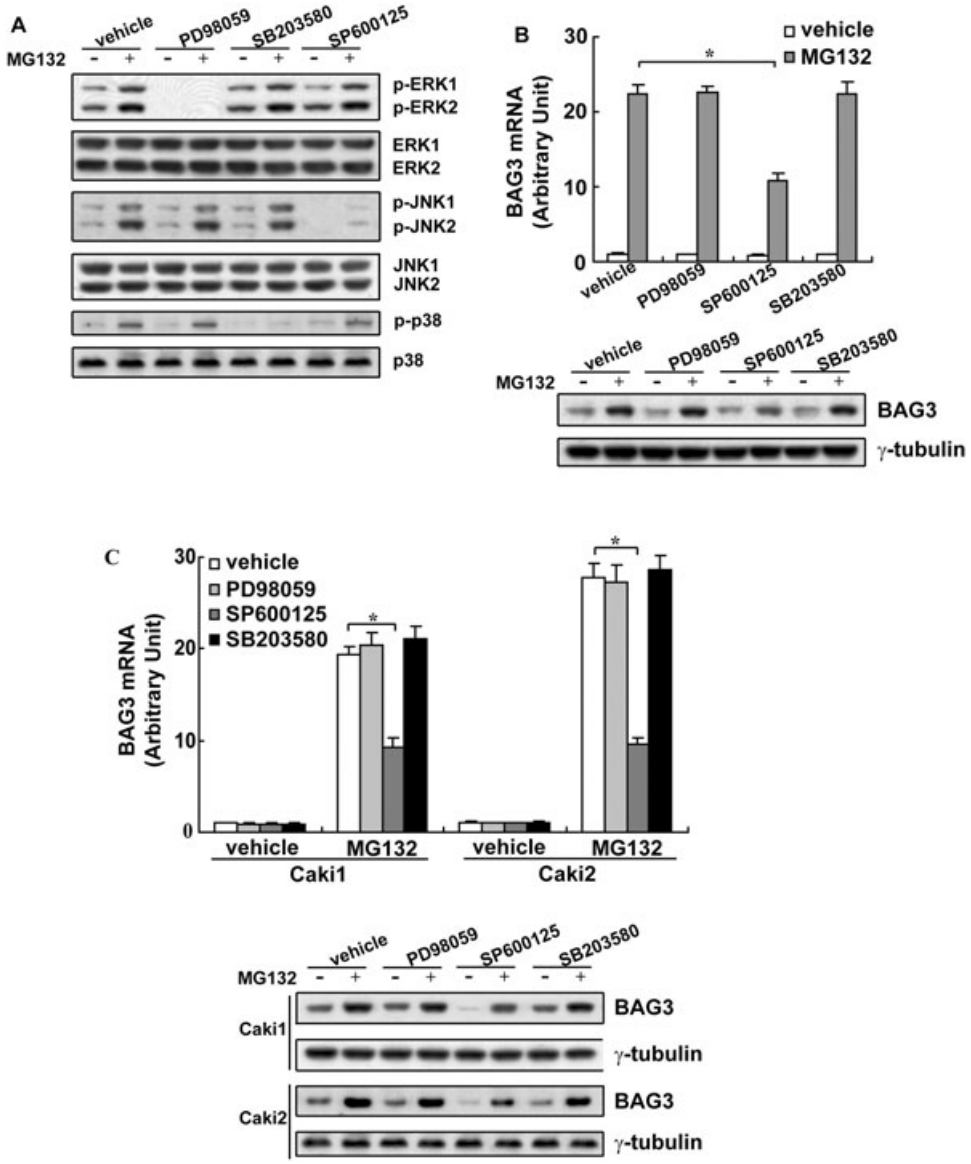

Figure 2 Effect of pharmacological inhibitors on BAG3 induction by MG132. (A) A498 cells were pre-treated with vehicle, PD98059 (10 μM), SB203580 (10 μM) or SP600125 (20 μM) for 1 h. The cells were then incubated with MG132 for 8 h, and Western blot was performed using the indicated antibodies. (B) A493 cells were pre-treated with various inhibitors for 1 h, subsequently treated with MG132 for additional 8 h, and real-time polymerase chain reaction was performed. (C) Caki1 and Caki2 cells were treated as (B) and BAG3 mRNA was measured using real-time polymerase chain reaction. *$P < 0.001$.

increased time-dependently to a maximum at 8–12 h. JNK and its major substrate c-Jun activation were seen 4 h after addition of MG132 and peaked at 8–12 h (Figure 1B).

Effects of MAPK inhibitors on MG132-induced BAG3 expression

Because studies have shown that the MAPK pathway is critical for the activation of gene expression upon various stimuli, we next sought to determine if the activation of these pathways by MG132 exposure influences BAG3 induction. To evaluate the roles of MAPKs in BAG3 induction by MG132, the small molecule inhibitors, PD98059, SB203580 and SP600125, were used as inhibitors for ERK, p38 kinase and JNK respectively. To confirm that the inhibitors were functional in our model, cells were treated with vehicle or MG132, with or without the specific mitogenic inhibitors. The activation state of the pathways was then measured by Western blot analysis (Figure 2A). All these inhibitors specifically inhibited the activity of their respective target proteins and had no cross-reactivity with the other pathways examined (Figure 2A). As reported in other types of cancer cells (Wang *et al.*, 2008), real-time PCR confirmed that MG132 significantly induced BAG3 expression in A498 kidney cancer cells (Figure 2B). BAG3 induction by MG132 was significantly inhibited by pre-treatment with SP600125, but not in A498 cells pre-treated with PD98059 or SB203580 (Figure 2B). To assess whether regulation of BAG3 induction by these pathways was a cell line-specific response, these experiments were repeated using two other kidney cancer cell lines, Caki1 and Caki2 (Figure 2C). Both cell lines displayed induction of BAG3 in response to MG132 treatment

that was significantly suppressed by SP600125 pre-treatment, whereas PD98059 and SB203580 had little effect on the levels of BAG3 mRNA (Figure 2C). These results indicate that JNK pathways are implicated in MG132-induced BAG3 expression in kidney cancer cells.

Role of JNK pathway in activation of BAG3 transcription by MG132

To confirm that JNK activity was involved in the MG132-induced BAG3 expression in A498 cells, we investigated whether administration of a JNK inhibitor or expression of dominant-negative JNK could affect MG132-induced activation of BAG3 gene. Pre-treatment of cells with SP600125 inhibited BAG3 luciferase reporter activity in a dose-dependent manner (Figure 3A). In addition, overexpression of JNK1 increased BAG3 luciferase reporter activity induced by MG132, whereas a DN-JNK1 dramatically suppressed BAG3 luciferase reporter activity (Figure 3B). The data thus confirm that the JNK signalling pathway was involved in activation of BAG3 transcription by MG132. Earlier, we had shown that MG132 induced BAG3 expression via activation of heat shock factor 1 (HSF1: Du *et al.*, 2009). We further performed chromosomal IP assay to investigate whether the JNK pathway affected the binding of HSF1 to MG132-responsive elements on the *BAG3* promoter (Figure 3C). Treatment with MG132 recruited HSF1 proteins to the MG132-responsive sites located on the *BAG3* promoter, whereas the signal was significantly suppressed by pre-treatment of the cells with SP600125 (Figure 3C). These results suggested that SP600125 inhibited BAG3 induction by MG132 via suppressing the interaction of HSF1 with MG132-responsive sites of the *BAG3* promoter.

Effects of JNK inhibition or BAG3 overexpression on MG132 cytotoxicity

We then investigated whether the activation of the JNK pathway affects the apoptotic response to proteasome inhibition. Whereas treatment with the JNK inhibitor SP600125 alone had little effect, when used in combination with MG132, it significantly increased levels of apoptosis induced by MG132 in A498 cells (Figure 4A). The sensitizing effects of

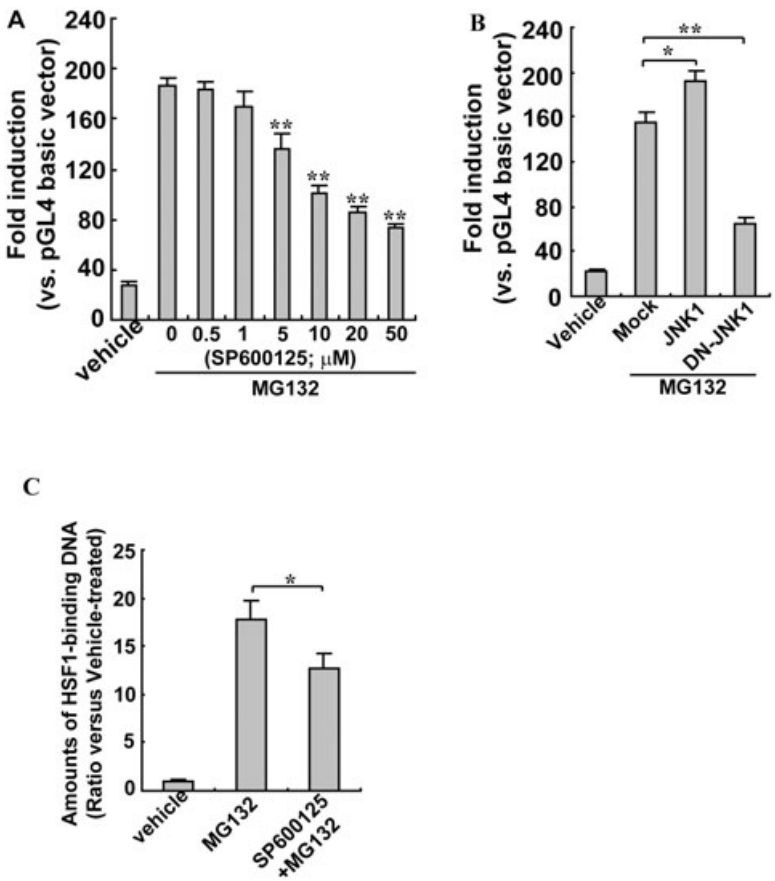

Figure 3 c-Jun N-terminal kinase (JNK) pathways are involved in activation of the BAG3 promoter. (A) A498 cells were transfected with reporter plasmid containing the BAG3 promoter. At 24 h after transfection, the cells were pre-treated with the indicated concentration of SP600125 for 1 h, and then treated with 2 μM MG132 for an additional 4 h. Cells were harvested and luciferase and *Renilla* assays were performed. *$P < 0.05$; **$P < 0.001$ versus treatment with MG132 alone. (B) Cells were co-transfected with the reporter plasmid containing the BAG3 promoter with mock, JNK1 or dominant-negative JNK1 (DN-JNK1) expression plasmid for 24 h, then treated with 2 μM MG132 for additional 4 h and luciferase activity was measured. The data are expressed as fold effect from three independent assays where luciferase activity was normalized with *Renilla* expression. *$P < 0.05$; **$P < 0.001$. (C) Cells were pre-treated with vehicle or SP600125 for 1 h, then exposed to 2 μM MG132 for 8 h; cross-linked chromatin was extracted and immunoprecipitated with an anti-HSF1 antibody. Immunoprecipitated DNA was amplified by real-time polymerase chain reaction. *$P < 0.05$.

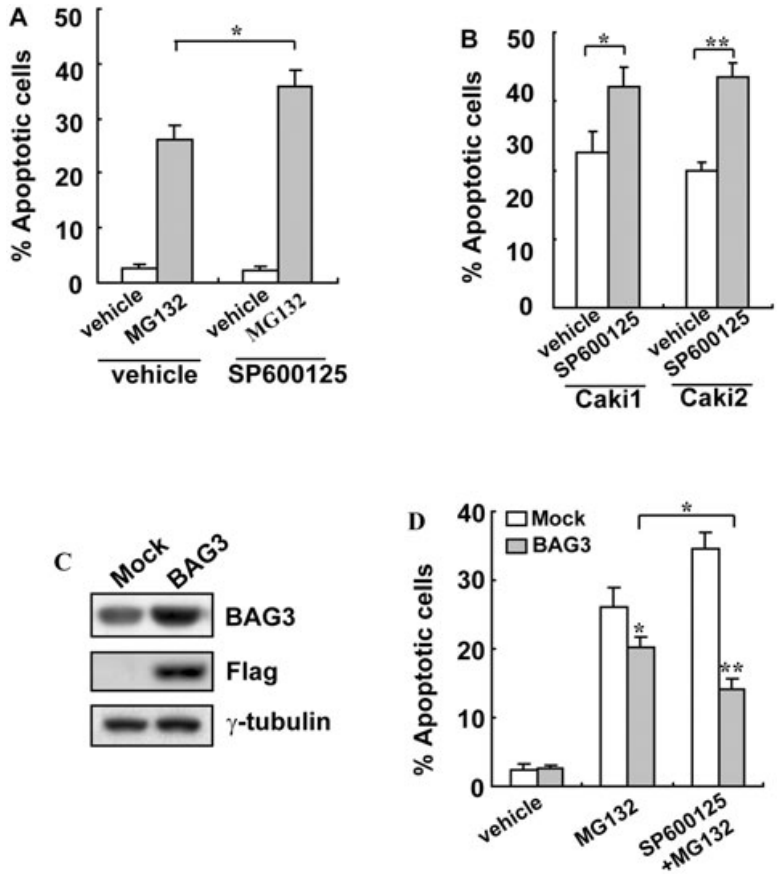

Figure 4 Effects of the c-Jun N-terminal kinase pathway or of overexpression of BAG3 on the apoptotic response of kidney cancer cells to MG132. (A) A498 cells were pre-treated with vehicle, SP600125 (20 μM) for 1 h, subsequently treated with 2 μM MG132 for additional 24 h and apoptotic cells were analysed. (B) Caki1 or Caki2 cells were treated as (A) and apoptotic cells were analysed. (C) A498 cells were transfected with mock or BAG3-expressing plasmid; total cellular protein was analysed by Western blot. (D) 24 h after transfection with mock or BAG3-expressing plasmid, A498 cells were pre-treated with vehicle or SP600125 for 1 h, subsequently treated with MG132 for additional 24 h and apoptotic cells were analysed. *$P < 0.05$; **$P < 0.001$.

this inhibitor on the apoptotic responses of Caki1 and Caki2 cells to MG132 were broadly similar to the responses observed in A498 cells (Figure 4B), suggesting that the JNK signal pathway might possess anti-apoptotic roles in kidney cancer cells treated with MG132.

Our previous studies indicated that proteasome inhibitors induced BAG3 expression (Wang *et al.*, 2008). Importantly, we have also shown that blocking BAG3 up-regulation in thyroid cancer cells using RNAi strategy can enhance cytotoxicity of proteasome inhibitors (Wang *et al.*, 2008). In the current study, we found that activation of JNK was involved in induction of BAG3 by MG132. To confirm the potential role of BAG3 in sensitizing effect of the JNK inhibitor, BAG3 was ectopically overexpressed in A498 cells (Figure 4C) and the cells were then treated with a combination of MG132 and SP600125. The results (Figure 4D) showed that, in the presence of BAG3 overexpression, the pro-apoptotic effects of MG132 were slightly, though significantly, less, than in the mock-transfected cells. However, the addition of SP600125 to MG132 now decreased this apoptotic effect in BAG3-overexpressing cells, instead of potentiating it, as in normal cells (see Figure 4A).

Discussion

Proteasome inhibitors represent a class of drugs that have anti-cancer activity through a variety of cellular mechanisms, including induction of apoptosis, interference with cell cycle progression, inhibition of angiogenesis, and the suppression of NF-κB (Voorhees and Orlowski, 2006). However, we have previously shown that proteasome inhibitors also induced BAG3 expression at the transcriptional level, and its induction suppressed anti-tumour effects of proteasome inhibitors (Wang *et al.*, 2008). Similar to our findings, several other distinct anti-apoptotic responses to proteasome inhibitor treatments have been described. Induction of heat shock proteins [Hsp27, Hsp70 (Hsp72) and Hsp90] has been implicated in proteasome inhibitor-induced anti-apoptotic signalling (Meriin *et al.*, 1998; Robertson *et al.*, 1999; Mitsiades *et al.*, 2002; Chauhan *et al.*, 2003; Hideshima *et al.*, 2004). Orlowski *et al.* (2002) found that proteasome inhibitors increased the cellular levels of mitogen-activated kinase phosphatase-1, a specific deactivator of pro-apoptotic JNK activity in breast cancer cells (Small *et al.*, 2004; Shi *et al.*, 2006). The suppression of these various responses by small molecule inhibitors

or small interfering RNA significantly enhanced the apoptotic response of various cell types to proteasome inhibition (Meriin *et al.*, 1998; Robertson *et al.*, 1999; Mitsiades *et al.*, 2002; Chauhan *et al.*, 2003; Hideshima *et al.*, 2004; Small *et al.*, 2004; Wang *et al.*, 2008). Evaluation of the mechanisms underlying induction of these anti-apoptotic molecules may contribute to the development of improved therapies using proteasome inhibitors.

In the current study, we investigated the role of MAPKs in the induction of BAG3 by MG132. Our data showed that p38 and ERK inhibitors had little effect on the induction of BAG3 by MG132 but that inhibition of JNK significantly reduced BAG3 expression by MG132. Several earlier studies have looked at the effects of bortezomib or MG132 treatment on molecular signalling in different cancer types. In liver, breast and pancreatic cancer cells treated with a proteasome inhibitor, JNK and ERK showed very similar patterns of activation to those described here in kidney cancer cells (Yang *et al.*, 2004; Codony-Servat *et al.*, 2006; Lauricella *et al.*, 2006; Sloss *et al.*, 2008).

The JNK pathway has been associated with the induction of apoptosis in response to various cellular stresses such as treatment with anti-cancer drugs. We did not expect JNK activation to be involved in BAG3 induction and that inhibition of JNK activation would enhance apoptotic responses of kidney cancer cells to proteasome inhibition. Meriin *et al.* (1998) had suggested that the proteasome inhibitor-induced JNK activation would be pro-apoptotic in lymphoid tumours. However, a growing body of evidence suggests that JNK activation is also central to anti-apoptotic and growth-promoting effects. For instance, JNK has been shown to be activated and/or overexpressed in an anti-apoptotic manner in various cancers, and JNK inhibition has been shown to increase anti-cancer effects of proteasome inhibitors in pancreatic cancer cells, suggesting an anti-apoptotic role for JNK in this cell type (Malicet *et al.*, 2003; Li *et al.*, 2007; Sloss *et al.*, 2008). The JNK pathway is also reported to be involved in the activation of Hsp70 (Kim *et al.*, 2005), one of whose most important functions is to prevent apoptosis. Although the reported results to date may seem contradictory, they may more likely represent tissue and cell type-specific differences in the response to different stimuli.

In the current study, our data showed that the JNK pathway was related to the activation of BAG3 expression in kidney cancer cells. These data indicate that the JNK pathway might play opposing roles in the induction of cellular apoptosis and maintenance of cellular homeostasis, suggesting that JNK triggers and signals to cellular apoptosis, as well as to the pathways leading to the cell survival.

Acknowledgements

This work was supported by National Natural Science Foundation of China (30870522) and Shenyang Outstanding Talent Foundation to H-Q.W.

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