Proteasome inhibition sensitizes hepatocellular carcinoma cells to TRAIL by suppressing caspase inhibitors and AKT pathway

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The ubiquitin–proteasome pathway is responsible for regulating cell cycle proteins, tumor-suppressor molecules, oncogenes, transcription factors, and pro- and anti-apoptotic proteins. The aim of this study is to evaluate the effects of proteasome inhibitors on human hepatocellular carcinoma (HCC) cells. HCC cells SK-Hep1, HLE and HepG2 were treated with the proteasome inhibitors MG132 and MG115. Our data showed that both inhibitors induce apoptosis in the three cell types tested in a dose-dependent manner. Moreover, subtoxic levels of MG132 and MG115 sensitized HCC cells to TRAIL-induced apoptosis. To investigate the mechanism of increased TRAIL sensitivity in HCC cells, we first examined surface expression of TRAIL and its receptors. MG132 upregulated TRAIL and its receptors (TRAIL-R1 and -R2) in SK-Hep1 and HLE, whereas MG115 upregulated them in SK-Hep1. MG132 downregulated expression of X-linked inhibitor of apoptosis protein (XIAP) in SK-Hep1 and HLE, and of survivin in all three cell-types. MG115 downregulated expression of XIAP in SK-Hep1, and survivin in SK-Hep1 and HepG2. Furthermore, MG132 downregulated phospho-AKT and its downstream target phospho-BAD, indicating that MG132 activated the mitochondrial apoptosis pathway by inhibiting phosphorylation of AKT and BAD. In conclusion, proteasome inhibitors induced apoptosis and augmented TRAIL sensitivity via both the IAP family and AKT pathways. Thus, combining proteasome inhibitors with a TRAIL agonist may provide a new therapeutic strategy for HCC. *Anti-Cancer Drugs* **17:261–268 © 2006 Lippincott Williams & Wilkins.**

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Introduction

Apoptosis, which can be mediated via extrinsic or intrinsic pathways, is essential for maintaining cellular homeostasis in the liver [1,2]. Dysregulation of apoptosis pathways contributes to hepatic diseases such as hepatocellular carcinoma (HCC), viral hepatitis, autoimmune hepatitis, ischemia reperfusion injury, iron or copper deposition disorders, toxic liver damage and acute liver failure [3,4]. HCC is usually resistant to chemotherapy and irradiation, and its cellular properties appear to be tightly linked to anti-apoptotic behavior at the molecular level [5]. Therefore, sensitization of cells to induction of apoptosis with specific drugs may be critically important to establish a therapeutic strategy for HCC.

Recently, the ubiquitin–proteasome pathway was shown to be responsible for regulating cell cycle proteins, tumor-suppressor molecules, oncogenes, transcription factors, and pro- and anti-apoptotic proteins. The 26S proteasome is a multicatalytic threonine protease with three distinct catalytic activities. The ubiquitin–proteasome protein degradation pathway is comprised of ubiquitin, a three-enzyme ubiquitination complex, the intracellular protein ubiquitination targets and the proteasome that is the organelle of protein degradation [6,7]. Proteasome inhibitors that interact with the proteasome–ubiquitin pathway have been reported as novel anti-cancer drugs [8,9]. Transformed cells are more sensitive to apoptosis induced by proteasome inhibition than normal cells [10,11]. This effect on cancer cells seem to be mediated through activation of apoptosis pathways, including stabilization of IκBα (an inhibitor of the NF-κB transcription factor), downregulation of FLICE inhibitory protein (FLIP) or dysregulation of p53 [7,11,13]. Clinically, Bortezomib, which is a small molecule with a boronate moiety linked to a dipeptide, has recently been approved by the US Food and Drug Administration for treatment of relapsed multiple myeloma [14–16]. Bortezomib has a highly specific inhibitory effect on the chymotryptic activity of the proteasome. Although multiple models to explain the anti-cancer effect of proteasome inhibitors have been proposed, the mechanism(s)

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by which the inhibitors exert an effect on cancer cells has not been elucidated. In addition, there is likely to be value in learning what happens when multiple therapeutic approaches, including the use of proteasome inhibitors, are combined.

TRAIL, a member of tumor necrosis factor TNF family, selectively induces apoptosis in various transformed cell lines. However, interestingly, several tumor cell lines and most normal cells are resistant to TRAIL-mediated apoptosis [17]. This selective toxicity for cancer cells is the basis of the current enthusiasm for TRAIL as a potential target of novel anti-cancer therapeutics [18]. Previous studies suggested that HCC cells may be resistant to TRAIL-mediated apoptosis despite the fact that they express TRAIL receptors [19,20].

Recently, some studies have suggested that proteasome inhibition sensitizes TRAIL-mediated apoptosis in cancer cell lines [21]. However, the precise mechanism of sensitization of cancer cells to TRAIL-mediated apoptosis by proteasome inhibition has not been well elucidated. Therefore, we investigated the effects of the proteasome inhibitors MG132 and MG115 on HCC cells, and evaluated the mechanism by which these agents sensitize HCC cells for TRAIL-mediated apoptosis.

Material and methods

Materials

The proteasome inhibitors carbobenzoxy-L-leucyl-L-leucinal (MG132) and carbobenzoxy-L-leucyl-L-leucyl-L-norvalinal (MG115) were obtained from Merck (Darmstadt, Germany), dissolved in DMSO at a stock concentration of 100 mmol/l, and stored at −20°C. Fresh dilutions in medium were made for each experiment.

Cell lines

The human HCC cell lines SK-Hep1 and HepG2 were purchased from the ATCC (Rockville, Maryland, USA). The HCC cell line HLE (JCRB0404) was purchased from the Health Science Research Resources Bank (Osaka, Japan). HCC cell lines were cultured in DMEM (Sigma-Aldrich, St Louis, Missouri, USA) at 37°C. All media were supplemented with 1% penicillin/streptomycin (Gibco/BRL, Grand Island, New York, USA) and 10% heat-inactivated FCS (Gibco/BRL).

Antibodies and reagents

The following antibodies were used: anti-human caspase-3 and c-FLIP polyclonal antibody from MBL (Nagaya, Japan), hILP/XIAP and Bcl-X from Transduction (Lexington, Kentucky, USA), survivin from Santa Cruz Biotechnology (Santa Cruz, California, USA), AKT antibody, phospho-AKT (Ser473) and phospho-BAD (Ser136) antibody from Cell Signaling Technology (Beverly, Massachusetts, USA). For FACS analysis, the following antibodies were used: phycoerythrin (PE)–anti-human DR4, PE–anti-human DR5, PE–anti-human TRAIL, PE–anti-mouse and human IgG (eBioscience, San Diego, California, USA). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma (St Louis, Missouri, USA).

Detection of apoptosis and cell proliferation

In total, 2×10^5 HCC cells were cultured in chamber slides for 24 h, followed by addition of 0.5 or 1 μmol/l of MG132. After incubation for 24 h, cell nuclei were stained with 4′,6-diamino-2-phenylindole (DAPI; Sigma). Cell viability was determined by MTT assay with the Cell Titer96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, Wisconsin, USA).

FACS analysis

HCC cell lines were incubated with several concentrations of MG132 for 24 h, detached from the plates with EDTA, and incubated with the surface-expressed TRAIL receptors and control mIgG. Cells were analyzed with FACScan cytometer and CellQuest software (Becton-Dickinson, Franklin Lakes, New Jersey, USA).

Immunoblotting

HCC cell lines were detached from the plates, washed with PBS and lysed in lysis buffer (50 mmol/l Tris–HCl, pH 8, 150 mmol/l NaCl, 5 mmol/l EDTA, 1% NP-40 and 1 mmol/l PMSF) according to the manufacturer's instructions. After 30 min incubation on ice, the lysates were centrifuged at 15 000 *g* at 4°C. Supernatant was collected and the protein concentration was measured according to Bio-Rad (Hercules, California, USA) protein assay protocol. Between 20 and 40 μg of protein was electrophoresed on 7.5–20% gradient SDS–PAGE gels and transferred to Immobilon PVDF transfer membranes (Millipore, Billerica, Massachusetts, USA).

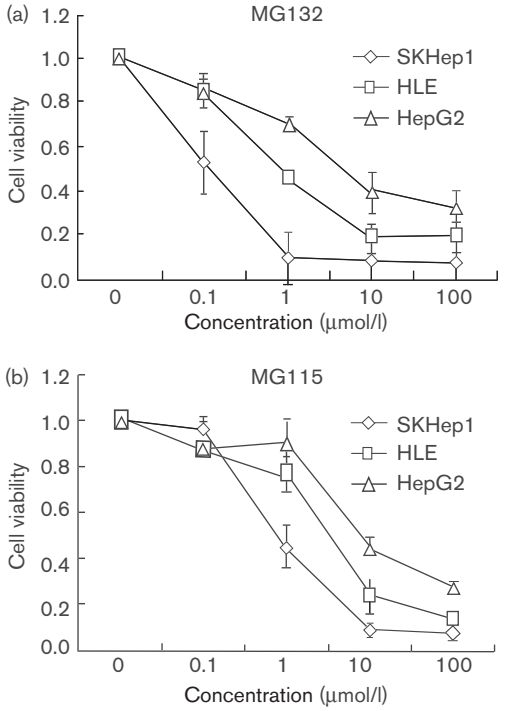
Results

The proteasome inhibitors MG132 and MG115 induced apoptosis in HCC cells

To investigate the change in viability of HCC cells in response to MG132 and MG115, we incubated SK-Hep1, HLE and HepG2 with different concentrations of MG132 and MG115 for 24 h. Cell viability in these cells was reduced by MG132 and MG115 in a concentration-dependent manner, with SK-Hep1 the most affected among the three cell types (Fig. 1). Next, to investigate if MG132 and MG115 induced apoptosis in HCC cells, we looked at DAPI staining 24 h after treatment with MG132 and MG115. We observed typical apoptotic features, including nuclear condensation and nuclear fragmentation (Fig. 2a and b).

Apoptotic cell death usually results from the action of caspases and since caspase activation is essential for the apoptotic response we decided to next examine caspase

Fig. 1



MG132 and MG115 induced cell death in human HCC lines. SK-Hep1, HLE and HepG2 were treated with 0–100 μmol/l MG132 (a) or MG115 (b) and analyzed after 24 h for cell viability by the MTT assay. A representative result from three independent experiments is shown.

activation in HCC cells treated with MG132. To do this, we analyzed caspase-8 and -3 by immunoblotting. Both caspase-8 and -3 were strongly upregulated in SK-Hep1 and HLE cells treated with MG132, and they showed a slight upregulation in HepG2 cells, in a dose-dependent manner (Fig. 3).

MG132 and MG115 augmented TRAIL-induced apoptosis in HCC cells

TRAIL can induce apoptosis in cancer cells via the caspase pathway, although previous studies have suggested that HCC cells in particular may be resistant to TRAIL-mediated apoptosis [19,20]. To examine whether MG132 and MG115 affect TRAIL sensitivity in HCC cells, we incubated SK-Hep1, HLE and HepG2 with different concentrations of TRAIL for 24 h after pre-treatment with subtoxic concentrations of MG132 and MG115. Combining MG132 and TRAIL decreased cell viability in the three HCC cells in a dose-dependent manner that was especially pronounced in SK-Hep1. Indeed, the treatment combination had a synergistic effect on cell viability in SK-Hep1, whereas in HLE and HepG2 the effect was additive (Fig. 4). To assess whether the decrease in cell viability was caused by apoptosis, we examined DAPI-stained cells. We observed an increase in the number of apoptotic cells in the SK-Hep1 population that had been treated with proteasome inhibitors and TRAIL (Fig. 2c and d). These results suggest that MG132 and MG115 augment TRAIL sensitivity in some HCC cells.

MG132 upregulated TRAIL receptors (TRAIL-R1 and -R2)

In order to assess the mechanism of increased TRAIL sensitivity in HCC cells, we examined cell surface expression of TRAIL and TRAIL receptors in SK-Hep1, HLE and HepG2 using flow cytometry analysis. We incubated SK-Hep1, HLE and HepG2 with subtoxic concentrations of MG132 and MG115 for 24 h. MG132 upregulated TRAIL and TRAIL receptors in SK-Hep1 and HLE. MG115 upregulated TRAIL and TRAIL receptors only in SK-Hep1. In HepG2, neither MG132 nor MG115 affected the expression of TRAIL or TRAIL receptors (Fig. 5).

MG132 and MG115 downregulated inhibitors of apoptosis

We next sought to determine the relative expression levels of apoptosis-related proteins. To this end, we analyzed expression of X-linked inhibitor of apoptosis (XIAP) and survivin, which are members of the IAP family, by immunoblotting. MG132 downregulated expression of XIAP in SK-Hep1 and HLE in a concentration-dependent manner, but not in HepG2. In contrast, MG115 downregulated XIAP only in SK-Hep1. MG132 downregulated survivin in all three cell lines; MG115 downregulated survivin only in SK-Hep1 and HepG2 (Fig. 6). The levels of other IAP family proteins, including Bcl-X and c-FLIP, were not significantly affected by treatment with the proteasome inhibitors (data not shown).

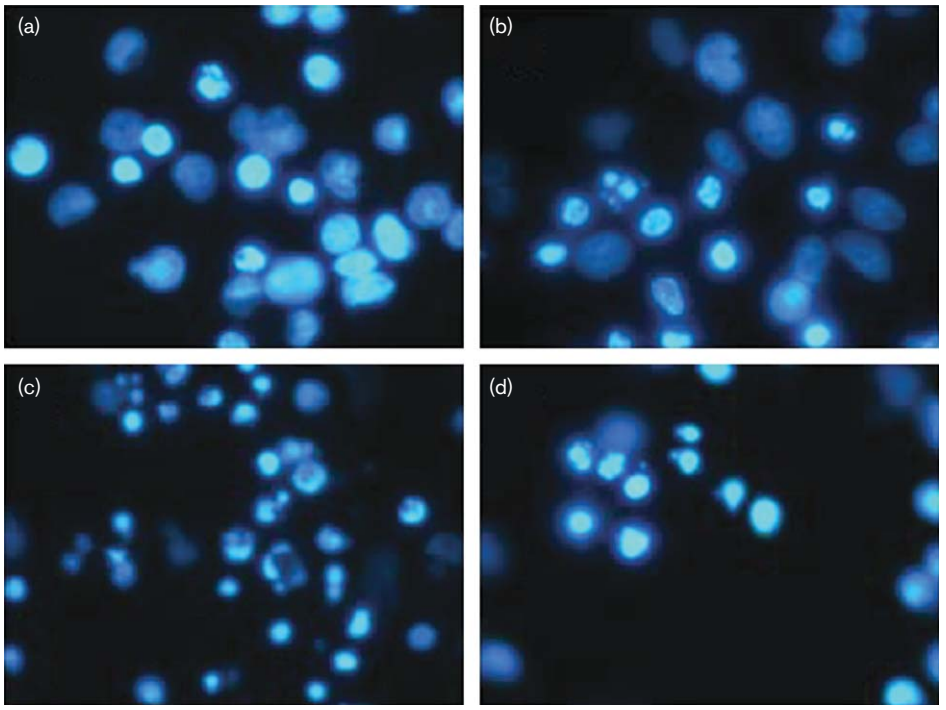
MG132 downregulated phosphorylation of AKT

Finally, we investigated the state of the AKT pathway, since the pathway plays a critical role in controlling the balance between cell survival and apoptosis [22]. To investigate whether MG132 treatment affects the AKT pathway, we incubated SK-Hep1 with different concentrations of MG132 for 24 h. We chose SK-Hep1 because among the HCC cell-types we tested, the SK-Hep1 cell line seemed to be the most sensitive to inhibitor-induced apoptosis. We determined the levels of both AKT and phospho-AKT by immunoblotting. In addition, we looked at phosphorylated BAD to determine at which point in the AKT pathway the treatment exerts its effect. Phosphorylation of AKT and BAD was downregulated by MG132 in a dose-dependent manner in SK-Hep1 (Fig. 7).

Discussion

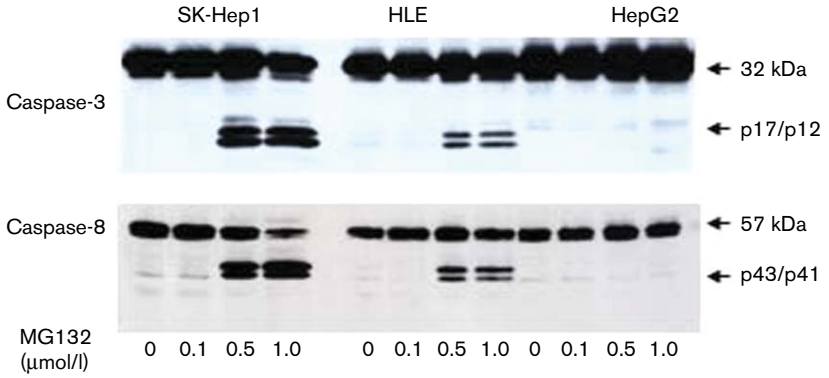
The results reported here clearly demonstrate that the proteasome inhibitors MG132 and MG115 induced apoptosis in a dose-dependent manner in the three HCC cell types tested, with the most pronounced effect observed in SK-Hep1 cells. Our results also showed that

Fig. 2



MG132 and MG115 induced apoptosis in SK-Hep1 cells and combination treatment with TRAIL synergistically augmented apoptosis. The cells were incubated with (a) 0.5 μmol/l MG132, (b) 0.5 μmol/l MG115, (c) 0.5 μmol/l MG132 + 10 ng/ml TRAIL and (d) 0.5 μmol/l MG115 + 10 ng/ml TRAIL for 24 h. Then, cell nuclei were visualized with DAPI staining of DNA.

Fig. 3



Increased caspase-3 and -8 cleavage after treatment with MG132. The HCC cells were incubated with MG132 at the indicated concentrations, lysed, and then 30 μg protein was analyzed by 7.5–20% gradient SDS–PAGE and immunoblotting. The same blot was incubated with antibodies that recognize caspase-8 and -3. A representative result from at least three independent experiments is shown.

the HCC cell death induced by MG132 and MG115 was caspase dependent. MG132 and MG115 are peptide aldehydes, which are substrate analogs and potent transition state inhibitors, primarily of the chymotrypsin-like activity of the proteasome [6]. These proteasome inhibitors can readily enter cells. Since they disrupt degradation of proteins involved in cell cycle control and tumor growth, the inhibitors can have dramatic effects on cell growth and viability [6]. Inhibiting the degradation of cell cycle regulators and growth-promoting proteins has been reported to affect tumor growth and cause tumor cells to undergo apoptosis [7–9,12]. Proteasome inhibitors act at many levels to enhance apoptosis signaling: by inhibition of NF-κB activity, increased activity of p53 and

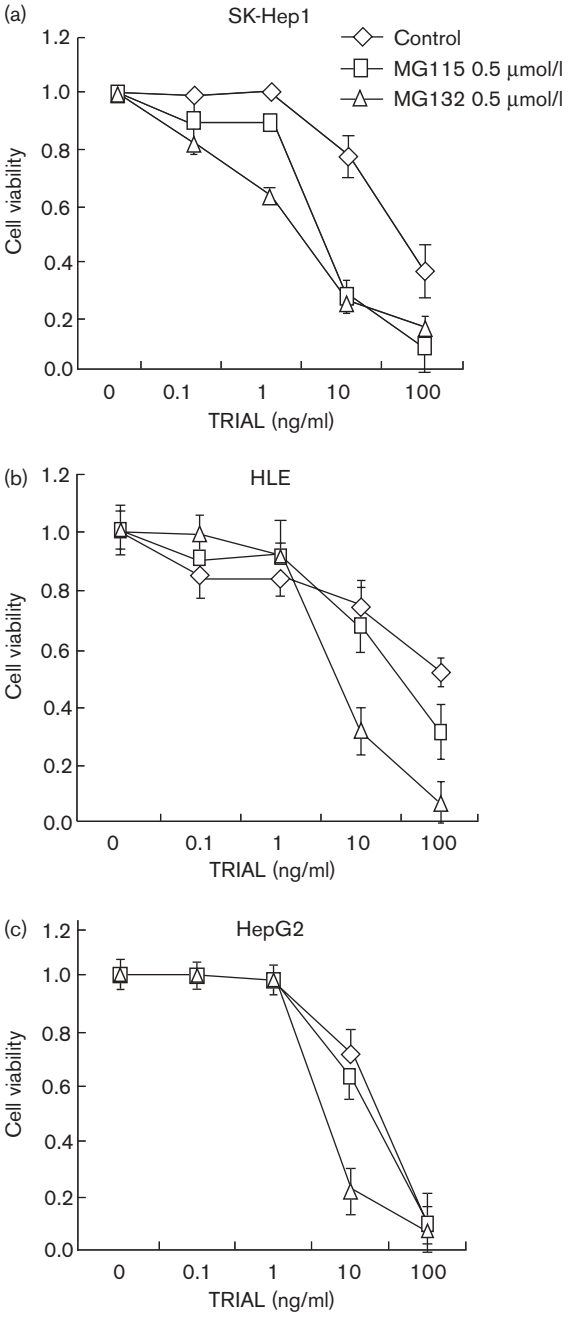
Bax proteins, and accumulation of cyclin-dependent kinase inhibitors p27 and p21 [7,12]. Bortezomib, the first proteasome inhibitor to enter clinical trials, has been shown to induce apoptosis in a variety of hematologic and solid malignancy models both *in vivo* and *in vitro* through stabilization of p53, p21, Bax and IκB, resulting in NF-κB inhibition [14–16]. Lactacystin, a proteasome inhibitor originally isolated from actinomycetes, induces apoptosis of human monoblastic U937 cells [23]. Inhibition of proteasome function by MG132 also induced apoptosis through upregulation of p53, Bax and $p27^{kip1}$ in gastric cancer cells, regardless of whether they were wild-type or mutant for p53 [24]. However, the mechanism of induction of apoptosis differs among different cell lines and the precise mechanism in HCC has been not fully elucidated.

Previous studies have shown that although most HCC cell lines are resistant to TRAIL, co-treatment with chemotherapeutic agents or irradiation sensitized TRAIL-resistant cells to TRAIL [19,20]. Thus, we decided to determine if MG132 and MG115 affect TRAIL sensitivity in HCC cells (Fig. 4). In our study, a subcytotoxic level of MG132 augmented TRAIL-induced apoptosis synergistically and in a dose-dependent manner in the three HCC cells, especially in SK-Hep1. However, MG115 augmented TRAIL sensitivity in SK-Hep1 synergistically, but only additively in HLE and HepG2. Among these cell lines, SK-Hep1 seems to be most sensitive to TRAIL-mediated apoptosis by proteasome inhibition.

Recently, several studies also suggested that proteasome inhibition sensitizes TRAIL-mediated apoptosis in some cell lines. NF-κB activation is efficiently blocked by proteasome inhibitors in lymphoid cells. Since binding of TRAIL to its receptors activates NF-κB, it is tempting to speculate that proteasome inhibition enhances TRAIL-mediated apoptosis principally by blocking NF-κB [25–28]. However, other studies have revealed that NF-κB activation does not protect all cells from TRAIL-mediated apoptosis and that Bortezomib can sensitize a mouse myeloid leukemia to apoptosis independent of any effects on NF-κB activation [29]. In leukemia cells, the level of c-FLIP, an anti-apoptotic protein, decreases after treatment with Bortezomib. Other studies have shown that proteasome inhibition can modulate the balance between pro- and anti-apoptotic members of the Bcl-2 family, resulting in accumulation of the pro-apoptotic protein Bik [25]. However, the mechanism also differs depending on tumor cell lines.

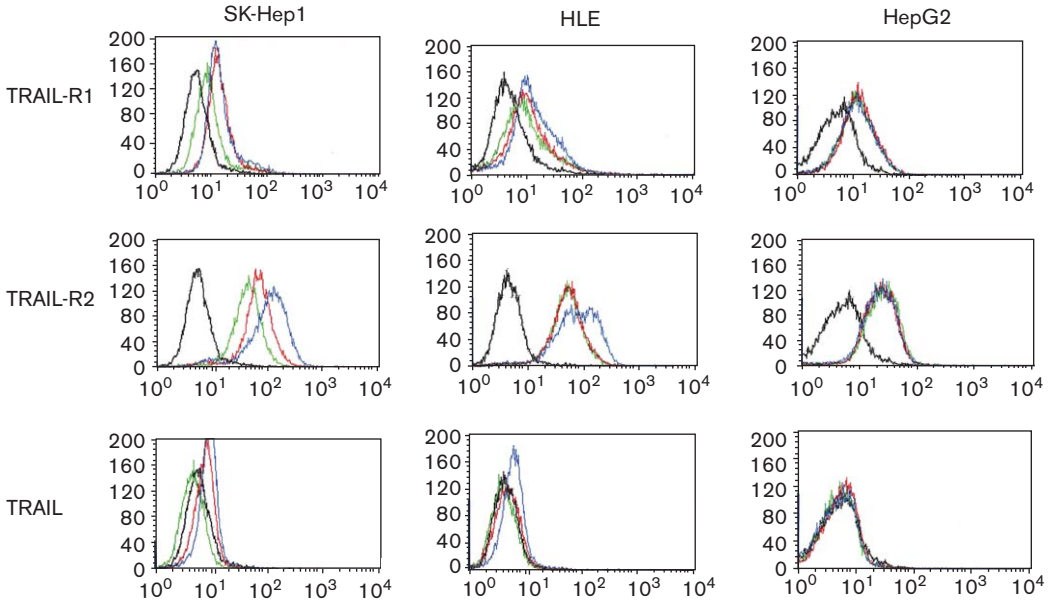
Here, we found that each of the inhibitors was able to upregulate TRAIL and its receptors in at least a subset of the cell types we tested. These result suggested that upregulation of TRAIL receptors by proteasome inhibition may be one of the major reasons for sensitization to TRAIL-induced apoptosis. Previous reports also suggested that proteasome inhibition increases the expression of TRAIL receptors, and then regulates the recruitment of FADD and caspase-8 to the death-

Fig. 4



Treatment with both proteasome inhibitors and TRAIL augmented cell death in HCC cells. SK-Hep1 (a), HLE (b) and HepG2 (c) were incubated with 0.5 μmol/l MG132 and 0.5 μmol/l MG115 and TRAIL at the indicated concentrations for 24 h. Then, cell viability was analyzed by the MTT assay. The data shown are representative of three independent experiments.

Fig. 5



MG132 and MG115 upregulated TRAIL and TRAIL receptors. The HCC cells were incubated with 0.5 μmol/l MG132 (blue lines) and 0.5 μmol/l MG115 (red lines) for 24 h, and then were stained with PE–anti-human TRAIL-R1, TRAIL-R2 and TRAIL. The control cells were incubated without stimulation (green lines). Autofluorescence and non-specific staining was determined using cells incubated with PE–control mouse IgG (black lines).

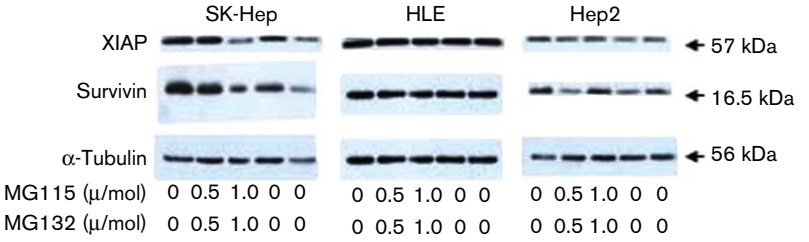
inducing signaling complex in HCC and other tumor cell lines [26]. Another report suggested that MG132 induced TRAIL-R1 expression via upregulation of CCAAT/enhancer binding protein homologous protein (CHOP) in prostate cancer DU145 cells [30]. Moreover, another group provided evidence that the increase in TRAIL receptor protein levels is associated with ubiquitination of the TRAIL-R2 protein [27]. Previous reports also suggested that induction of TRAIL receptor mRNA following DNA damage is dependent on the presence of wild-type p53 [31]; however, in our study, SKHep1 and HLE, with mutant p53, exhibited receptor upregulation, although HepG2, with wild-type p53, did not show a significant change in expression of TRAIL receptors. These results provide support for the idea that upregulation of TRAIL-R1 and -R2 by MG132 and MG115 in SK-Hep1 and HLE might be p53 independent.

We also looked at expression of apoptosis-related proteins. We found that each of the inhibitors was able to downregulate XIAP and survivin in at least a subset of the cell types we tested. We have shown that XIAP is overexpressed in human HCC, and that it is a principal inhibitor of apoptosis through its ability to inhibit caspase-3, -7 and -9 [32]. Previous reports suggested that XIAP, which can directly bind to caspases and act as a ubiquitin ligase for caspases, might exert its caspase-inhibitory role via its ubiquitin–protein ligase activity [33,34]. From our results, we conclude that downregulation of protein levels of XIAP by MG132 and MG115 is one of the causes of the augmentation of TRAIL-induced apoptosis in HCC cells that we observed.

MG132 also downregulated survivin in the three HCC cell lines we tested, whereas MG115 downregulated survivin only in SK-Hep1 and HepG2 (Fig. 6). Protein levels of the other caspase inhibitors, including Bcl-X and c-FLIP, were not significantly affected by treatment with the proteasome inhibitors. Survivin, a human IAP protein, is expressed ubiquitously during embryonic and fetal development, but is undetectable in most normal adult tissues. However, survivin is expressed at high levels in human cancers [35,36]. Survivin can bind to caspase-3 and -7, and overexpression of survivin blocks apoptosis induced by caspase-3 and -7. We consider downregulation of survivin another important cause of the augmentation of TRAIL-induced apoptosis we observed in HCC cells after treatment with proteasome inhibitors.

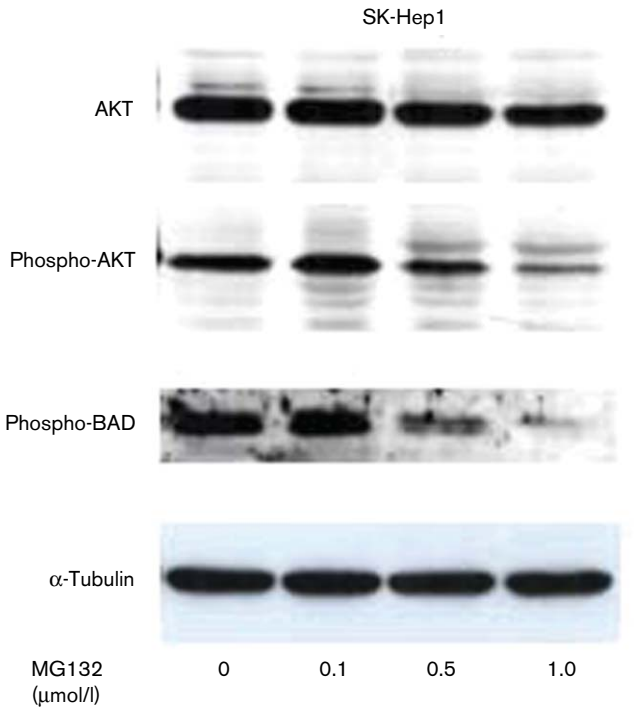
Finally, we investigated the AKT pathway, since the pathway also plays a critical role in controlling the balance between cell survival and apoptosis. AKT promotes cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets, including BAD and caspase-9 [22]. Our study demonstrated that the levels of phosphorylated AKT and downstream factor phosphorylated BAD were reduced in SK-Hep1 in a dose-dependent manner after treatment with MG132 (Fig. 7). Previous reports have shown that AKT, the serine/threonine kinase that mediates survival signals, is

Fig. 6



MG132 downregulated IAP protein levels. The HCC cells were incubated with MG132 at the indicated concentrations. The cellular lysates were analyzed by SDS–PAGE and immunoblotting. Subsequently, the same blot was incubated with antibodies that recognize XIAP or survivin. The data shown are representative of several independent experiments.

Fig. 7



MG132 downregulated the AKT pathway. SK-Hep1 was incubated with MG132 at the indicated concentrations. The cellular lysates were analyzed by SDS–PAGE and immunoblotting. Subsequently, the same blot was incubated with antibodies that recognize AKT, phospho-AKT (Ser473) or phospho-BAD (Ser136).

overexpressed in several malignancies. A number of AKT substrates that are components of intrinsic cell death machinery have been identified, including glycogen synthase kinase-3, the Bcl-2 family member BAD, the protease caspase-9 and Forkhead transcriptional factors. In each case, phosphorylation of AKT suppresses their pro-apoptotic function. BAD is a pro-death member of the Bcl-2 family that initiates apoptosis by binding to Bcl-X_L on the outer mitochondrial membrane, causing the release of cytochrome C into the cytosol. AKT phosphorylates BAD on Ser136, promoting association of BAD with 14-3-3 proteins in the cytosol and inactivating its pro-apoptotic function. Our data suggest that proteasome inhibition downregulated phosphorylation of AKT and its downstream target BAD in HCC cells. Moreover, by inhibiting phosphorylation of AKT and BAD, MG132 may have also activated the mitochondrial apoptosis pathway.

In conclusion, we demonstrated that proteasome inhibitors can induce apoptosis and augment TRAIL sensitivity by upregulation of TRAIL receptors, and downregulation of IAPs and the AKT pathway. These results suggest that combining proteasome inhibitors with a TRAIL agonist may be prove to be an effective new strategy for treatment of HCC.

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