Antioxidants block proteasome inhibitor function in endometrial carcinoma cells

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We have recently demonstrated that proteasome inhibitors can be effective in inducing apoptotic cell death in endometrial carcinoma cell lines and primary culture explants. Increasing evidence suggests that reactive oxygen species are responsible for proteasome inhibitor-induced cell killing. Antioxidants can thus block apoptosis (cell death) triggered by proteasome inhibition. Here, we have evaluated the effects of different antioxidants (edaravone and tiron) on endometrial carcinoma cells treated with aldehyde proteasome inhibitors (MG-132 or ALLN), the boronic acid-based proteasome inhibitor (bortezomib) and the epoxyketone, epoxomicin. We show that tiron specifically inhibited the cytotoxic effects of bortezomib, whereas edaravone inhibited cell death caused by aldehyde-based proteasome inhibitors. We have, however, found that edaravone completely inhibited accumulation of ubiquitin and proteasome activity decrease caused by MG-132 or ALLN, but not by bortezomib. Conversely, tiron inhibited the ubiquitin accumulation and proteasome activity decrease caused by bortezomib. These results suggest that edaravone and tiron rescue cells of proteasome inhibitors from cell death, by inhibiting blockade of proteasome caused by MG-132 and ALLN or bortezomib, respectively. We also tested other antioxidants, and we found that vitamin C inhibited bortezomib-induced cell death. Similar to tiron, vitamin C inhibited cell death by blocking the ability of bortezomib to inhibit the proteasome. Until now, all the antioxidants that blocked proteasome inhibitor-induced cell death also blocked the proteasome inhibitor mechanism of action.
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Introduction

During the last few years, a number of studies have proved that proteasome inhibitors are effective anticancer drugs. Among patients with haematological malignancies, those with multiple myeloma are currently being treated with the proteasome inhibitor bortezomib (Velcade, formerly known as PS-341) [1–6]. Ongoing preclinical studies have suggested that proteasome inhibitors show antitumour activity against solid tumours, including carcinomas of the breast [7], lung [8], colon [9], bladder [10], ovary [11], pancreas [12] and others. A recent study in our laboratory has demonstrated that proteasome inhibitors can also be effective for inducing apoptotic cell death in endometrial carcinoma cell lines and primary culture explants [13].

Proteasome inhibitors can be classified into different groups on the basis of their chemical structures and modes of action: [14] (i) the boronic acid-based proteasome inhibitors such as bortezomib, (ii) the aldehyde-based proteasome inhibitors such as MG-132, MG-115, ALLN, PSI and glyoxal or (iii) the epoxyketones such as epoxomicin. The vast majority of the currently available proteasome inhibitors preferentially block the chemotrypsin-like activity of the proteasome.

Recent evidences suggest that proteasome inhibitors might cause cell death by the induction of reactive oxygen species (ROS) in several malignancies such as non-small lung cancer cells [15], head and neck squamous carcinoma cells [16] and mantle-cell lymphoma cells [17]. These studies have also demonstrated that different antioxidants can inhibit proteasome-induced cytotoxicity in some cell types. Recent studies have, however, also demonstrated that the proteasome-inhibitor action of bortezomib on melanoma cells [18] or on different cell lines [19] might be blocked by antioxidants like tiron or vitamin C.

We have previously demonstrated that proteasome inhibitors are effective in inducing death in endometrial carcinoma cell lines and primary tumour explants.

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Here, we have assessed the effects of different antioxidants on proteasome-inhibitor cytotoxicity on endometrial carcinoma cell lines. Surprisingly, we have found a different effect of several antioxidants on different proteasome inhibitors (bortezomib, MG-132, ALLN and epoxomicin). For example, tiron completely inhibits proteasome-induced apoptosis caused by bortezomib, but has no effects on the other inhibitors. Conversely, edaravone blocks MG-132-induced and ALLN-induced apoptosis, but does not have significant effects on apoptosis induced by bortezomib/epoxomicin. The evaluation of the effects of proteasome inhibitors has demonstrated that tiron inhibits the ability of bortezomib to inhibit the proteasome. In contrast, edaravone blocks the effects of aldehyde-based proteasome inhibitors such as MG-132 and ALLN.

Materials and methods

Reagents, plasmids and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) and monoclonal antibody to tubulin and tiron were from Sigma (St Louis, Missouri, USA). MCI-186 (edaravone) the proteasome inhibitors MG-132, epoxomicin and ALLN and the fluorogenic proteasome substrate III were from Calbiochem (La Jolla, California, USA). Bortezomib (Millenium Pharmaceuticals, Cambridge, Massachusetts, USA) was obtained from the Department of Pharmacy (Hospital Arnau de Vilanova, Lleida, Spain). Antibodies to active caspase-3 and caspase-9 were obtained from Cell Signalling (Beverly, Massachusetts, USA). Antibody to ubiquitin was from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Peroxidase-conjugated antimouse and antirabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden).

Cell lines, culture conditions and transfection

The Ishikawa 3-H-12 cell line was obtained from the American Type Culture Collection (Manassas, Virginia, USA). KLE cells were a gift from Dr Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid, Spain). RL-95 and HEC-1-A cells were a gift from Dr Reventos (Hospital Vall d'Hebron, Barcelona, Spain). The melanoma cell lines were a gift from Rosa M. Martí. All cell lines were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% foetal bovine serum (Invitrogen Inc., Carlsbad, California, USA), 1 mmol/l HEPES (Sigma), 1 mmol/l sodium pyruvate (Sigma), 2 mmol/l L-glutamine (Sigma) and 1% of penicillin/streptomycin (Sigma), at 37°C with saturating humidity and 5% CO_2.

Cell-viability assays and assessment of apoptosis

Cell viability was determined by MTT assay. Endometrial adenocarcinoma cells were plated onto M96-well plates at 15×10^3 cells/well. After the indicated treatments, the cells were incubated for 2–3 h with 0.5 mg/ml of MTT reagent and lysed with dimethyl sulphoxide. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmond, California, USA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0.5 mg/ml to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Western blot analysis

Endometrial adenocarcinoma cell lines were washed with cold phosphate-buffered saline and were lysed with lysis buffer (2% SDS; 125 mmol/l Tris–HCl, pH 6.8). Protein concentrations were determined with the Protein assay Kit (Bio-Rad). Equal amounts of proteins were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, Massachusetts, USA). Nonspecific binding was blocked by incubation with Tris-buffered saline/Tween solution (20 mmol/l Tris–HCl, pH 7.4; 150 mmol/l NaCl; 0.1% Tween-20) with 5% of nonfat milk. Membranes were incubated with the primary antibodies overnight at 4°C. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

Proteasome-activity assay

Endometrial carcinoma cell lines were plated onto M24 multiwell dishes at 15×10^5 cells/well. The cells were treated with the experimental conditions for 8 h. After treatment, cells were rinsed with phosphate-buffered saline and lysed in 50 µl of cytoplasmic lysis buffer (50 mmol/l HEPES, pH 8; 1% NP-40; 150 mmol/l NaCl; 1 mmol/l EDTA and 1 mmol/l ethylene glycol tetraacetic acid). Nuclei were removed by centrifugation at 10 000 rpm for 5 min. Protein concentrations were determined with the Protein Assay Kit. A total of 10–20 µg of protein was transferred to a 90-µl final volume of assay buffer (20 mmol/l HEPES, pH 7; 9, 0.5 mmol/l EDTA; 5 mmol/l $MgCl_2$ and 2 mmol/l ATP). After 10 min at 37°C, 10 µl containing 200 µmol/l of the fluorogenic proteasome substrate III Suc-Leu-Leu-Val-Tyr-AMC (final concentration of substrate 20 µmol/l) was added to each sample. Fluorescence was read every 15 min in a microplate fluorimeter at 380 nm excitation wavelength and 460 nm emission.

Results

Tiron inhibits the cytotoxic effects of bortezomib and edaravone inhibits the effects of aldehyde-based proteasome inhibitors

We have previously demonstrated that proteasome inhibitors are effective in inducing apoptosis of endometrial carcinoma cells. In contrast, some antioxidants have been shown to inhibit cell death caused by proteasome inhibitors. We first tested the effects of tiron and edaravone on cell death caused by different types of proteasome inhibitors, which use different chemical mechanisms for inhibiting the 20S proteasome. We have

used the aldehyde-based proteasome inhibitors, MG-132 and ALLN; the dipeptide boronic acid, bortezomib; and the peptide epoxyketone, epoxomicin. The ishikawa (IK) endometrial carcinoma cell line was treated with the four proteasome inhibitors in addition to increasing doses of either tiron or edaravone, and we assessed cell viability by MTT assay 24 h after treatment. The results showed that tiron treatment caused a dose-dependent inhibition of the decrease in cell viability caused by bortezomib, but had no effect on cell death caused by treatment with the other three proteasome inhibitors (Fig. 1a). Conversely, edaravone inhibited the decrease in cell viability caused by MG-132 and ALLN in a dose-dependent manner; however, it did not have any effect on cell death induced either by bortezomib or by epoxomicin (Fig. 1b).

Tiron and edaravone inhibit apoptotic features induced by bortezomib and aldehyde proteasome inhibitors, respectively

We have also addressed the effects of tiron and edaravone on apoptotic nuclear morphology caused by proteasome inhibition. IK cells were treated with the four proteasome inhibitors, alone or in the presence of either tiron or edaravone, and cells were stained with Hoechst 24 h after treatment. In agreement with the results obtained with the viability assays, the addition of tiron dramatically reduced the number of nuclei displaying apoptotic morphology in the cultures treated with bortezomib, but did not show any effect on the number of apoptotic nuclei in the cultures treated with epoxomicin, MG-132 or ALLN (Figs 2a and b). In contrast, edaravone inhibited the appearance of apoptotic nuclei on MG-132-treated and ALLN-treated cells, but it did not cause any reduction in the bortezomib-treated or epoxomicin-treated cultures (Figs 2a and b). To rule out the possibility that such effects were specific to a cell line, we performed the experiments on another endometrial carcinoma cell lines (Fig. 2b), and we obtained identical results.

We next analysed the effects of tiron and edaravone on caspase activation in endometrial carcinoma cell lines under proteasome inhibitor treatment. IK cells were treated with bortezomib, MG-132, ALLN or epoxomicin, in the presence or absence of tiron and edaravone. After 24 h, we analysed caspase activation by Western blot. All four proteasome inhibitors caused activation of caspase-3 and caspase-9, as seen by Western blot analysis of procaspase processing and generation of active fragments (Fig. 2c). Caspase activation was completely abolished in conditions containing bortezomib along with tiron, but not in conditions containing any of the other three proteasome inhibitors along with tiron (Fig. 2c). In contrast, edaravone inhibited caspase-9 and caspase-3 processing induced by MG-132 or ALLN, but did not inhibit caspase processing caused by bortezomib or epoxomicin.

Ubiquitin accumulation by bortezomib and aldehyde-based proteasome inhibitors is inhibited by tiron and edaravone, respectively

We next assayed the effects of either tiron or edaravone on the function of proteasome inhibitors by using two different readouts. First, we examined the accumulation of ubiquitinated proteins by Western blot, using an antibody that recognizes ubiquitin. Ubiquitinated product accumulation results from the inability of proteasome to degrade such products. Time-course incubation of IK cells with MG-132 resulted in an increasing amount of ubiquitinated proteins being present in cell protein extracts (Fig. 3a). Similar results were obtained with the other proteasome inhibitors.

Treatment of IK cells with each of the four proteasome inhibitors produced a similar amount of accumulation of ubiquitinated products. IK cells under cotreatment with tiron and bortezomib, however, showed amounts of ubiquitinated products that were identical to the normal levels of untreated cells (Fig. 3b). This inhibition of accumulation of ubiquitinated proteins was not observed with cotreatment with tiron or any of the other three proteasome inhibitors. Similarly, edaravone inhibited the accumulation of ubiquitinated proteins caused by MG-132 or ALLN, without any effects on ubiquitin accumulation caused by bortezomib or epoxomicin (Fig. 3b).

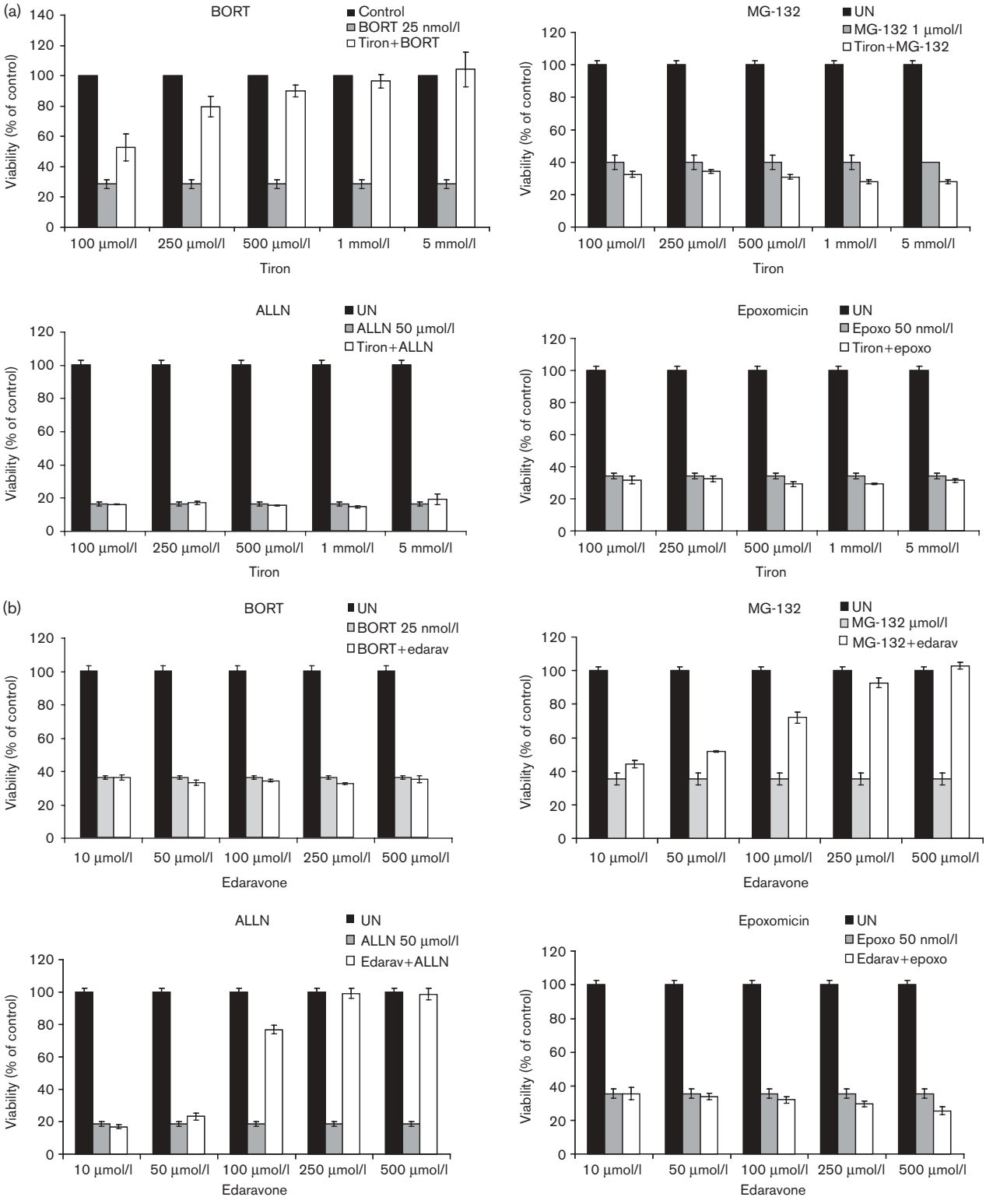
To determine whether these effects were specific for a precise tumour cell type, we extended the study to three additional endometrial carcinoma cell lines (RL-95/2, HEC-1-A and KLE) and one melanoma cell line (M36). As shown in Figs 3c and d, bortezomib was inhibited by tiron. In contrast, this action was performed on MG-132 and ALLN inhibition by edaravone, both in carcinoma and in melanoma cell lines.

Tiron and edaravone blocked proteasome-activity inhibition caused by bortezomib and aldehyde-based proteasome inhibitors

To ascertain whether the ubiquitin accumulation was the result of proteasome inhibition, we assayed the proteasome activity by incubation of cell lysates with the fluorogenic substrate, Suc-Leu-Leu-Val-Tyr-AMC. Such a substrate is used to measure the chymotrypsin-like peptidase activity of the 20S proteasome. Treatment of IK cells with each of the four proteasome inhibitors resulted in a significant reduction of proteasome activity, by this assay. Bortezomib and epoxomicin were more efficient than MG-132 and ALLN in inhibiting the proteasome (Fig. 4a).

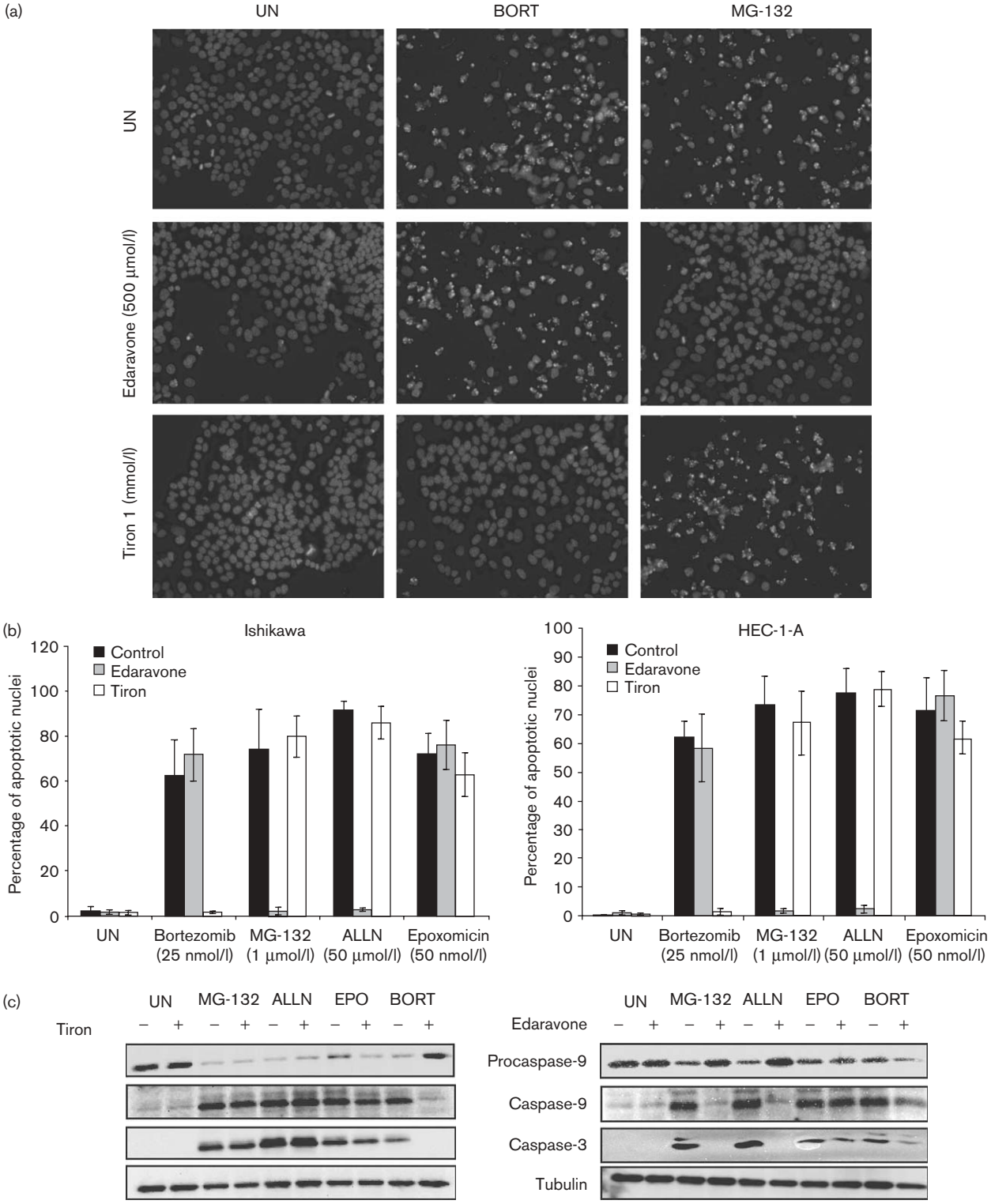
To assess the effects of antioxidants on proteasome-activity inhibition, we assayed proteasome activity in cell lysates obtained from cultures cotreated with either tiron or edaravone and with each of the proteasome inhibitors

Fig. 1



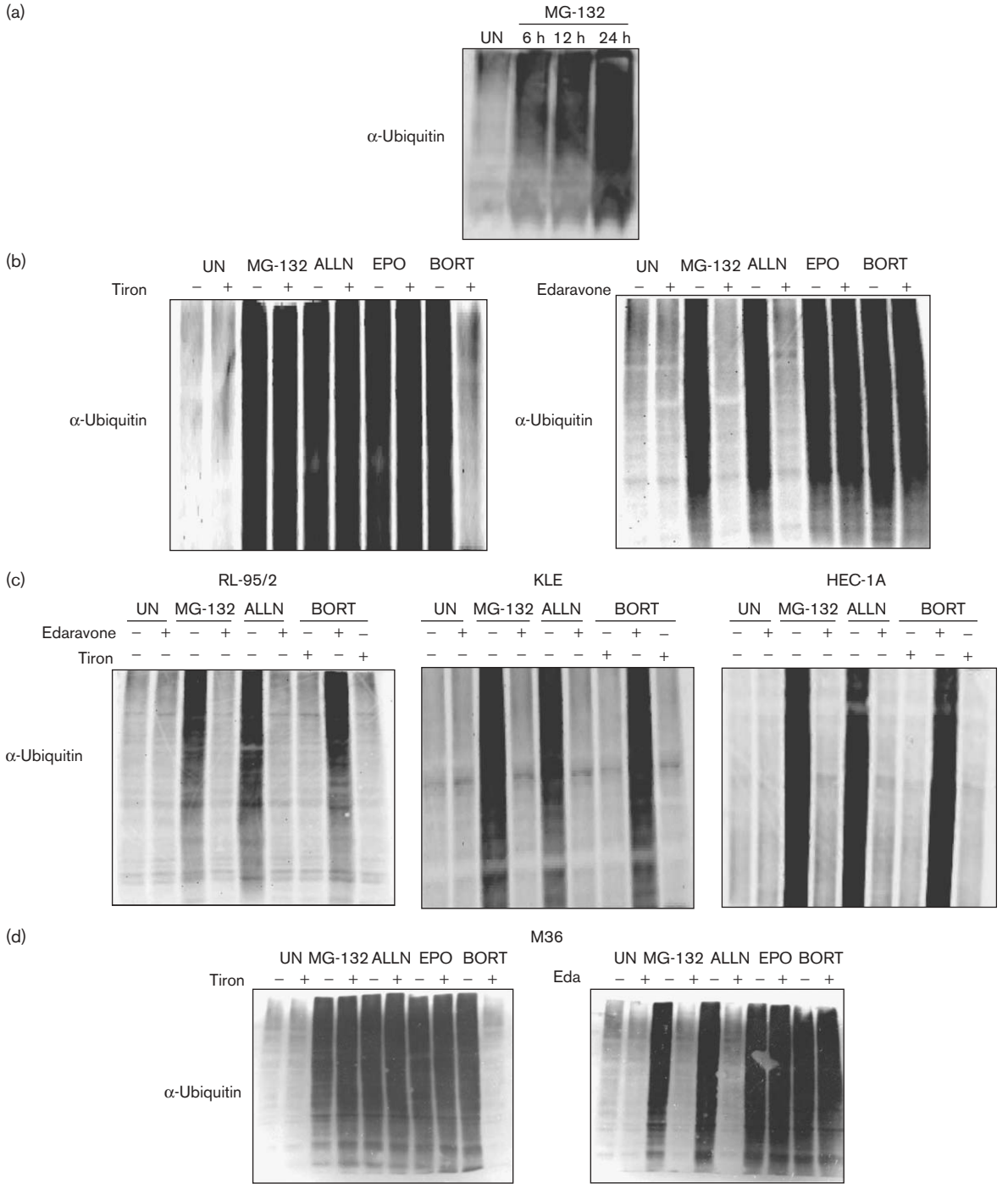
Differential effects of tiron and edaravone on reduction of cell viability caused by structurally different proteasome inhibitors. (a) IK cells were treated with 25 nmol/l bortezomib (top left), 1 µmol/l MG-132 (top right), 50 µmol/l ALLN (bottom left) or 50 nmol/l epoxomicin (bottom right), alone or in combination with increasing doses of tiron. Cell viability was assessed by MTT assay 24 h after treatment. Results are expressed as percentage of viability. (b) IK cells were treated with 25 nmol/l bortezomib (top left), 1 µmol/l MG-132 (top right), 50 µmol/l ALLN (bottom left) or 50 nmol/l epoxomicin (bottom right), alone or in combination with increasing doses of edaravone. Cell viability was assessed by MTT assay 24 h after treatment. Results are expressed as percentage of viability. BORT, bortezomib; IK, ishikawa; UN, untreated.

Fig. 2



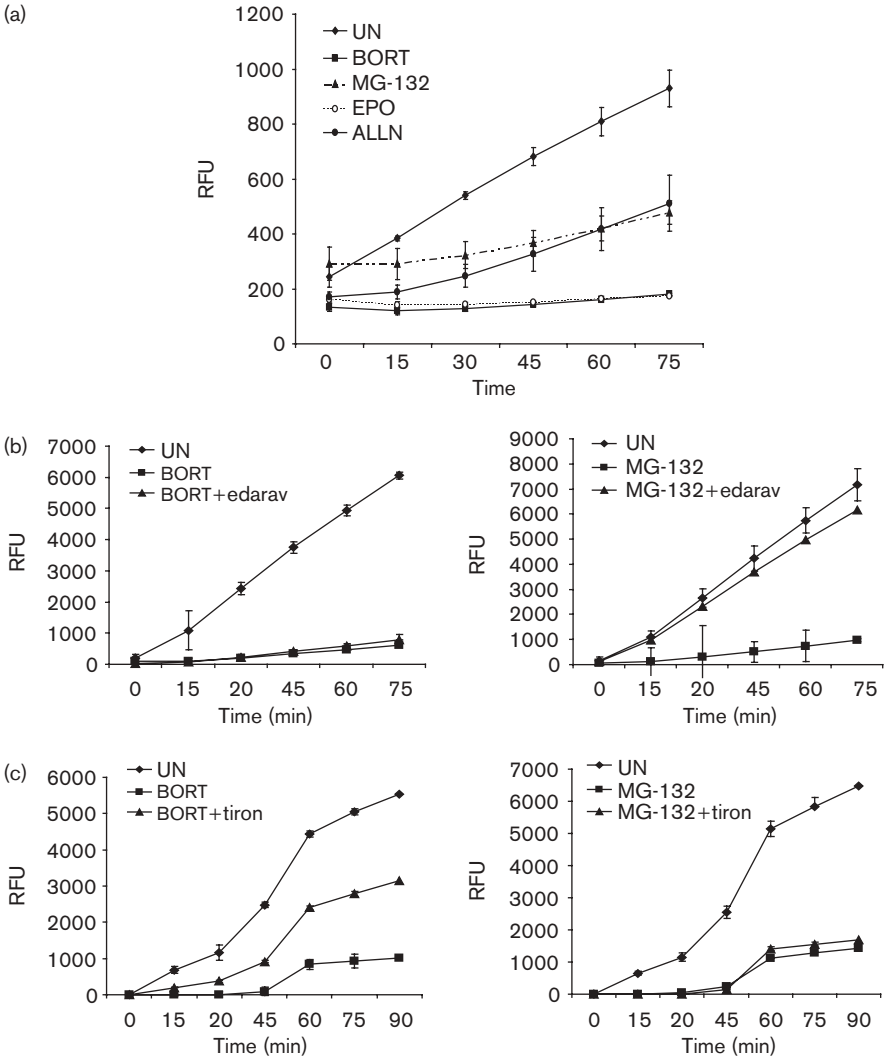
Tiron and edaravone block apoptotic nuclear morphology and caspase activation observed after treatment with proteasome inhibitors. (a) Representative micrographs showing Hoechst staining of IK cells treated for 24 h with either bortezomib 25 nmol/l or MG-132 1 μmol/l, alone or together with edaravone 500 μmol/l or tiron 1 mmol/l. (b) Quantification of IK (left) or HEC-1-A (right) nuclei displaying apoptotic morphology. IK or HEC-1-A cells were treated with the indicated proteasome inhibitors and doses, alone or in combination with edaravone or tiron. Results are expressed as percentage of apoptotic nuclei over control condition. (c) IK cells were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l of tiron (left panel) or 500 μmol/l edaravone (right panel). After 24 h, cells were harvested in lysis buffer. Cell lysates were analysed by Western blot with antibodies to caspase-9, active caspase-3. Membranes were reprobed with antibodies to tubulin to ensure equal protein amounts. IK, ishikawa.

Fig. 3



Tiron and edaravone block ubiquitin accumulation induced by bortezomib-based and aldehyde-based proteasome inhibitors, respectively. (a) IK cells were treated for 6, 12 or 24 h with MG-132 and lysed. Protein lysates were analysed by Western blot with antiubiquitin antibodies. (b) IK cells were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l tiron (left panel) or 500 μmol/l edaravone (right panel). After 24 h, cells were harvested in lysis buffer. Cells lysates were analysed by Western blot with antibodies to ubiquitin. (c) RL-95/2, KLE and HEC-1A endometrial carcinoma cell lines were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l tiron or 500 μmol/l edaravone. After 24 h, cells were harvested in lysis buffer. Cells lysates were analysed by Western blot with antibodies to ubiquitin. (d) M36 melanoma cell lines were treated with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lanes (+), proteasome inhibitors were incubated with 1 mmol/l tiron (left panel) or 500 μmol/l edaravone (right panel). After 24 h, cells were harvested in lysis buffer. Cell lysates were analysed by Western blot with antibodies to ubiquitin. IK, ishikawa.

Fig. 4



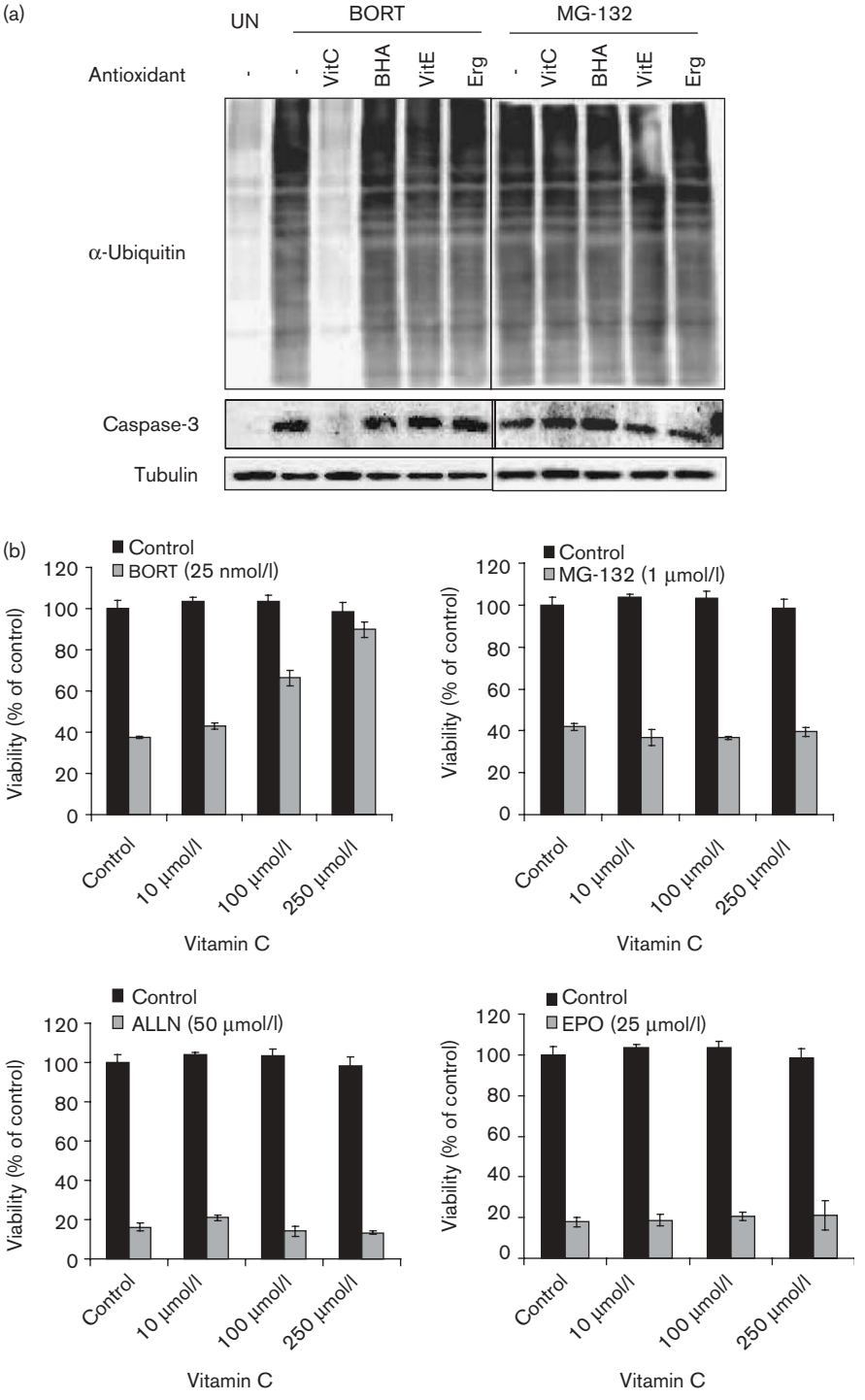
Effects of edaravone and tiron on activity of proteasome inhibitors on endometrial carcinoma cells. (a) Proteasome inhibitors reduce proteasome activity. IK cells were treated for 24 h with 1 μmol/l MG-132 (MG), 50 μmol/l ALLN, 50 nmol/l epoxomicin (EPO), 25 nmol/l bortezomib (BORT) or were left untreated (UN). Cell lysates were incubated in the presence of the proteasome substrate as described in Materials and methods. Results are expressed as relative fluorescence units (RFUs) over time of incubation with the substrate. (b) Edaravone inhibits MG-132 blockade of proteasome activity. IK cells were treated for 24 h with 1 μmol/l MG-132 (right graph) or 25 nmol/l bortezomib (BORT) (left graph), alone or in the presence of edaravone. Cell lysates were incubated in the presence of the proteasome substrate, as described in Materials and methods. Results are expressed as RFUs. (c) IK cells were treated for 24 h with 1 μmol/l MG-132 (right graph) or 25 nmol/l bortezomib (BORT) (left graph) alone or in the presence of tiron. Cell lysates were incubated in the presence of the proteasome substrate, as described in Materials and methods. Results are expressed as RFUs. IK, ishikawa.

included in this study. Treatment of IK cell with MG-132 in addition to edaravone returned the activity of the proteasome to normal levels, indicating that edaravone blocks the proteasome inhibitory function of aldehyde-based proteasome inihibitors (Fig. 4b). In contrast, edaravone had no effects on inhibition caused by bortezomib. Tiron also insignificantly reduced the blockade of the proteasome caused by the bortezomib treatment of cells, but not that caused by the MG-132 treatment of cells (Fig. 4c).

Effects of other antioxidants on cell killing caused proteasome inhibition on endometrial carcinoma

To generalize the effects of antioxidants on proteasome inhibitors, we extended our study to other antioxidants. IK cells were treated with bortezomib or MG-132 in the presence or absence of the following antioxidants: vitamin C, vitamin E, butylated hydroxyanisole or ergothioneine. Cell lysates were collected 24 h later, and protein extracts were analysed by Western blot for ubiquitin accumulation

Fig. 5



Effects of antioxidants on the viability and activity of proteasome inhibitors. (a) IK cells were treated with 1 μmol/l MG-132 or 25 nmol/l bortezomib (BORT) or were left untreated (UN). In the indicated lines, proteasome inhibitors were coincubated with the antioxidant vitamin C (VitC), butylated hydroxyanisole (BHA), vitamin E (VitE) or ergothioneine (Erg). After 24 h, cells were harvested in lysis buffer. Cells lysates were analysed by Western blot with antibodies to ubiquitin. (b) IK cells were treated with 25 nmol/l bortezomib (top left), 1 μmol/l MG-132 (top right), 50 μmol/l ALLN (bottom left) or 50 nmol/l epoxomicin (bottom right), alone or in combination with increasing doses of vitamin C. Cell viability was assessed by MTT assay 24 h after treatment. Results are expressed as percentage of viability. IK, ishikawa.

or caspase-3 activation. Out of all the combinations, we found that vitamin C inhibited caspase 3 activation in bortezomib-treated cells, but not in MG-132-treated ones. Such inhibition correlated with the inhibition of ubiquitin accumulation (Fig. 5a). These results further support the hypothesis that all the antioxidants that were found to prevent proteasome-induced cell death also blocked the ability of the proteasome inhibitor to block the proteasome. Results obtained with vitamin C on cell viability were confirmed by the MTT analysis of IK cells treated with bortezomib, MG-132, ALLN or epoxomicin (Fig. 5b).

Discussion

We have previously demonstrated that proteasome inhibitors are effective tools for inducing apoptosis of endometrial carcinoma cell lines and primary explants [13]. Here, we have demonstrated that different antioxidants can block the cytotoxicity induced by different proteasome inhibitors on endometrial carcinoma cells. Although tiron and vitamin C inhibit the cell death induced by the boronic acid-containing bortezomib, edaravone inhibits cell killing caused by the aldehyde-based proteasome inhibitors, MG-132 or ALLN. In contrast, edaravone does not have any effect on cell death triggered by bortezomib, but completely blocks cell death caused by treatment with MG-132 or ALLN. In addition, we have found no effects of any of the antioxidant substances that have been tested on epoxomicin-induced cell death. We have also seen that the antiapoptotic process is not caused by the modification of pathways downstream of the proteasome, but by interfering with the ability of the proteasome inhibitor to block the proteasome.

Recent findings have suggested that bortezomib and other proteasome inhibitors can increase the production of either endoplasmic reticulum stress or ROS, which is ultimately responsible for proteasome-inhibitor cytotoxicity [15,16,20]. For that reason, several works have approached the effects of antioxidants and radical scavengers in the process of apoptosis induced by proteasome inhibitors. Some studies have demonstrated that ROS scavengers such as glutathione (GSH)-reduced ethyl ester or *N*-acetylcysteine (NAC) block the features of bortezomib-induced apoptosis in mantle-cell lymphoma [17], whereas others have demonstrated that NAC or GSH do not affect apoptosis induced by bortezomib [18,19,21]. One of these free radical scavengers is tiron. Tiron has a potent antioxidant activity because of its ability to scavenge O_2^- radicals. Tiron has been shown to inhibit cell death induced by bortezomib in head and neck squamous cell carcinoma [16] and in pancreatic cancer cells [22]. Recent findings have suggested that ROS scavengers such as tiron can inhibit proteasome-induced cell death, whereas others like butylated hydroxyanisole do not. It has also been shown that antioxidants such as tiron or vitamin C can selectively interfere with and inhibit the function of bortezomib as a proteasome inhibitor. As a result, tiron or vitamin C can inhibit the cytotoxic action of bortezomib [18,19,21]. Both vitamin C and tiron have been shown to abrogate bortezomib activity via direct interaction and to form a biologically inactive complex with bortezomib [19]. In agreement with these reports, we have found that tiron and vitamin C inhibited bortezomib action in endometrial carcinoma cells, but have no effects on other proteasome inhibitors like the aldheyde-based MG-132 or ALLN, or the epoxyketone, epoxomicin. In addition to these data, we have evaluated the effects of edaravone on cytotoxicity induced by proteasome inhibitors. Edavarone is a free radical scavenger that primarily scavenges hydroxyl and peroxyl radicals and has the antioxidant ability to inhibit lipid peroxidation Edaravone has been proved to improve the clinical outcome of patients after acute myocardial infarction [23,24] and acute cerebrovascular injury [25,26]. Moreover, edaravone has been demonstrated to exhibit antitumourogenic activity [27,28]. Interestingly, our results demonstrate that although edaravone inhibits MG-132-induced or ALLN-induced cell death, it does not inhibit cell death caused by bortezomib or epoxomicin. Edaravone completely abolishes cytotoxicity, and decreases the number of apoptotic nuclei and the levels of caspase activation. We found, however, that edaravone inhibited the accumulation of ubiquitinated products, suggesting that it could block the proteasome-inhibiting action of MG-132. This possibility was further analysed by a proteasome-activity assay. In agreement with the results obtained with ubiquitin accumulation, we demonstrated that edaravone inhibited the action of MG-132. In summary, some antioxidants or ROS scavengers, such as tiron or vitamin C, might block the action of boronic acid-based proteasome inhibitors, such as bortezomib. In contrast, other antioxidants, such as edaravone, might block aldehyde-based proteasome inhibitors, without any effects on other types of proteasome inhibitors. Therefore, a careful study of the activity of the proteasome should be made when using antioxidants or free radical scavengers to block proteasome-inhibitor cytotoxicity.

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