Chemical Blockage of the Proteasome Inhibitory Function of Bortezomib

IMPROT ON TUMOR CELL DEATH

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The proteasome inhibitor bortezomib is emerging as a potent anti-cancer agent. Still, recent clinical trials have revealed a significant secondary toxicity of bortezomib. Consequently, there is much interest in dissecting the mechanism of action of this compound to rationally improve its therapeutic index. The cytotoxic effect of bortezomib is frequently characterized by interfering with downstream events derived from the accumulation of proteasomal targets. Here we identify the first chemical agent able to act upstream of the proteasome to prevent cell killing by bortezomib. Specifically, we show that the polyhydroxyl compound Tiron can function as a competitive inhibitor of bortezomib. This effect of Tiron was surprising, since it is a classical radical spin trap and was expected to scavenge reactive oxygen species produced as a consequence of bortezomib action. The inhibitory effect of Tiron against bortezomib was selective, since it was not shared by other antioxidants, such as vitamin E, MnTBAP, l-N-acetyl-cysteine, and FK-506. Comparative analyses with nonboronated proteasome inhibitors (i.e. MG132) revealed a specificity of Tiron for bortezomib. We exploited this novel feature of Tiron to define the “point of no return” of proteasome inhibition in melanoma cells and to block cell death in a three-dimensional model of human skin. Cells from T-cell lymphoma, breast carcinoma, and non-small cell lung cancer were also responsive to Tiron, suggesting a broad impact of this agent as a bortezomib blocker. These results may have important implications for the analysis of bortezomib in vivo and for the design of drug mixtures containing proteasome inhibitors.

The 26 S proteasome is a multicatalytic protease responsible for most nonlysosomal intracellular protein degradation (1–3). Interestingly, multiple pro-oncogenic factors are controlled by the proteasome. Those include transcriptional factors, cyclins, cyclin-dependent kinase inhibitors, cytokines, and a series of pro- and antiapoptotic factors (including Bim or Mcl-1, respectively) that can function as rheostats of cell fate (4). Therefore, inhibition of the proteasome is emerging as a potent cellular tool to selectively kill tumor cells by exploiting their altered genetic background (5, 6). Experimental and clinical support for this therapeutic strategy has been provided by bortezomib (Velcade™, formerly known as PS-341) (7).

Bortezomib is a cell-permeable dipeptide boronic acid that can reversibly inhibit the chymotryptic-like proteolytic activity of the β5 subunit of the proteasome (8, 9). A salient pharmacological feature of bortezomib is its ability to bypass classical multidrug resistance mechanisms (9, 10). For example, we have recently shown that cell lines isolated from early, intermediate, and advanced melanoma (a paradigm of chemoresistant tumor types) can be efficiently killed by bortezomib in conditions that maintain the viability of their normal cellular counterparts (11). Similar antineoplastic activity has been reported in a variety of solid and hematological cancers (6, 12, 13). Based on these results, multiple randomized clinical trials are currently under way. In particular, a high response rate has been reported in multiple myeloma, where the United States Food and Drug Administration granted approval for the use of bortezomib in patients with relapsed and refractory tumors (14).

Despite the efficacy of bortezomib in hematological malignancies, clinical trials and experimental mouse models suggest that solid tumors are likely to require additional combination therapies for long term responses (11, 12, 15, 16). The secondary toxicity of bortezomib poses also an important clinical limitation (e.g. grade 3 thrombocytopenia and peripheral neuropathy can affect 10–30% of patients). Although these adverse effects can be managed with supportive measures, they compromise dose and treatment schedules (17). Therefore, there is a large interest in defining the mode of action of bortezomib to rationally design therapeutic strategies that can improve its anti-cancer effect.

The identification of the key effectors of the cytotoxic effect of bortezomib as well as cellular or chemical factors able to oppose or inhibit its action has been complicated by the fact that the proteasome controls the half-life of the vast majority (up to 80%) of cellular proteins (5, 7). Most frequently, the impact of bortezomib in normal or tumor cells is determined by interfering with events suspected to act downstream of proteasome inhibition. Those range from stress kinases to survival signals or apoptotic factors (18). In addition, the dysregulation of mitochondrial functions and release of ROS3 are also common processes induced by bortezomib (5, 7). Thus, as early as 3–4 h after bortezomib treatment, tumor cells from non-small cell lung cancer or squamous carcinoma dysregulate the mitochondrial membrane potential (ΔΨm).

3 The abbreviations and trivial names used are: ROS, reactive oxygen species; AMC, 7-amido-4-methylcoumarin; Bz-423, 1,4-benzodiazepine; DCF, 2',7'-dichlorofluorescein; DIOC(3), 3',3'-dihexyloxacarbocyanine iodide; FK-506, tacrolimus; L-N-acetyl-cysteine; MnTBAP, manganeses(II)meso-tetrakis(4-benzoic acid) porphyrin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide; Tiron, 4,5-dihydroxy-1,3-benzene disulfonic acid; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Vit E, vitamin E; GST, glutathione S-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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Inhibition of the Antitumoral Activity of Bortezomib

and accumulate a significant amount ROS, subsequently leading to caspase activation (19, 20).

The response of melanoma cells to bortezomib is intriguing. We and others have recently shown that bortezomib is able to bypass the traditional resistance of these tumor cells to standard chemotherapeutic agents, in part by an effective induction of the Bcl-2 homology domain 3-only proapoptotic protein NOXA (11, 21). Despite a fast activation of NOXA (detectable by 6 h post-treatment), the processing of the apoptotic effectors, the caspas, was relatively slow (11). Thus, it was possible that, compared with other cell types, in melanoma cells bortezomib was not as effective an inducer of events such as ROS production that can cooperate with caspase activation.

To establish the contribution of ROS to bortezomib-induced melanoma cell death, we performed a comparative kinetic analysis of $\Delta \psi_{m}$ and ROS levels in normal melanocytes and melanoma cells. In addition, to prove the requirement of ROS for the cytotoxic effect of bortezomib, cells were treated with the antioxidants manganese(III)meso-tetakis(4-benzoic acid) porphyrin (MnTBAP), vitamin E (Vit E), and L-N-acetylcysteine (L-NAC) or the inhibitor of superoxide anion (O$_2^-$) production tauracilium (FK-506). The polyhydroxylyl compound disodium 4,5-dihydroxybenzene-1,3-disulfonate (Tiron) was also included in this study as an example of a potent antioxidant (22, 23) that has been recently used to validate a critical role of ROS as an early mediator of cell death induced by bortezomib (19, 20).

Surprisingly, of all the ROS scavengers tested, only Tiron blocked bortezomib-induced cell death (in a selective manner and in multiple cell types). This protective effect was specific for bortezomib, since Tiron had no impact on cell death and accumulation of ubiquitinated proteins by MG132, a different class of proteasome inhibitor. The unexpected mode of action of Tiron was exploited to address mechanistic aspects of the antineoplastic activity of bortezomib and to reduce its toxicity toward normal cells. In addition, we developed an artificial skin reconstruction model to analyze the selectivity of bortezomib for tumor cells and the protective effect of Tiron in a system that recapitulates the architecture of the human skin in vivo.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Bortezomib was obtained from Millennium Pharmaceuticals Inc. (Cambridge, MA); MG132 was from Calbiochem (EMD Biosciences, San Diego, CA); Tiron, L-NAC, and Vit E were obtained from Sigma; FK-506 was from Fujisawa Pharmaceutical (Osaka, Japan), and MnTBAP was from Alexis Corp. (San Diego, CA). 2′,7′-Dichlorodihydrofluorescein diacetate, 3,3′-dihexyloxycarbocya- nine iodide (DiOC$_6$ (3)), and carbonyl cyanide $m$-chlorophenylhydrazone were from Molecular Probes, Inc. (Eugene, OR). 1,4-benzodiazepine (Bz-423) was synthesized as previously described (24). Antibodies against Mcl-1 (S-19), CHOP (GADD 153 (R-20)) and ubiquitin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-p53 was from Novocastra Laboratories (Newcastle, UK); anti-Smac and anti-tubulin and $\beta$-actin (clone AC-74) were from Sigma.

**Cell Culture**—Normal human melanocytes, fibroblasts, and keratinocytes were isolated from foreskins essentially as previously described (25). Melanocytes and keratinocytes were maintained in 154 media with the corresponding melanocyte or keratinocyte growth supplements (Clonetics). Fibroblasts were grown in Dulbecco’s modified Eagle’s medium. Jurkat (human acute leukemia cell line), MDA-MB-231 (human breast carcinoma cells) and H460 (human non-small cell lung cancer cells) were obtained from the American Type Culture Collection (Manassas, VA). Jurkat and H460 were grown in RPMI 1640 (Invitrogen), MDA-MB-231 and the metastatic melanoma cell lines SK-Mel-19 and SK-Mel-103 were grown in Dulbecco’s modified Eagle’s medium (Invitrogen). Medium for fibroblasts and tumor cells was supplemented with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen).

**Cell Viability**—The percentage of dead cells at the indicated times and drug concentrations was estimated by standard trypan blue exclusion or by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based assays as described elsewhere (26). For simplicity, control samples, which were incubated only with solvent (0.05% Me$_2$SO), are indicated as nontreated.

**Protein Immunoblotting**—To monitor changes in protein expression, 2 × 10$^5$ cells were treated with the proteasome inhibitor bortezomib (50 nM) in the absence or presence of Tiron (1 mM) or L-NAC (10 mM). At the indicated time points, whole cell lysates were prepared by Laemmli extraction. 50 mg of protein were subjected to electrophoresis in 12 or 4–15% gradient SDS gels and subsequently transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). The corresponding antibody signal was visualized with the ECL detection system (Amershams Biosciences).

**Preparation of Mitochondrial Extracts**—To analyze the release of mitochondrial factors from the mitochondria, the indicated melanoma cells were treated with 50 nM bortezomib. Floating and adherent cells were pooled at different times post-treatment. Cytosolic and membrane-rich fractions were prepared by digitonin extraction as described in Ref. 11. Standard protein immunoblotting was used to visualize changes in the localization of Smac as an example of a mitochondrial proapoptotic factor released upon bortezomib treatment.

**Detection of Intracellular ROS**—Normal melanocytes or melanoma cells were seeded in 6-well plates at a density of 2 × 10$^4$ cells/well. 18 h after plating, cells were treated with 50 nM bortezomib, and 30 min before the indicated collection times, they were loaded with 100 $\mu$m 2′,7′-dichlorodihydrofluorescein diacetate. Cell pellets were washed twice in phosphate-buffered saline, and the fluorescence of the oxidized product, 2′,7′-dichlorofluorescein (DCF), was measured by a FACSCalibur flow cytometer as described elsewhere (27). Cells treated with 20 $\mu$m Bz-423 were used as positive controls for ROS release.

**Mitochondrial Membrane Potential ($\Delta \psi_{m}$)**—Normal melanocytes or melanoma cells (2 × 10$^4$) were treated with 50 nM bortezomib for 3, 6, 9, 12, 18, and 24 h. 30 min before collection, cells were labeled with 20 nM DiOC$_6$ (3). DiOC$_6$ (3)-dependent fluorescence was monitored on a FACSCalibur flow cytometer as previously described, using cells treated with carbonyl cyanide $m$-chlorophenylhydrazone (1 mM) as a reference control for disruption of $\Delta \psi_{m}$ (27).

**Proteasome Inhibition Assays**—To analyze the impact of Tiron on the ability of bortezomib to block the proteasome in intact cells, the chymotryptic activity of the proteasome was estimated essentially as previously described (28). Briefly, 3 × 10$^6$ melanoma cells were incubated for various periods of time in the presence or absence of 50 nM bortezomib or 1 mM Tiron (as single agents or in combination). Floating and adherent cells were collected by trypsinization and washed twice in phosphate-buffered saline. Cell pellets were extracted by incubation with 100 $\mu$l of lysis buffer (50 mM HEPES, 5 mM CHAPS, 0.5 mM EDTA, 0.035% SDS, pH 7.5) for 1 h on ice. Samples were then centrifuged at 14,000 rpm for 10 min, and supernatants were isolated. Protein (40 $\mu$g) was added to 90 $\mu$l of lysis buffer, plates were warmed for 10 min at 37°C, and 10 $\mu$l of the succinyl-Leu-Leu-Val-Tyr-AMC substrate (BACHEM, King of Prussia, PA) was added to a final concentration of 150 $\mu$m. The resultant fluorescence of the liberated 7-amido-4-methylcoumarin (AMC) dye was measured by a FACSCalibur flow cytometer.
was then measured on a Cytofluor® multiwell plate reader series 4000 (Applied Biosystems, Framingham, MA) set at an excitation wavelength of 380 nm and emission of 460 nm.

Mechanistic Analyses of the Inhibitory Effect of Tiron on Bortezomib—Proteasome-enriched fractions were prepared by affinity purification using the human proteasome isolation kit from Calbiochem (EMD Biosciences, San Diego, CA). Essentially, 10⁷ cells were lysed in Buffer A (50 mM HEPES, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 2 mM ATP). 500 µg (at a concentration of 1 mg/ml) were incubated at 4°C (2 h) with 100 µl of GST-control agarose beads or with beads conjugated to GST fused to the ubiquitin-like domain of HHR23-B (UbLHRB), which has a high affinity for the proteasome (29–31). Samples were centrifuged for 1 min at 14,000 rpm. The supernatant (herein referred to as GST-eluate) was stored to determine the remaining activity in proteasome-depleted fractions. The affinity matrix was washed twice in Buffer A and finally resuspended in 100 µl of reaction buffer (25 mM HEPES, 500 µM EDTA, pH 7.6). Increasing volumes of the GST-control or GST-UbLHRB (and of the eluates) were incubated with 10 µM succinyl-Leu-Leu-Val-Tyr-AMC in a final volume of 100 µl. The intrinsic chymotryptic activity of the corresponding fractions was estimated by fluorimetry as indicated above. To determine the mode of action of Tiron (i.e. to differentiate between competitive and noncompetitive inhibition), the intensity of AMC fluorescence at each concentration of bortezomib (Fi) was used to estimate the percentage inhibition of the proteasome (40–100) of the indicated cells treated as in C. Data are presented as the fraction of cells with reduced DIOC₃(3)-dependent fluorescence. The inset corresponds to an example of DIOC₃(3) fluorescence intensity in line SK-Mel-103 treated for 6 h with bortezomib (blue line) or vehicle control (red line). Carboxyl cyanide m-chlorophenylhydrazone (CCCP) (1 mM; black line) was included as a positive control for ΔΨₘ disruption. DMSO, Me₂SO.

Cell Cycle Analyses—2 × 10⁶ normal melanocytes were treated with vehicle control (0.05% Me₂SO), bortezomib (50 nM), Tiron (1 mM), or the combination of bortezomib plus Tiron (50 nM and 1 mM, respectively). 24 h post-treatment, cell cycle analyses were conducted after a 2-h incubation of cells in labeling solution (50 µg/ml propidium iodide (Sigma), in phosphate-buffered saline containing 0.2% Triton and 10 µM succinyl-Leu-Leu-Val-Tyr-AMC in a final volume of 100 µl. The intrinsic chymotryptic activity of the corresponding fractions was estimated by fluorimetry as indicated above. To determine the mode of action of Tiron (i.e. to differentiate between competitive and noncompetitive inhibition), the intensity of AMC fluorescence at each concentration of bortezomib (Fi) was used to estimate the percentage inhibition of the proteasome (40–100) of the indicated cells treated as in C. Data are presented as the fraction of cells with reduced DIOC₃(3)-dependent fluorescence. The inset corresponds to an example of DIOC₃(3) fluorescence intensity in line SK-Mel-103 treated for 6 h with bortezomib (blue line) or vehicle control (red line). Carboxyl cyanide m-chlorophenylhydrazone (CCCP) (1 mM; black line) was included as a positive control for ΔΨₘ disruption. DMSO, Me₂SO.

FIGURE 1. Kinetic analysis of ROS production by bortezomib. A and B, early events induced by bortezomib (Bor; 50 nM) in melanoma cells. A, induction of NOXA in whole cell extracts of the indicated melanoma lines estimated by protein immunoblotting. B, release of Smac/Diablo from the mitochondria. Shown are protein immunoblots corresponding to mitochondrial (SmacMito) and cytosolic-enriched (SmacCyto) fractions. Actin is shown as a loading control. C, ROS production in a pool of normal melanocytes (as) and the melanoma cell lines SK-Mel-19 (●) and SK-Mel-103 (■) upon treatment with bortezomib (50 nM). ROS was estimated by flow cytometry as an increase in fluorescence of the oxidized product DCF. The inset represents the fluorescence signal of line SK-Mel-103 treated with bortezomib (blue line) or vehicle control (red line). Fluorescence distributions are also shown for the benzodiazepine Bz-423 (20 µM) (black line), a positive control for ROS generation (27). All analyses were performed in triplicate, and the results are expressed as percentage of cells with increased DCF fluorescence ± S.E. D, changes in the ΔΨₘ of the indicated cells treated as in C. Data are presented as the fraction of cells with reduced DIOC₃(3)-dependent fluorescence. The inset corresponds to an example of DIOC₃(3) fluorescence intensity in line SK-Mel-103 treated for 6 h with bortezomib (blue line) or vehicle control (red line). Carboxyl cyanide m-chlorophenylhydrazone (CCCP) (1 mM; black line) was included as a positive control for ΔΨₘ disruption. DMSO, Me₂SO.

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Propidium iodide fluorescence was measured using a FACSCalibur flow cytometer. Cell cycle distribution was determined with Cell Quest software (BD Biosciences).

Artificial Human Skin—Human skin equivalents were generated as previously reported (32) by reconstituting epidermal and dermal equivalent using freshly isolated preparations of human melanocytes, keratinocytes, and fibroblasts. Transformed fibroblasts were generated by retrovirus-mediated transduction of the oncogene H-RasV12 and the adenoviral protein E1A essentially as previously described (33). For the generation of dermal equivalents, an acellular layer of 1.35 mg/ml type I collagen (Collaborative Biomedical, Bedford, MA), in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum was deposited onto tissue culture inserts (Transwell, Costar, Cambridge, MA) in 6-well plates. This layer was subsequently covered with 3 ml of fibroblast mix (containing normal and transformed fibroblasts at a ratio of 10:1) in collagen (1.125 × 10^5/ml) and left submerged for 5 days in 10% Dulbecco’s modified Eagle’s medium. Neonatal foreskin keratinocytes and melanocytes were subsequently seeded onto the concave center of the dermal reconstructs at a ratio of 1:15 (with a total of 1.5 × 10^5 cells/reconstruct). Cell stratification was achieved as previously described. After 10–14 days of air exposure, skin reconstructs were processed for treatment and analysis of cell death. In brief, skin reconstructs were treated for 48 h with 50 nM bortezomib in the presence or absence of 1 mM Tiron. Tissue specimens were harvested and processed for standard cryosection and visualization of the skin architecture by hematoxylin and eosin. Cell death in tissue sections was determined with an APO-BrdU TUNEL assay kit (Molecular Probes, Inc., Eugene, OR).

RESULTS

Induction of ROS by Bortezomib in Melanoma Cells—We have previously determined that nanomolar concentrations of bortezomib (10–100 nM) can efficiently kill metastatic melanoma cells while maintaining the viability of normal melanocytes (11). Under those conditions, melanoma cell death is preceded by the induction of the proapoptotic protein NOXA (Fig. 1) and the subsequent release of apoptogenic factors from the mitochondria (Fig. 1B). To determine the extent of ROS production and its contribution to the cytotoxic effect of bortezomib, cells were treated with a 50 nM dose of this compound, and changes in the fluorescence of the redox-dependent dye 2′,7′-dichlorodihydrofluorescein diacetate (34) were determined as a function of time. Metastatic melanoma cells SK-Mel-19 and SK-Mel-103 were included as a reference for normal cells.

Consistent with their resistance to proteasome inhibition, normal melanocytes showed no induction of DCF-dependent fluorescence by µg/ml RNase A). Propidium iodide fluorescence was measured using a FACSCalibur flow cytometer. Cell cycle distribution was determined with Cell Quest software (BD Biosciences).

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Consistent with their resistance to proteasome inhibition, normal melanocytes showed no induction of DCF-dependent fluorescence by
bortezomib even 48 h after treatment (Fig. 1C). Melanoma cells were also relatively refractory to bortezomib-mediated ROS release (particularly SK-Mel-19; see Fig. 1C). No significant DCF fluorescence was detected prior to 6 h (i.e., preceding the accumulation of NOXA). In these tumor cells, ROS production was visualized 12–18 h post-bortezomib treatment (Fig. 1C). In contrast, the benzodiazepine Bz-423, a potent inhibitor of the mitochondrial F1F0-ATPase and thus a promoter of O2\textsuperscript{−} production (27, 35), was in fact able to completely shift the fluorescence of 2',7'-dichlorodihydrofluorescein diacetate in melanoma cells (see inset in Fig. 1C). Therefore, although melanoma cells have the capacity of responding to potent ROS inducers, they maintain low levels of ROS in response to bortezomib until late times after treatment.

To confirm the maintenance of the mitochondrial physiology at early time points after bortezomib treatment, the mitochondrial membrane potential (ΔΨ\textsubscript{m}) was analyzed at different intervals after drug addition. Reduction in the intensity of fluorescence of the potentiometric cationic dye DiOC\textsubscript{6}(3) was used as a surrogate for the dissipation of ΔΨ\textsubscript{m} (34). As shown in Fig. 1D, melanocytes were remarkably effective at maintaining ΔΨ\textsubscript{m}. In melanoma cells, there was membrane depolarization, but it occurred 12–18 h after treatment. Compare, for example, the limited changes in ΔΨ\textsubscript{m} of bortezomib-treated cells with the strong depolarization effect of the positive control carbonyl cyanide m-chlorophenylhydrazone (inset in Fig. 1D).

Differential Effects of l-NAC and Tiron on Bortezomib-induced Melanoma Cell Death—Although the production of ROS in SK-Mel-19 or -103 did not significantly precede the release of proapoptotic mitochondrial factors, it could still serve as a positive feedback loop to enhance the killing activity of bortezomib. If this were the case, ROS scavengers would be expected to reduce the cytotoxic effect of bortezomib. To test this hypothesis, cell killing was determined in the presence or absence of l-NAC or Tiron. l-NAC is a sulfhydryl donor and scavenger of hydroxyl radical (HO\textsuperscript{•}), superoxide (O2\textsuperscript{−}), and hydrogen peroxide (H2O2) (36) and has been shown to inhibit bortezomib-induced apoptosis in cells from multiple myeloma (37), Jurkat, myelomonocytic leukemia (38), and gastrointestinal adenocarcinoma (39). Tiron is also an O2\textsuperscript{−} scavenger (22, 23) reported to block apoptotic cell death by bortezomib in cells from non-small cell lung cancer (19) and squamous carcinoma (20).

l-NAC and Tiron were well tolerated in the melanoma lines studied up to concentrations of 10 and 2 mM, respectively (see example in Fig. 2A, c, k, g, and o). Interestingly, l-NAC did not affect the response of melanoma cells to bortezomib (Fig. 2A, d and l). However, l-NAC was active as an ROS scavenger, since it could block death induced by cisplatin (Fig. 2A, f and n), whose toxicity relies in part on the induction of ROS (40–42). In contrast to l-NAC, Tiron was unexpectedly effective against bortezomib-induced death (Fig. 2A, h and p). For example, Tiron reduced bortezomib-induced death of SK-Mel-103 from 80% to less than 5% at 36 h post-treatment (Fig. 2B). This protection was sustained, since no significant amount of dead cells was observed even 48 h after treatment (Fig. 2B, right). Similar sustained protection was observed for SK-Mel-19 (Fig. 2B, left).

No Inhibitory Effect of FK-506 and the Antioxidants Vitamin E or MnTBAP against the Cytotoxic Effect of Bortezomib—Given the disparity between Tiron and l-NAC, we decided to extend the ROS scavenger studies to additional compounds (FK-506, Vit E, and MnTBAP), with a broader spectrum of action. FK-506 has been described to block O2\textsuperscript{−} production by the ubiquinol-cytochrome c reductase component of the mitochondrial respiratory chain complex III (43). Vit E is a highly effective lipid peroxyl radical scavenger that prevents toxic effects of O2\textsuperscript{•−} (molecular oxygen), HO\textsuperscript{•}, and HO\textsuperscript{−} (hydroxyl anion) (44). MnTBAP is a classical O2\textsuperscript{−} dismutase mimetic (45, 46). Dose-response curves were performed to identify conditions where these compounds behaved as effective antioxidants by blocking maximal ROS production by Bz-423 (Fig. 1C, inset). As indicated by standard MTT-based assays, 1 μM FK-506, 100 μM Vit E, or 100 μM MnTBAP maintained melanoma cell viability (see example for line SK-Mel-103 in Fig. 3A). In these conditions, FK-506, Vit E, and MnTBAP conferred a nearly 80% protection against Bz-423 (Fig. 3B). In clear contrast, none of these compounds affected the kinetics or extent of the antitumor effect of bortezomib (Fig. 3C).

Tiron Counteracts the Ability of Bortezomib to Induce the Accumulation of Proteasome Targets—Since l-NAC, MnTBAP, or Vit E overlap with Tiron in their O2\textsuperscript{−} scavenger activity, it was likely that Tiron had another function in addition to its antioxidant properties. Therefore, we tested the possibility that instead of acting downstream of the proteasome by sequestering ROS, Tiron acted at an upstream step, by preventing the access of bortezomib to the proteasome. To this end, proteasome function was addressed by standard protein immunoblotting. As we previously reported (11), treatment of normal melanocytes and tumor melanoma cells with bortezomib leads to the increase in the intracellular amount of ubiquitinated proteins as well as classical proteasome targets such as Mcl-1, p53, and CHOP (Fig. 4A). Surprisingly, Tiron prevented the accumulation of these factors both in the case of normal melanocytes and melanoma cells (see Fig. 4A). Therefore, the
proteasome of bortezomib-treated cells was still functional in the presence of Tiron.

In contrast to bortezomib and as expected for a standard antioxidant, treatment of melanoma cells with l-NAC had no detectable impact on the extent and kinetics of accumulation of ubiquitinated proteins and proteasome targets by bortezomib (Fig. 4B). Moreover, consistent with Tiron acting upstream of the proteasome and l-NAC at a downstream step, Tiron was highly effective at blocking caspase processing by bortezomib, whereas l-NAC was not (Fig. 4C).

The Protective Effect of Tiron Is Specific to Bortezomib—To determine whether Tiron was acting directly at the level of the proteasome (e.g. by providing steric hindrance to the catalytic subunits), melanoma cells were treated with MG132, a potent proteasome inhibitor but structurally unrelated to bortezomib (9). Interestingly, Tiron had no effect on the ability of MG132 to inhibit the proteasome and accumulate ubiquitinated proteins (Fig. 5, insets) or to kill melanoma cells (Fig. 5). These results illustrate the selectivity of Tiron for bortezomib and support a novel mechanism to counteract its proteasome-inhibitory function.

**Tiron Protects the Proteasome from the Inhibitory Effect of Bortezomib**—Altogether, the results presented above are compatible with a functional proteasome when Tiron is coadministered with bortezomib. To evaluate this possibility, the proteolytic status of the proteasome was analyzed by means of the release a fluorescent moiety (AMC) from a pentapeptide with a chymotryptic–like consensus sequence recognized by the proteasome (see "Experimental Procedures" and Ref. 28). In the clinic, bortezomib is used at concentrations leading to 70–80% inhibition of the proteasome (10). In cultured melanoma cells, this extent of inactivation is achieved 1–2 h after treatment (see Fig. 6, A and B). Simultaneous treatment with Tiron restored AMC-cleaving activity (i.e. proteasomal function). Thus, in the presence of Tiron, the proteasome remained active 8 h after the addition of bortezomib, and minimal changes in activity were observed at longer incubation times. 8–12 h after treatment with bortezomib and Tiron, the proteasome still retained 70% of its intrinsic activity (Fig. 6, A and B; results not shown). Therefore, Tiron is the first cell-permeable and nontoxic compound found to prevent the inhibition of the chymotryptic activity of the proteasome by bortezomib.

**Mode of Action of Tiron** —To determine whether the effect of Tiron on the activity of bortezomib was direct or it required an additional cellular component, enriched proteasomal fractions were prepared as previously reported (29–31). In short, agrose-GST beads conjugated to the ubiquitin-like moiety of HHR23B (namely UbL25) were used to concentrate the proteasome (29–31). The efficiency of the purification and the range of proteasome extracts providing a linear emission of AMC-dependent fluorescence is shown in Fig. 6, C and D. Thus, the chymotryptic activity of GST-UbL25-containing fractions was 8-fold higher than that corresponding to GST-controls (Fig. 6C). Bortezomib was able to inhibit 85% of the activity of GST-UbL25-bound proteasome (Fig. 6D). In these conditions, Tiron was also highly effective as a bortezomib inhibitor, allowing for dose-dependent recovery of the intrinsic proteolytic activity (Fig. 6D). Therefore, these results further support a direct interference of Tiron in the proteasome-inhibitory function of bortezomib.
We next determined the impact of Tiron on the $K_{\text{app}}$ or the maximal activity ($I_{\text{max}}$) of bortezomib (to define the mode of action of Tiron). As shown in Fig. 6C, increasing the concentration of Tiron from 0 to 50 nM primarily affected the IC$_{50}$ of bortezomib, which shifted from 10 to 80 nM. The apparent $I_{\text{max}}$ of bortezomib was not significantly altered by Tiron. Therefore, curves were fitted to a competitive model involving one binding site (Fig. 6E). Whereas the $I_{\text{max}}$ was maintained in the order of 90–95% at the different doses of Tiron tested, $K_{\text{app}}$ increased 8-fold (not shown). The same interpretation was achieved by the classical representation of Lineweaver-Burk double reciprocal plots. Thus, all curves converged on the y axis as expected for a direct competitive inhibition of Tiron on bortezomib (Fig. 6F).

The Protective Effect of Tiron Is Not Tumor Type-specific—A corollary of the data presented above is that the previously reported ROS-dependent effect of Tiron against bortezomib in cells such as H460 (non-small cell lung cancer) was not properly interpreted. In fact, we found that Tiron also prevented the bortezomib-dependent global accumulation of ubiquitinated proteins as well as direct proteasome targets (Fig. 7). Interestingly, similar protective effects of Tiron were observed with other malignant cell lines of different cellular origin. For example, Tiron blocked bortezomib-induced cell death and accumulation of proteasomal targets in T cell leukemia (Jurkat) or breast carcinoma (MDA-MB-231) (Fig. 7). Thus, Tiron represents a novel inhibitor of bortezomib with a broad efficacy in a variety of tumor cell types.
Exploiting the Inhibitory Features of Tiron to Define the Point of No Return in the Cytotoxic Effect of Bortezomib—The proteasome controls the half-life or the vast majority of the cellular proteins. Although bortezomib can effectively inhibit the proteasome within 1 h after treatment (Fig. 6, A and B), it is unclear at what point proteasome inhibition becomes irreversible and the cells commit to apoptosis. To address the “point of no return” in bortezomib-driven melanoma cell death, SK-Mel-103 melanoma cells were treated with 50 nM bortezomib, and Tiron (1 mM) was added either simultaneously (time \( t/11005 \) 0 h) or at progressively increased time points. Cell death was estimated by trypan blue exclusion at \( t/11005 \) 36 h. Interestingly, full protection by Tiron was achieved only within the first 6 h after bortezomib treatment (Fig. 8 A). The addition of Tiron at \( t/11005 \) 8 h significantly reduced the protective effect (dropping from 95 to 60%), which was nearly lost if bortezomib was left in the medium for 10 h (Fig. 8A). Importantly, the reduction in the protection by Tiron occurred once NOXA was accumulated in the melanoma cells (Fig. 8B) and before a significant release of ROS, \( \Delta \psi_m \) depolarization (Fig. 1, C and D), and the release of Smac from the mitochondria (Fig. 1B). In summary, the efficacy and specificity of Tiron for bortezomib offered an excellent experimental tool for a kinetic analysis of cell fate after proteasome inhibition.

Protection of Normal Cells against Secondary Effects of Bortezomib—As indicated before, a major complication of clinical trials of bortezomib is its secondary toxicity to multiple organs (47), including the skin (48). Therefore, a compound able to protect normal cells from undesired effects of the proteasome could be of potential interest for patient management. As a proof of principle, we first determined the impact of Tiron on bortezomib-treated normal melanocytes. In this

![FIGURE 7. Broad spectrum of action of bortezomib in tumor cells. Jurkat, MDA-MB-231, and H460 cell lines were treated with bortezomib (Bor; 50 nM) in the presence or absence of Tiron (T; 1 mM), alone or in combination, for the indicated times. Levels of Mcl-1 and ubiquitinated (Ub) proteins were visualized by standard immunoblotting. Tubulin is shown as a control for equivalent gel loading and transfer. All of these cell types were protected by Tiron. NT, no treatment.](image)

![FIGURE 8. Mechanistic analysis of bortezomib-induced toxicity in normal and tumor cells by exploiting the inhibitory effect of Tiron. A and B, to determine the "point of no return" of bortezomib-driven melanoma cell death, SK-Mel-103 melanoma cells were treated with 50 nM bortezomib, and Tiron (1 mM) was either omitted or added at the indicated times. Cells were collected 36 h after bortezomib treatment. A, protective effect of Tiron estimated by trypan blue exclusion. Note that whereas full protection can be provided if Tiron is added 4–6 h after bortezomib treatment, the protective effect is lost thereafter. B, impact of Tiron on the accumulation of NOXA as a function of time. Lanes 1 and 2 correspond to cells treated with vehicle control (NT) or bortezomib, respectively, in the absence of Tiron. Lanes 3–8 correspond to cells where Tiron was added at the indicated times post-treatment with bortezomib. C, protection of normal skin melanocytes. Cell cycle distribution determined by flow cytometry of propidium iodide-stained cells 24 h after treatment with vehicle control (NT), 50 nM bortezomib (Bor), or a combination of both agents. For each experimental condition the percentage of cells at the G0/G1, S, and G2/M phases of the cell cycle is shown. In the presence of Tiron, the fluorescent profiles of control and bortezomib-treated cells are indistinguishable.](image)
context, we have previously shown that melanocytes respond to bortezomib by halting proliferation at the G2/M phases of the cycle. To determine the protective effect of Tiron against these normal cells, freshly isolated melanocyte populations (passage 4) were treated with bortezomib and/or Tiron, and cell cycle distribution was determined by propidium iodide staining and flow cytometry. As shown in Fig. 8C, Tiron maintained the proliferative status of melanocytes in the presence of bortezomib. Thus, the amount of cells in G0/G1, S, and G2/M phases of bortezomib-treated melanoma cells coincubated with Tiron was nearly indistinguishable from vehicle controls (Fig. 8C).

Analysis of the Cytotoxic Effect of Bortezomib and the Protection by Tiron in a Three-dimensional Skin Model—It is well known that cell-cell and cell-matrix interactions affect the response of normal and tumor cells to chemotherapeutic agents (49). In fact, simplified cell monolayer cultures are not necessarily predictive of drug response in vivo (50). Therefore, the development of tractable experimental models where the potency of bortezomib could be modulated by Tiron could provide a novel venue for preclinical studies. To this end, we engineered artificial human skin to contain nests of oncogenically transformed cells in the dermal equivalent (as a model of early tumorigenic events in the skin; see Fig. 9, black arrows). The reconstructs were submerged in vehicle control or in 50 nM bortezomib for 48 h and subsequently processed for histological analysis of cell architecture (hematoxylin-eosin staining) and for detection of cell death by standard TUNEL analyses. In these conditions, bortezomib led to a massive collapse of the nests (see the arrow in Fig. 9b), accompanied by a positive TUNEL signal (Fig. 9f). Similar results were obtained with malignant melanoma cells (not shown). However, a secondary toxicity was also observed for normal fibroblasts and keratinocytes (see positive TUNEL staining in the dermis and epidermal equivalents, respectively, Fig. 9f, white arrows). Interestingly, adding Tiron at the time of bortezomib treatment (t = 0 h) completely blocked death of the transformed cells as well as preventing the secondary toxicity of bortezomib on normal fibroblasts and keratinocytes (Fig. 9c, g and h). The addition of Tiron at t = 10 h could no longer protect the transformed cells from bortezomib (Fig. 9, d and h). Normal cells, however, could sustain proteasome inhibition for longer periods of time (up to 18 h) prior to bortezomib addition (Fig. 9, g and h; results not shown). In summary, Tiron is the first example of a highly permeable and effective inhibitor of bortezomib that can allow for a tractable analysis of the point of no return of proteasome inhibition in normal and tumor cells in the context of three-dimensional organ models.

DISCUSSION

Here we report a novel strategy to specifically antagonize bortezomib by the polyhydroxyl compound Tiron. Instead of its previously reported function downstream of the proteasome (by acting as a ROS scavenger) (19, 20), Tiron was found to display an unexpected role at an upstream level. Specifically, we showed that Tiron is a competitive inhibitor of bortezomib, which maintains the proteasome in its active form (see model in Fig. 10).

Proteasome inhibitors have been widely used to address mechanistic analyses of protein stability and regulation in a variety of essential cellular processes. Classical aldehyde-based agents such as MG-132 or PSI have been instrumental in identifying key mediators of apoptosis, cell cycle progression, and response to oxidative stress in normal and tumor cells (51). More recently, the substitution of the highly reactive aldehyde groups by boronated moieties has lead to the generation of inhibitors with increased affinity for the catalytic subunits of the proteasome and reduced multidrug resistance sensitivity (9, 10). These features have also enhanced the selectivity toward tumor cells (9). Among this novel class of proteasome inhibitors, the dipeptidyl boronic acid bortezomib is emerging as a prototype of highly effective anti-cancer agents in vitro and in vivo.

Whereas considerable effort has been devoted to address the downstream effects of bortezomib (5, 7), it is remarkable that mechanisms of inactivation of this compound in living cells are poorly understood. Hepatic cytochrome P450 enzymes have been reported to inactivate bortezomib by deboronation and subsequent hydroxy-
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| Expected Mode of action (Standard antioxidants) | Tiron |
|-----------------------------------------------|-------|
| Indirect inhibition                            | Direct (competitive) inhibition |
| ![Diagram](image1.png)                        | ![Diagram](image2.png) |

**FIGURE 10. Model of action of Tiron.** Tiron was expected to act as a classical antioxidant by blocking ROS production and interfering with bortezomib downstream of the proteasome (left panel). However, here we showed that Tiron exerts its action directly by blocking the action of bortezomib (right panel). Therefore, no inhibition of the proteolytic activity of the proteasome (and the subsequent accumulation of ubiquitinated targets) is observed in cells coincubated with bortezomib and Tiron. As a result, Tiron-treated cells maintain their normal physiology, both in terms of cell cycle progression and cell survival. See “Discussion” for additional details.

However, interactions with other drugs currently used in combination strategies in clinical studies have not yet been reported. This is important, because long term responses in solid aggressive tumors, including melanoma, are likely to require additional targeting of cellular functions in addition to proteasome inhibition by bortezomib (11, 15, 16).

Our results are highly unanticipated, because Tiron has been described as a potent \( \text{O}_2^- \) scavenger, and therefore it has been frequently used to determine the implication of ROS in biological processes. In particular, Tiron has been reported to block ROS-driven apoptosis in experimental models of drug response in renal cells (53), colorectal carcinoma (54), pancreatic carcinoma (55), or prostate (56) cancer. In addition, Tiron, acting as a ROS scavenger, was shown to abolish the increased intracellular \( \text{O}_2^- \) production in experimental models of diabetes (57–59), as well as pathologies associated with oxidant stress and ischemia in vivo (60, 61). Moreover, Tiron was used as a ROS scavenger in bortezomib-treated tumor cells (19, 20). If Tiron had behaved simply as an antioxidant, we would have expected a normal accumulation of proteasomal targets after bortezomib treatment. This was the scenario observed for L-NAC in the presence of bortezomib (Fig. 10, left). However, our results clearly indicate that Tiron actually prevents the inhibition of the proteasome and thus acts upstream of the mitochondria and ROS production (Fig. 10, right). These results emphasize the incorrect/misleading conclusions that can be derived from interpreting the effect of Tiron as a ROS scavenger.

Intriguingly, NMR spectroscopy and high pressure liquid chromatography-based chromatographic analyses have shown that polyhydroxyl compounds such as Tiron bind to boronic acid with high affinity (62, 63). Thus, \(^{11}\text{B} \) NMR spectra indicated the formation of a 1:1 boric acid–Tiron complex at pH 3–10, with a maximum efficiency of binding at pH 4–8 (63). Moreover, anion exchange resins containing Tiron have been reported to remove trace amounts of boron from aqueous solutions (64). Based on these features, Tiron has been used to separate out boric acid/borate in water purification processes (63–65). The ability of Tiron to bind boronic acid may underlie the competitive inhibitory mechanism found here for bortezomib. The specificity of Tiron for boronic
derivatives of proteasome inhibitors is supported by the fact that Tiron had no impact on the activity of the unrelated aldehyde-containing MG132. By virtue of its preventing the inhibition of the proteasome, Tiron is a more potent blocker of bortezomib-induced cell death than other agents that interfere with downstream events. For example, inhibitors of stress kinases (e.g. c-Jun N-terminal kinase or p38 inhibitors) or apoptotic caspases (e.g. benzylxycarbonyl-VAD-fluoromethylketone) can delay bortezomib-induced cell death but cannot abrogate it (66). Moreover, the activity of Tiron first shown here in organotypic skin models and in a broad variety of tumor types could provide a novel approach for kinetic and mechanistic analyses aimed at defining the point of commitment of tumor cells in response to proteasome inhibition.

In summary, the substitution of aldehyde moieties for boronic acid in peptidyl-based compounds has lead to the generation of proteasome inhibitors of a higher potency (9). However, the boronic acid that has provided an improved stability and affinity of bortezomib for the proteasome and reduced its recognition by multidrug resistance drug pump systems (9) may provide a vulnerable point for inactivation by Tiron or polyhydroxyl-containing compounds. Therefore, we propose that structures with vicinally positioned hydroxyl groups should be avoided in the rational design of compounds aimed at synergizing with the anti-cancer activity of bortezomib. In addition, the permeability of Tiron and its efficacy in a three-dimensional human skin equivalent could provide a tractable model to address secondary toxicities of bortezomib (48) as well as to better define its mode of action in the context of surrounding cell-cell and cell-matrix interactions.

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