Targeted inhibition of the immunoproteasome is a potent strategy against models of multiple myeloma that overcomes resistance to conventional drugs and nonspecific proteasome inhibitors

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Proteasome inhibition is a validated strategy for therapy of multiple myeloma, but this disease remains challenging as relapses are common, and often associated with increasing chemoresistance. Moreover, nonspecific proteasome inhibitors such as bortezomib can induce peripheral neuropathy and other toxicities that may compromise the ability to deliver therapy at full doses, thereby decreasing efficacy. One novel approach may be to target the immunoproteasome, a proteasomal variant found predominantly in cells of hematopoietic origin that differs from the constitutive proteasome found in most other cell types. Using purified preparations of constitutive and immunoproteasomes, we screened a rationally designed series of peptidyl-aldehydes and identified several with relative specificity for the immunoproteasome. The most potent immunoproteasome-specific inhibitor, IPSI-001, preferentially targeted the $\beta 1_i$ subunit of the immunoproteasome in vitro and in cellulo in a dose-dependent manner. This agent induced accumulation of ubiquitin-protein conjugates, proapoptotic proteins, and activated caspase-mediated apoptosis. IPSI-001 potently inhibited proliferation in myeloma patient samples and other hematologic malignancies. Importantly, IPSI-001 was able to overcome conventional and novel drug resistance, including resistance to bortezomib. These findings provide a rationale for the translation of IPSIs to the clinic, where they may provide antimyeloma activity with greater specificity and less toxicity than current inhibitors. (Blood. 2009;113:4667-4676)

Introduction

The ubiquitin-proteasome pathway is used by eukaryotes as the major mechanism for regulated intracellular proteolysis. As such, it plays an important role in cellular homeostasis, and is critical to several processes, including angiogenesis, cell-cycle progression, DNA repair, programmed cell death, and the stress response.[1,2] This pathway has become a validated target for cancer therapy with the approval of the proteasome inhibitor bortezomib as a single agent for mantle cell lymphoma[3-5] and multiple myeloma (MM).[6-8] Modulation of proteasome function is also a rational approach to achieve chemosensitization, and bortezomib with pegylated liposomal doxorubicin has recently been shown to be superior to bortezomib alone against MM.[9] These successes have led to studies of the role of bortezomib in the up-front setting in myeloma, and to the introduction in early phase studies of a second generation of irreversible proteasome inhibitors, such as carfilzomib[10] and NPI-0052.[11,12]

In its assembled form, the proteasome consists of a core 20S particle with 4 stacked rings of 7 subunits each. Most cells express the constitutive proteasome variant, in which the 20S core is capped on both ends by a 19S activator complex, which aids in stabilization, ATP-dependent removal of ubiquitin, and unfolding/shuttling of proteins into the core. The inner 2 rings contain β subunits numbered 1 through 7, of which β1, β2, and β5 participate in proteolysis.[11-13] These encode the 3 major catalytic activities of

the proteasome: chymotrypsin-like (ChT-L), trypsin-like (T-L), and post–glutamyl peptide hydrolyzing, or caspase-like (C-L) activities. One variant of the proteasome that has been described is the immunoproteasome, which differs from the constitutive proteasome in part through the replacement of β1, β2, and β5, with distinct subunits known as $\beta 1_i$, $\beta 2_i$, and $\beta 5_i$, to form the $20S_i$ core.[14,15] This variant, which also contains different regulatory subunits known as 11S or PA28,[16] has been found to be highly expressed in cells of hematopoietic origin in several species.[17-19] Studies of $20S_i$ function have revealed that it generates peptide fragments with more hydrophobic and basic amino acids at the C-terminus,[20] which are better suited for presentation to major histocompatability class I molecules, providing the rationale for its name. However, the $20S_i$ also participates in many constitutive proteolytic processes,[17,19,21-24] and conversely the 20S proteasome may in some cases generate immunogenic epitopes,[25] suggesting that each proteasome variant may provide both housekeeping and specialized functions.

The role that the $20S_i$ plays outside of the immune system is just beginning to be elucidated, with expression levels being correlated to several disease states, including Huntington disease,[26] Alzheimer disease,[27] and macular degeneration.[28] Diseases associated with an aberrant immune response, such as inflammatory bowel disease[29,30] and rheumatoid arthritis,[31] have also been identified as having

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increased $20S_i$ expression. This may be due in part to the ability of inflammatory cytokines, such as tumor necrosis factor and interferon-γ, to induce expression and preferential incorporation of $β1_i$, $β2_i$, and $β5_i$ into the proteasome.[16] Preferential expression of $20S_i$ has also been observed in MM,[32] suggesting that specific immunoproteasome inhibition could be a promising therapeutic strategy. While bortezomib, carfilzomib, and NPI-0052, as well as other proteasome inhibitors, have been validated in myeloma and other model systems, all of these appear to target both the constitutive and immunoproteasomes indiscriminately. This lack of specificity may in part explain some of the side effects of these agents, such as peripheral neuropathy and gastrointestinal effects, which may be due to targeting of the constitutive proteasome in these tissues. In contrast, by selectively inhibiting the $20S_i$, it may be possible to maintain antimyeloma and antilymphoma efficacy while reducing these toxicities, thereby increasing the therapeutic index.

In this work, we describe the identification of a series of immunoproteasome-specific inhibitors (IPSIs) using an in vitro screen, and characterization of a lead compound, IPSI-001, which showed the greatest selectivity between proteasome variants. IPSI-001 induced cell death preferentially in $20S_i$-containing cells in a dose-dependent fashion through activation of both intrinsic and extrinsic apoptotic pathways. This agent also induced apoptosis in patient-derived myeloma samples, as well as samples from patients with other hematologic malignancies. Importantly, we found that IPSI-001 was able to overcome resistance not only to conventional chemotherapeutics, but also to bortezomib. Although this first generation of IPSIs in a peptidyl-aldehyde backbone has lower potency, they will be useful for proof-of-principle studies, and can be used as a backbone upon which other chemistries can be added, such as boronic acids, to enhance their potency further. Taken together, these data provide a rationale for further study of IPSIs as possible anticancer drugs, especially in the myeloma setting.

Methods

Cell models and cell culture

RPMI 8226, ANBL-6, KAS-6/1, Sup-B8, 8226.Dox40, 8226.LR5, 8226.BR, MM1.S, MM1.R, peripheral blood mononuclear cells (PBMCs), and patient-derived cells were cultured in RPMI 1640 with 2 mM L-glutamine (Invitrogen, Carlsbad, CA). SK-N-MC, HeLa, BT-474, HT-29, and Hs294T cells were grown in DMEM containing L-glutamine and 110 mg/L sodium pyruvate (Invitrogen). SK-N-MC cells were further supplemented with 1× MEM nonessential amino acids. Human pancreatic carcinoma cells (HPACs) were cultured in a 1:1 mixture of Dulbecco modified Eagle and Ham F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate supplemented with 0.002 mg/mL insulin, 40 ng/mL hydrocortisone, and 10 ng/mL epidermal growth factor. Single donor human umbilical vein endothelial cells (HUVECs) were maintained with endothelial cell growth medium (PromoCell, Heidelberg, Germany). All cell lines except HUVECs were supplemented with 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) and maintained in 5% CO_2 at 37°C. Interleukin-6 (IL-6)–dependent cells and purified plasma cells from patients were supplemented with 1 ng/mL IL-6 (R&D Systems, Minneapolis, MN). Patient samples were collected under a protocol approved by the University of North Carolina at Chapel Hill Institutional Review Board. Informed consent was obtained in accordance with the Declaration of Helsinki. Mononuclear cells from bone marrow aspirates or peripheral blood were isolated by density gradient centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ). Malignant cells were then isolated by immunomagnetic bead positive selection in a Midi magnetic-activated cell separation (MACS) LS column following the manufacturer's

protocol (Miltenyi Biotec, Auburn, CA).[10] LNCaP and PC-3 prostate cancer cell pellets were a gift from Dr Young Wang (University of North Carolina at Chapel Hill). MOLT-4, U937, AML-193, and CCRF-CEM cell pellets were a gift from Dr Beverly Mitchell (Stanford Cancer Center, Stanford, CA).

Proteasome purification and competitive binding assays

Isolation of 20S and $20S_i$ preparations from bovine pituitary and spleen was performed as described previously.[33,34] Competitive binding profiles of IPSI-001 using [^{14}C]-3,4-dichloroisocoumarin as an active-site probe,[33] and competitive binding profiles using VS-L$_3$-AHx$_3$-danysl,[10,35] were obtained as described previously.

Inhibitor synthesis

Peptidyl-aldehyde proteasome inhibitors were synthesized by oxidation of the corresponding peptidyl alcohols using a modification of the dimethyl sulfoxide–carbodiimide reaction described by Pfitzner and Moffatt, as described previously.[33,34]

Measurement of proteasome activity

The ChT-L activity in purified proteasome preparations was determined using the substrate carbobenzoxy-glycyl-glycyl-phenylalanyl-para-amino-benzoate. ChT-L activity releases the para-aminobenzoate, which is then quantified as previously described.[33,34] C-L activity was determined with the substrate carbobenzoxy-leucyl-leucyl-aspartyl-2-naphthylamide in an analogous fashion.[33,34] Studies of 20S and $20S_i$ proteasome activity in vitro in cellular extracts, or in intact cells in culture (in cellulo), were performed as described previously.[36]

Immunoblotting

Whole-cell extracts were prepared and separated by denaturing gel electrophoresis as described previously.[37] The antibodies used included antiactin, anti-JNK, antiubiquitin, and antirat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax, anti–p-JNK (Thr138/Tyr185), and anti–poly-(ADP-ribose) polymerase (PARP; EMD Biosciences, San Diego, CA), anti–HSC-70 antibodies (Stressgen, San Diego, CA), and antimouse and antirabbit secondary antibodies (Amersham Biosciences).

Cell viability and apoptosis assays

The WST-1 reagent was used to determine cellular viability, whereas detection of apoptotic DNA fragmentation was performed using the Cell Death Detection ELISA^{PLUS} kit (both from Roche Diagnostics, Indianapolis, IN). Fold increase in DNA fragmentation is presented as the mean relative to vehicle-treated controls as described previously.[10] Caspase-3, -8, and -9 activation was evaluated using fluorogenic substrates (BioMol, Plymouth Meeting, PA). Externalization of phosphatidyl-serine was examined by annexin V (BioVision, Mountain View, CA) and TO-PRO-3 (Invitrogen) staining, followed by analysis using fluorescence-activated cell sorting (FACS). Drug-specific apoptosis was calculated as follows: [(% live in control − % live in treated)/% live in control]. Changes in the mitochondrial transmembrane potential were determined using the JC-1 dye (Invitrogen). All of these assays were performed as described previously,[10] and according to the manufacturer's specifications. In some cases, colorimetric substrates (R&D Systems) were used to examine caspase activation initially following the same protocol as the fluorogenic assays, except samples were then read in a multilabel plate reader (Victor 3V; Perkin-Elmer, Waltham, MA) at 405 nm. Results were expressed as fold activation over the vehicle control.

Results

IPSI-001 inhibits the immunoproteasome

To identify potentially isoform-specific proteasome inhibitors, the 20S and $20S_i$ were purified to homogeneity from pituitary and

Table 1. Inhibitory potency of a panel of peptidyl-aldehydes against proteasome preparations

Inhibitor/activity	Immunoproteasome, K_i (μM)	Constitutive proteasome, K_i (μM)
Z-LLL-CHO (MG-132)		
ChT-L	5.1 ± 0.9 (6)	6.9 ± 0.84 (6)
Z-FL-CHO (bortezomib-aldehyde)		
ChT-L	2.1 ± 0.29 (9)	5.55 ± 0.47 (6)
Z-LnL-CHO (IPSI-001)		
ChT-L	1.03 ± 0.17 (3)	105 ± 1.3 (3)
BrAAP	1.45 ± 0.15 (8)	239 ± 14 (6)
Z-FF-CHO (IPSI-002)		
ChT-L	4.0 ± 0.27 (6)	56 ± 4.4 (3)
BrAAP	1.46 ± 0.38 (9)	NI
Z-hFF-CHO (IPSI-003)		
ChT-L	2.74 ± 0.33 (6)	40*
BrAAP	3.19 ± 0.11 (3)	NI
Z-LF-CHO (IPSI-004)		
ChT-L	5.6 ± 0.2 (3)	154*
BrAAP	4.07 ± 0.17 (3)	NI
Z-AF-CHO (IPSI-005)		
ChT-L	24 (2)	380 (2)
BrAAP	24 (2)	NI*

Dixon plots were used to obtain K_i values. Assays were carried out at 3 substrate concentrations and at 6 different inhibitor concentrations without preincubation of the enzyme with inhibitors, and the number of determinations is shown in parentheses. Substrate concentrations were 0.5 to 4.0 mM for the ChT-L activity using Z-GGF-pAB.

Standard single-letter abbreviations are used for amino acid residues where applicable.

ChT-L indicates chymotrypsin-like activity; BrAAP, branched-chain amino acid preferring; hF, homophenylalanine; IC_{50}, concentration inducing 50% target inhibition; IPSI, immunoproteasome-specific inhibitor; K_i, inhibitory constant; nL, norleucinal; pAB, para-amino-benzoate; Z, carbobenzoxy; and NI, not inhibited

*IC_{50} instead of K_i.

spleen tissues, respectively.[33] These preparations were then used to screen a panel of rationally designed peptidyl-aldehyde inhibitors using substrates specific for the ChT-L and C-L activities.[34] The peptidyl-aldehyde version of bortezomib, Z-FL-CHO, showed K_i's of 2.1 and 5.55 μM against the ChT-L activity of the $20S_i$ and 20S proteasomes, respectively (Table 1). By increasing the hydrophobicity of the amino acid in the P1, or C-terminal position, however, greater selectivity could be achieved for the immunoproteasome, in that inhibitory potency was maintained for the immunoproteasome, but decreased for the constitutive proteasome. This was most notable in the case of carbobenzoxy-leucyl-norleucinal (Z-LnL-CHO, or IPSI-001; Figure 1A), which showed a 100-fold preference for the immunoproteasome, with a K_i of 1.03 μM against the ChT-L $20S_i$ activity, compared with 105 μM against the constitutive proteasome (Table 1). IPSI-001 also had a preference for the branched chain amino acid preferring activity of the immunoproteasome, with a K_i of 1.45 μM compared with 239 μM for the constitutive proteasome (Table 1).

To study the binding characteristics of IPSI-001, the purified constitutive proteasome and immunoproteasome, as well as ANBL-6 myeloma cells, were exposed to increasing concentrations of this inhibitor. The purified immunoproteasome was then exposed to [14C]-3,4-dichloroisocoumarin, whereas cells were exposed to vinyl-sulfone-LLL-AHx3-dansyl, both of which bind available proteasome active sites. Protein extracts were then separated by electrophoresis, and subjected either to autoradiography for the former, or to Western blotting with an antidansyl antibody for the latter. Preincubation of purified immunoproteasomes with IPSI-001 prevented subsequent binding of the radioactive probe only to subunit $\beta 1_i$, without an impact on $\beta 2_i$ or $\beta 5_i$ (Figure 1B right panel), and no binding was seen to constitutive proteasome subunits (Figure 1B left panel). Similarly, in ANBL-6 myeloma
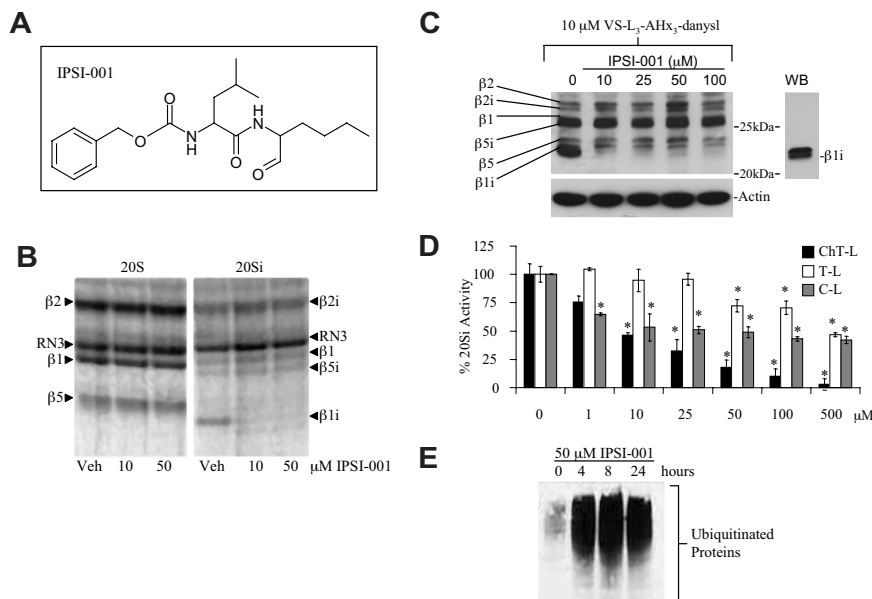

Figure 1. IPSI-001 inhibits the immunoproteasome. (A) Chemical structure of IPSI-001. (B) Competitive binding experiment to determine subunit binding profile of IPSI-001 in purified proteasomes from bovine pituitaries (20S) and spleen (20Si). Purified proteasomes were incubated with 10 or 50 μM IPSI-001 or vehicle (Veh) for 20 minutes, and then [14C]-3,4-dichloroisocoumarin was added for an additional 30 minutes. Proteasomes were then separated by denaturing gel electrophoresis and the binding profile was identified using autoradiography. Subunit identifications were made based on the known electrophoretic migration patterns of immunoproteasome and constitutive proteasome subunits. (C) Competitive binding experiment to determine the subunit binding profile of IPSI-001 in ANBL-6 cells. Cells were treated for 4 hours with IPSI-001, followed by addition of the VS-L3-AHx3-dansyl reagent that binds to all unoccupied catalytic subunits. Protein extracts were then separated by denaturing gel electrophoresis and probed by Western blotting using an antidansyl antibody to visualize binding patterns. Antiactin was used as a loading control and anti-$\beta 1_i$ was used to confirm the relative position of $\beta 1_i$. (D) In vitro assay for the 3 major catalytic activities of the proteasome in ANBL-6 cell lysates (5 μg) exposed to increasing concentrations of IPSI-001 in triplicate. Representative data are shown as the mean plus or minus standard deviation (SD) from 3 independent experiments. *$P < .05$ for proteasome inhibitory activity over control. (E) Western blot analysis of the accumulation of polyubiquitinated proteasome substrates in response to 50 μM IPSI-001 treatment in ANBL-6 cells over a period of 24 hours.
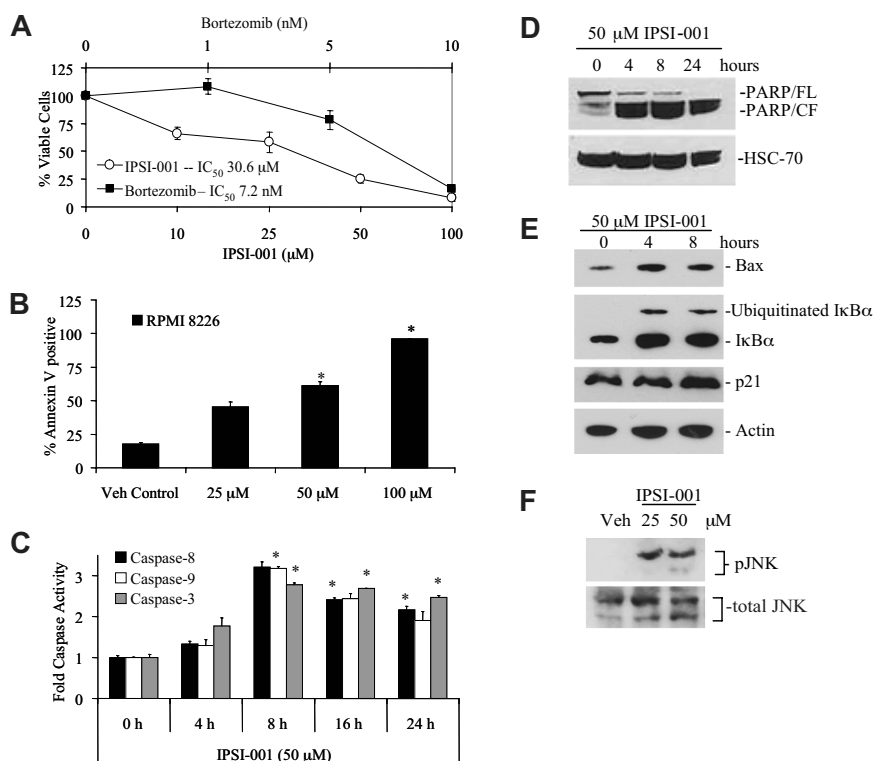
Figure 2. Cell death and molecular changes associated with IPSI-001. (A) WST-1 cytotoxicity assay in ANBL-6 cells treated for 18 hours with bortezomib or IPSI-001. The data shown are the mean plus or minus SD from 3 independent experiments. (B) Flow cytometric analysis was performed after annexin V staining in RPMI 8226 cells treated for 24 hours with the indicated concentrations of IPSI-001, with each experiment performed in duplicate. Error bars indicate SD. *$P <$ .05 comparing treated cells over vehicle control. (C) Representative results from 2 independent experiments of a colorimetric activity assay for caspases in RPMI 8226 myeloma cell lysate (100 μg) treated for up to 24 hours with 50 μM IPSI-001. The data shown are the mean plus or minus SD from duplicate experiments. *$P <$ .05 for fold caspase activity over baseline. (D) Western blot analysis for the late-stage apoptotic marker PARP, where the cleaved fragment (CF) represents apoptotic cells compared with the full-length (FL) PARP, which is present in healthy, nonapoptotic ANBL-6 cells. HSC-70 levels were used as a loading control. (E) Protein expression of Bax and IκBα in RPMI 8226 cells after incubation with 50 μM IPSI-001 for 24 hours. Actin levels were used as a loading control. (F) Protein expression of phosphorylated JNK (phospho-JNK) and total JNK in RPMI 8226 cells treated with 25 or 50 μM IPSI-001 for 24 hours.

cells, which expressed a mixture of the 2 proteasome isoforms, IPSI-001 binding occurred exclusively to $\beta 1_i$ (Figure 1C), with no binding to other immunoproteasome or constitutive proteasome subunits.

The consequences of binding of IPSI-001 to proteasome function were studied using ANBL-6 extracts and substrates specific for the ChT-L, T-L, and C-L activities. Low concentrations of IPSI-001 resulted in inhibition of the ChT-L and C-L activities, with sparing of the T-L function (Figure 1D). Increasing concentrations of IPSI-001 provided a further, dose-dependent, decrease in ChT-L activity, though a plateau was reached in C-L activity where concentrations greater than 10 μM produced little additional inhibition. Finally, inhibition of the T-L activity was seen as well, though this occurred only at some of the highest concentrations tested. To examine the impact of immunoproteasome inhibition on cellular protein turnover, Western blotting was performed to examine ubiquitin-protein conjugate levels. Consistent with a role for the immunoproteasome in housekeeping-associated proteolytic functions, IPSI-001 induced a rapid increase in conjugates at 8 hours and lasting up to 24 hours (Figure 1E).

Cytotoxic and proapoptotic impact of IPSI-001

Nonspecific proteasome inhibitors such as bortezomib and carfilzomib that target both the constitutive and immunoproteasomes inhibit cellular proliferation and induce apoptotic cell death. To assess whether inhibition of the $20S_i$ was by itself sufficient to induce similar effects, ANBL-6 cells were exposed to IPSI-001. This reduced the viability of ANBL-6 (Figure 2A) in a dose-dependent fashion within a brief, 24-hour exposure time with a half-maximal inhibitory concentration (IC_{50}) of 30.6 μM, compared with that of bortezomib at 7.2 nM. Reduction in viability was accompanied by the induction of apoptosis, as measured by an increased content of annexin V–positive RPMI 8226 cells (Figure 2B), and by apoptotic DNA fragmentation in ANBL-6 cells (Figure S1A, available on the *Blood* website; see the Supplemental

Materials link at the top of the online article). IPSI-001's cytotoxic activity was compared with other proteasome inhibitors, such as epoxomicin and lactacystin, which are structurally similar to carfilzomib and NPI-0052, respectively (Figure S1B). As expected, IPSI-001 had an IC_{50} of 30.1 μM, whereas that of bortezomib was 5.8 nM.

Bortezomib and carfilzomib induce programmed cell death by activating both the intrinsic apoptotic pathway mediated by caspase-9, and the extrinsic pathway mediated by caspase-8–dependent signaling, which then converge on a common effector, caspase-3. We examined whether IPSI-001 also exerted its antiapoptotic effect through activation of intrinsic and extrinsic caspase pathways[38] and found that dual caspase-8 and -9 activation was seen in a time-dependent fashion as early as after 4 hours (Figure 2C), which then induced caspase-3 activity. Other consequences of immunoproteasome inhibition included cleavage of PARP (Figure 2D), and depolarization of the trans-mitochondrial membrane potential (Figure S2).

Several mechanisms contribute to the proapoptotic effects of bortezomib, including suppression of nuclear factor kappa B by stabilization of its inhibitor IκB, accumulation of proapoptotic Bax, and activation of c-Jun N-terminal kinase (JNK).[39-41] To evaluate the impact of IPSI-001 on these pathways, RPMI 8226 cells were treated with 25 or 50 μM IPSI-001. Increases in Bax and IκBα levels were observed as early as 4 hours later (Figure 2E). Similarly, activation of JNK was also seen (Figure 2F), suggesting that immunoproteasome-specific inhibitors have comparable mechanisms of action as do current nonspecific inhibitors.

Preferential activity of IPSI-001 in immunoproteasome-containing cells

One of the promising aspects of IPSIs is that they should relatively spare cells expressing predominantly constitutive proteasomes. To evaluate this hypothesis, lysates from a panel of cell lines representing solid tumors that predominantly
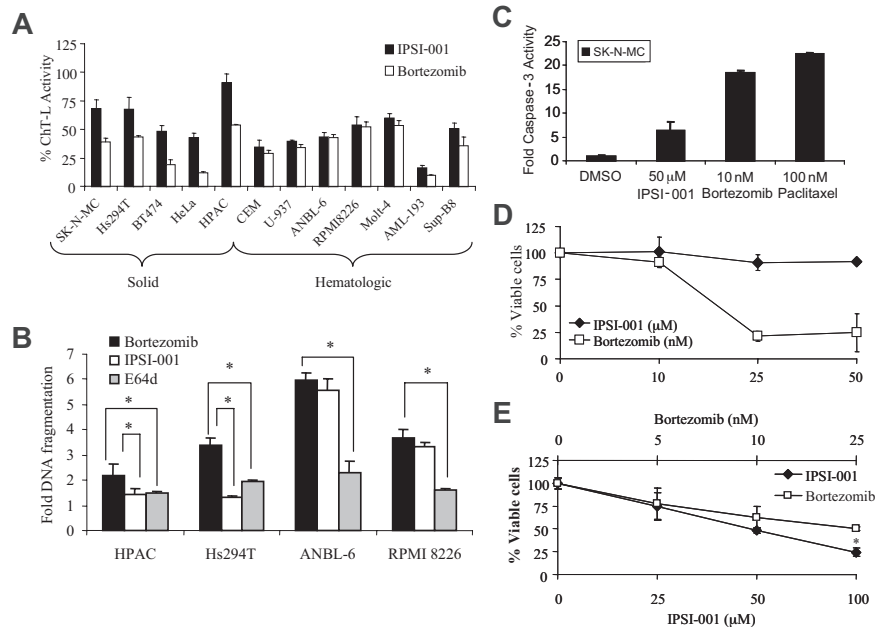
Figure 3. IPSI-001 preferentially inhibits the proteasome and induces death in lymphoid-derived cells. (A) The ChT-L activity was measured in vitro in solid tumor (SK-N-MC, Hs294T, BT474, HeLa, and HPAC; lanes 1-5) and hematologic cancer cells (CCRF-CEM, U-937, ANBL-6, RPMI 8226, MOLT-4, AML193, and SupB8; lanes 6-12) by exposing lysates to 50 μM IPSI-001 or 10 nM bortezomib for 15 minutes, followed by incubation with the specific ChT-L activity substrate. The data shown are the mean plus or minus SD from experiments performed in triplicate. (B) HPAC pancreatic adenocarcinoma cells, Hs294T melanoma cells, and ANBL-6 and RPMI 8226 human myeloma cells were exposed to vehicle, 10 nM bortezomib, 25 μM E64d, or 50 μM IPSI-001 for 24 hours. Apoptosis was evaluated using an enzyme-linked immunosorbent assay that detects apoptotic nuclear DNA fragmentation. Cell death was expressed as the fold increase in apoptosis over the vehicle control, which was arbitrarily set at 1.0. Representative data are shown from 3 independent experiments. *$P < .05$ comparing the inhibitors used. (C) Human SK-N-MC cells were treated for 24 hours with the indicated agents and then cell lysates (30 μg) were measured for caspase-3 activation using a fluorogenic substrate. Representative data are shown from 2 independent experiments, each performed in triplicate, as the mean plus or minus SD. (D) Human umbilical vein endothelial cells (HUVECs) were plated until they were 70% to 80% confluent, and then treated with increasing concentrations of IPSI (μM) or bortezomib (nM) for 24 hours, followed by evaluation by WST-1 for cell viability in triplicate. The data shown are the mean plus or minus SD from experiments performed in triplicate. (E) Human peripheral blood mononuclear cells (PBMCs) were plated and exposed to IPSI-001 and bortezomib. Viable cells were assayed 24 hours later using WST-1 reagent. *$P < .05$ for IPSI-001 treatment compared with bortezomib.

express 20S, and hematologic malignancies that predominantly express 20S$_i$ (Figure S3), were studied. Bortezomib at 10 nM was able to induce significant inhibition of the proteasome ChT-L activity in all of these cells (Figure 3A). When ISPI-001 was used at 50 μM, a concentration that inhibited the proteasome in hematologic cell lines to a comparable extent as bortezomib (Figure S1A), however, a lower impact on ChT-L function was seen in solid tumor cells, suggesting relative sparing of the 20S. Proapoptotic activity was then assessed in a smaller sample of 4 cell lines, including the 20S$_i$-containing ANBL-6 and RPMI 8226 myeloma cells, as well as the 20S-containing HPAC pancreatic carcinoma and Hs294T melanoma cells. Bortezomib induced DNA fragmentation in all of these models (Figure 3B), but IPSI-001 concentrations that gave comparable levels of apoptosis in the myeloma cells induced modest, if any, cell death in the solid tumor cells. IPSI-001 is also known as calpeptin, and was originally identified as an inhibitor of the sulfhydryl calcium–activated protease calpain.[42] Although the activity of calpains under conditions of normal cellular calcium concentrations is felt to be low,[42] it was important to rule out the possibility that the cell death being observed was due to an impact on calpain function. Therefore, cells were also treated with E64d, a cell-permeable agent that is a 20-fold more potent and specific calpain inhibitor than IPSI-001.[43] Little to no apoptosis was found in either the solid tumor or MM cell lines exposed to this agent, indicating that inhibition of calpain activity was not sufficient for apoptotic induction by IPSI-001.

Peripheral neuropathy is one toxicity of bortezomib against which there is no prophylaxis, and for which only symptomatic care and dose reduction or discontinuation is recommended. SK-N-MC cells, which are derived from an Askin tumor representing primitive neuroectodermal tissue, and are used to model neurotoxic drug effects,[44-47] were studied. Bortezomib induced a significant increase in caspase-3 activity in these cells, which was comparable with that following exposure to paclitaxel, another neurotoxic drug (Figure 3C). In contrast, IPSI-001 to some extent spared SK-N-MC cells, even at concentrations that had induced almost complete inhibition of the ChT-L activity in hematologic tumor cells. This was reflected by a much lower extent of induction of caspase-3 activity by IPSI-001. Although studies have not been done to directly correlate proteasome inhibitor-mediated neurotoxicity in these cells, these data should give an indication of the probable effect in neural cells. To further assess the impact of IPSI-001 on primary cells, human umbilical vein endothelial cells (HUVECs) were treated with either bortezomib or IPSI-001. A slight decrease in the viable cell population of approximately 10% was observed in HUVECs treated with IPSI-001 at concentrations up to 50 μM (Figure 3D). However, HUVECs incubated with bortezomib had approximately 75% cell death at concentrations higher than 25 nM. Finally, IPSI-001 activity against peripheral blood mononuclear cells (PBMCs) was investigated, and a dose-dependent decrease in cellular viability was observed similar to that after bortezomib exposure (Figure 3E). Together, these findings suggest that IPSIs may have an improved toxicity profile, with a lesser impact on proteasome function and cellular viability in nonhematopoietic tissues than bortezomib, but may induce similar cytopenic effects.
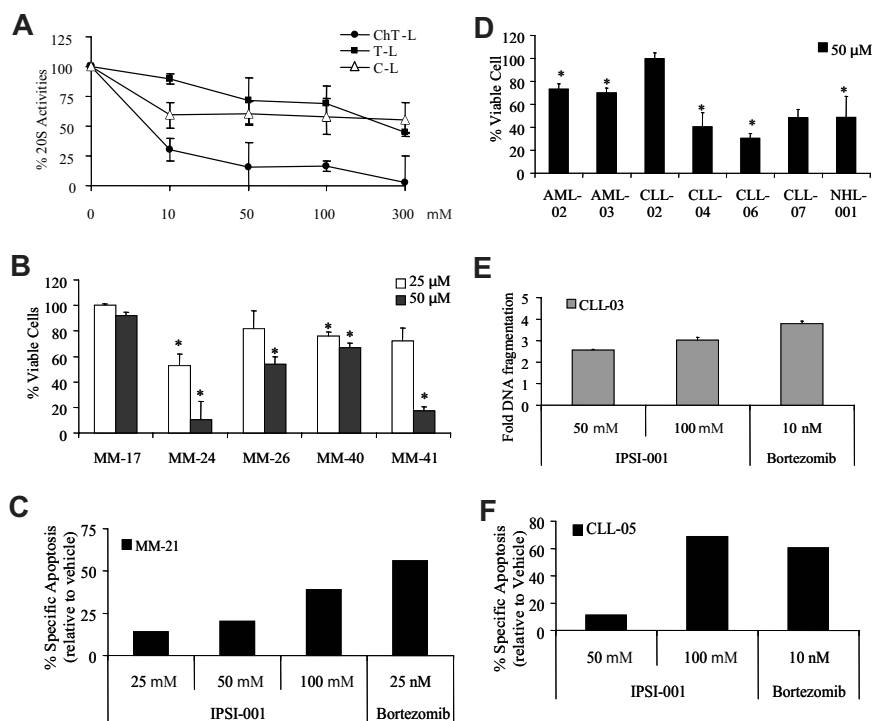
Figure 4. Multiple myeloma and other primary samples from patients with hematologic malignancies are sensitive to IPSI-001. (A) Cellular extracts (5 μg) from CD33+ acute myeloma leukemia (AML-01) patient cells were exposed to increasing doses of IPSI-001 and proteasome inhibition was assessed using fluorogenic substrates for the ChT-L, T-L, and C-L activities of the proteasome in triplicate. These data are similar to experiments performed in 3 additional CD19+ patient samples. The data shown are the mean plus or minus SD. (B) Purified CD138+ plasma cells from patient samples were treated with 25 and 50 μM IPSI-001 for 24 hours, and the effects on cell viability were examined using the WST-1 reagent in triplicate. All of the samples are from patients with chromosome 13 deletions (MM-17, MM-24, MM-26, MM-40, and MM-41). The data shown are the mean plus or minus SD. *P < .05 compared with vehicle control. (C) Apoptosis was measured in a patient-derived bone marrow aspirate purified for CD138+ cells, which were treated for 24 hours with IPSI-001 or bortezomib, and then evaluated for programmed cell death using annexin V/TO-PRO-3 staining. (D) Purified CD19+ cells or CD33+ cells from patient samples were treated with IPSI-001 for 24 hours, and the effects on cell viability were examined using the WST-1 reagent in triplicate. The data shown are the mean plus or minus SD. *P < .05 compared with vehicle alone treatment. (E) Purified lymphocytes from a CLL patient were treated with IPSI-001 for 24 hours. Apoptosis was evaluated using an enzyme-linked immunosorbent assay that detects apoptotic nuclear DNA fragmentation in triplicate. Cell death is expressed as the fold increase in apoptosis over the vehicle control, which was arbitrarily set at 1.0. The data shown are the mean plus or minus SD. (F) Purified lymphocytes from a CLL patient were treated with IPSI-001. Annexin V–positive cells were measured using flow cytometry and results are displayed as the percentage specific apoptotic population.

Effect of IPSI-001 in patient-derived samples

The activity of IPSI-001 against MM cell lines suggested that it could be active against primary plasma cells as well. Consistent with the previous in vitro data, IPSI-001 strongly reduced the ChT-L–like activity and modestly reduced C-L activity at low doses (Figure 4A). At higher IPSI-001 concentrations, further ChT-L inhibition was achieved, the C-L activity reached a plateau, and significant T-L inhibition was seen only at the highest drug levels. We then examined whether IPSI-001 had antiproliferative effects against purified patient plasma cells. All of the patient samples showed sensitivity to IPSI-001 treatment for 24 hours, with dose-dependent decreases in the live cell population, even in samples from patients with poor-risk disease harboring chromosome 13 deletions (Figure 4B). To confirm that this decrease was due to induction of apoptosis, flow cytometric analysis using annexin V staining was performed, and showed a dose-dependent increase in programmed cell death (Figure 4C). These experiments were performed on purified CD138+ plasma cells, and the effect of IPSI-001 on other clonal subsets of marrow-derived cells is not yet known.

In addition to activity against MM, bortezomib is preclinically active against other hematologic malignancies, so we also sought to examine the impact of IPSI-001 on other models. To that end, purified samples from patients with diffuse large B-cell non-Hodgkin lymphoma (NHL), chronic lymphocytic leukemia (CLL),

and acute myeloid leukemia (AML) were treated with 50 μM IPSI-001 for 24 hours, followed by evaluations for viability (Figure 4D). All disease types showed evidence of some level of susceptibility to IPSI-001–induced cell death. One exception was a patient with prolymphocytic leukemia (CLL-02), a variant of CLL that is generally more aggressive and less responsive to treatment that, like one myeloma patient sample (MM-17, Figure 4B), showed only a slight response. As was the case for the plasma cell samples, exposure to IPSI-001 in 2 patient CLL isolates resulted in activation of apoptosis (Figure 4E,F).

IPSI-001 overcomes chemotherapy resistance and synergizes with dexamethasone

Novel agents that can overcome chemoresistance to other drugs may have greater potential for clinical antitumor activity, and we therefore studied IPSI-001 in several drug-resistance models. RPMI 8226 wild-type cells were compared with their doxorubicin (Dox40)–resistant, melphalan (LR5)–resistant, and bortezomib-resistant (BR) counterparts, and MM1 dexamethasone-sensitive (S) cells were compared with dexamethasone-resistant (R) cells. Doxorubicin- and melphalan-resistant clones did show some decreased sensitivity to IPSI-001, especially at lower doses (Figure 5A), suggesting the possibility of some cross-resistance. However, higher concentrations of IPSI-001 induced substantial apoptosis (Figure 5A) and DNA fragmentation (Figure 5B). Dexamethasone-resistant MM1.R cells showed comparable responses
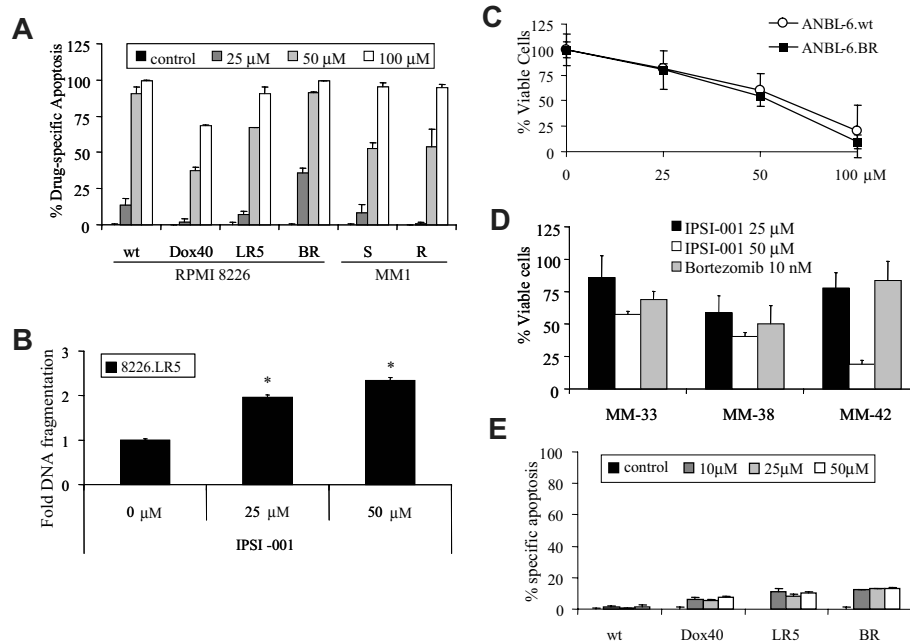
Figure 5. IPSI-001 is able to overcome chemotherapy resistance. (A) Drug-free for at least 48 hours; doxorubicin-, melphalan-, and bortezomib-resistant RPMI 8226; and dexamethasone-sensitive and-resistant MM1 multiple myeloma cell lines were exposed to increasing doses of IPSI-001 for 24 hours in duplicate, followed by determination of apoptotic cell populations using dual annexin V/TOPRO-3 staining. The data shown are the mean plus or minus SD from 3 independent experiments. (B) Melphalan-resistant 8226.LR5 cells were treated with IPSI-001 (25 and 50 μM) for 24 hours and apoptosis was measured using a commercial kit for apoptotic DNA fragmentation. The data shown are the mean plus or minus SD. *$P < .05$ compared with vehicle control. (C) ANBL-6.wt and ANBL-6.BR were compared for their sensitivity to IPSI-001 after a 24-hour exposure using WST-1 proliferation assay. The data shown are the mean plus or minus SD from experiments performed in triplicate. (D) Purified CD138^{+} plasma cells from patient samples were treated with 25 and 50 μM IPSI-001 for 24 hours, and the effects on cell viability were examined in triplicate. Cytogenetic studies showed that MM-33 had a chromosome 13 deletion and MM-38 had a chromosome 9 deletion and trisomy 11, whereas the MM-42 sample was not evaluated. The data shown are the mean plus or minus SD. (E) RPMI 8226 cell lines resistant to doxorubicin, melphalan, and bortezomib were treated with the calpain inhibitor E64d and analyzed for annexin V staining by flow cytometry. Representative results from 2 independent experiments are shown as the mean plus or minus SD.

to IPSI-001 as the dexamethasone-sensitive MM1.S cells (Figure 5B). Our group has also developed bortezomib-resistant (BR) myeloma cell lines that show significantly decreased sensitivity to bortezomib (Figure S4).[10] Notably, and in contrast to the previous data, 8226.BR cells were even more sensitive to IPSI-001 at lower doses than their wild-type counterparts, supporting the lack of any cross-resistance. The latter possibility was also studied in the independently derived, bortezomib-resistant ANBL-6.BR cells, and compared with wild-type cell populations. Comparable decreases in viable cell populations were found in both ANBL-6.wt and ANBL-6.BR cells after exposure to IPSI-001 (Figure 5C). Importantly, plasmacytes from 3 bortezomib-refractory patients all remained sensitive to 50 μM IPSI-001 and to a higher degree than comparable 10-nM bortezomib exposure, with no significant resistance found even in MM-33, a patient with chromosome 13 deletion (Figure 5D). Lastly, to rule out the possibility that it was the ability of IPSI-001 to inhibit calpains that allowed it to overcome resistance, E64d was again used as a control. Less than 20% apoptosis was observed in all cell lines tested, including wild-type cells (Figure 5E), despite the use of inhibitor levels that were 20-fold more potent than those of IPSI-001. These findings again support the assertion that it is the ability of IPSIs to target the immunoproteasome that is responsible for their activity

Antimyeloma agents are often used in combinations to enhance their antitumor activity, and addition of dexamethasone to bortezomib is one strategy that has shown improved efficacy.[48] To determine whether IPSI-001 works in concert with dexamethasone, ANBL-6 cells were treated simultaneously with both drugs for 48 hours. Compared with dexamethasone or IPSI-001 alone, the combination demonstrated a greater antiproliferative effect (Figure S5). Moreover, statistical analysis indicated that a moderate to high

degree of synergy was present between the 2 drugs at most concentrations tested (Table S1).

Discussion

Multiple myeloma remains an incurable disease that almost invariably relapses after initial therapy, and though subsequent remissions can be achieved, they typically grow shorter with each successive line of chemotherapy.[49] Although it is possible that the efficacy of proteasome inhibitors such as bortezomib is due to their lack of specificity for different proteasome isoforms, it is also likely that this broad inhibitory activity may contribute to the induction of treatment-emergent neuropathy, which can be dose limiting. This led us to consider that targeting the 20S$_i$, which is highly expressed in hematopoietic tissues and contains 3 different proteolytic subunits compared with the constitutive proteasome, could provide efficacy with decreased toxicity. Here, we report the identification of IPSIs that relatively spare the 20S, and the characterization of IPSI-001, the most potent of these agents. IPSI-001 preferentially inhibited immunoproteasome activity in vitro and in lymphoid-derived cell lines and patient-derived samples, induced extrinsic and intrinsic pathways of apoptosis, synergized with dexamethasone, and was able to overcome conventional drug resistance, as well as resistance to bortezomib itself.

IPSI-001 was identified in a screen of peptidyl-aldehyde compounds for favored ChT-L–inhibitory activity against 20S$_i$, and showed a 100-fold preference for this target over the comparable 20S activity (Table 1). In addition, IPSI-001 also exhibited 165-fold preference for the BrAAP activity of the 20S$_i$, which is

attributed to the $\beta 1$ and $\beta 1_i$ subunits[50] (Table 1). Although the potency of IPSI-001 is not sufficient to warrant in vivo testing or translation into the clinic, its properties will be useful for further validation of the immunoproteasome as a target. Expression of $20S_i$ has been found predominantly in immune tissues,[51] but lower levels have also been noted in the brain,[27] intestinal epithelium,[52] liver,[53] and ocular lens.[54] In the latter 2, however, expression seems to be related to specific disease states leading to production of inflammatory cytokines known to induce $20S_i$ subunits, suggesting that under normal conditions these would predominantly contain 20S. Moreover, peptide-based drugs such as bortezomib do not pass through the blood-brain barrier,[55] implying that central nervous system toxicity would be unlikely with IPSIs as well. This leaves intestinal epithelium as an area of concern for toxicity based on the known expression profiles, but our studies of cell lines derived from such tissues (Figure S3) showed a predominant expression of 20S. Taken together, these findings suggest that IPSIs may indeed have a modest systemic toxicity profile, though they may well retain the hematologic toxicity of agents such as bortezomib, or perhaps even exceed it based on the increased cell death observed in PBMCs treated with IPSI-001 (Figure 3E). Once clinically relevant IPSIs are developed, therefore, it will be important to closely evaluate their tissue distribution and proteasome binding. In addition, studies correlating the level and type of immunoproteasome subunits with the impact of IPSIs will be of great help in further determining their therapeutic index.

Our lead compound, Z-Leu-nLeu-CHO, is also known as calpeptin, which inhibits calpain I and calpain II, proteases important for cell motility and cell cycle progression that play roles in necrosis and apoptosis in some cell line models.[42] To rule out the possibility that calpain inhibition was responsible for the induction of apoptosis by IPSI-001 (Figure 3B), as well as its ability to overcome chemotherapy resistance (Figure 5D), we used the sulfhydryl protease inhibitor E64d as a control. These studies showed that calpain suppression was not sufficient to accomplish either of these outcomes by itself, and studies combining bortezomib with E64d showed that this approach did not overcome bortezomib resistance (not shown). In addition, some of the IPSIs we have identified are longer than dipeptides, making them less inhibitory for calpains, and these too overcome resistance (not shown). Ultimately, however, since peptidyl-aldehydes can react with free sulfhydryl groups, the role of calpain inhibition in these processes will be completely ruled out only with peptides based on other chemistries that reduce binding to sulfhydryl groups, such as boronic acids. It is also notable that recent data point to an increase in calpain activity in proteasome-inhibited cells,[56] as well as in a cisplatin-resistant melanoma cell line.[57] These findings suggest that IPSI-001, or other calpain inhibitors, may prove to be useful in combination therapies where the dual calpain/$20S_i$ inhibitory activity may be able to target more pathways of resistance activated by malignant cells.

The ChT-L subunit is the rate-limiting step of proteolysis by the proteasome. Kisselev et al proposed the "bite-chew" method of protein breakdown, whereby the incoming protein is cleaved at ChT-L sites (bite), and C-L sites are allosterically activated. The protein is then further degraded by the C-L and T-L sites (chew), whereas the ChT-L activity is allosterically inhibited, thus completing one cycle.[58] NPI-0052 has inhibitory properties against all 3 major proteolytic activities,[11] carfilzomib primarily inhibits the ChT-L activity at lower concentrations, but at higher concentrations the T-L and C-L activities are also affected,[10] and bortezomib's inhibitory effect is ChT-L > C-L≫T-L. We found that IPSI-001

bound potently and specifically to the $\beta 1_i$ subunit (Figure 1B,C) yet had an inhibitory profile similar to bortezomib (Figure 1D). Previous reports have generally attributed proteolytic activities of the $20S_i$ catalytic subunits to their 20S counterparts, a model in which $\beta 5_i$ should encode the ChT-L activity.[21,59] Of note, Ho et al recently reported development of a $\beta 1_i$ (LMP-2)–specific inhibitor that had greater growth inhibitory effects in cell populations with high $\beta 1_i$ expression.[60] However, the $\beta 1_i$-specific inhibitor developed by Ho et al was found to have ChT-L inhibitory activity, and the authors suggested that overlapping catalytic activities existed for the $\beta 1_i$ subunit. The phenomenon of simultaneous inhibition of the ChT-L activity has been reported by 2 other groups who developed constitutive $\beta 1$ inhibitors with moderate specificity whose activity was attributed to allosteric inhibition of the $\beta 5$ subunit.[61,62] Lastly, a report by Kisselev et al, using a highly specific $\beta 1$ inhibitor, demonstrated that the dual ChT-L and C-L inhibitory properties were due to (1) the $\beta 1$ inhibitor binding and inhibiting the C-L activity; (2) binding of the $\beta 1$ inhibitor to a noncatalytic regulatory site that inhibits the ChT-L activity; and (3) $\beta 1$ inhibitors allosterically stimulating the T-L activity.[63] Given the similar inhibitory profiles of IPSI-001 and the inhibitor developed by Ho et al, we postulate that these $\beta 1_i$ inhibitors also bind the noncatalytic, regulatory subunit proposed by Kisselev et al that is present in both 20S and $20S_i$ proteasomes. This possibility is further supported by increased binding potential of IPSI-001 to the $\beta 1_i$ subunit in relation to BrAAP activity (Table 1).

In general, plasma cell isolates from patients responded to exposure to IPSI-001 (Figure 4B), but in one patient sample, MM-17, a very modest effect was observed. This patient was found to have harbored disease with deletion of chromosome 13, which is a poor prognostic marker for myeloma, but one that typically responds well to bortezomib. However, deletions of chromosome 13 were also present in samples MM-24, MM-26, MM-33, MM-40, and MM-41, which did respond well. In addition, patient MM-17 had received 2 prior therapies, including bortezomib, and initially responded to therapy but had progressive disease off treatment. Three patients were clinically refractory to bortezomib at the time their cells were isolated (MM-33, MM-38, and MM-42), yet remained sensitive to IPSI-001. Interestingly, MM-33 and MM-42 had plasma cell leukemia that is typically an aggressive myeloma variant, but both responded well to IPSI-001.

In conclusion, our data indicate that the immunoproteasome is a rational target for anticancer therapy in MM. Agents based on IPSI-001, or some of the other IPSIs that have been identified, which would have enhanced potency without losing their specificity, should strongly induce apoptosis in MM and other hematologic malignancies. Moreover, they should relatively spare other tissues not expressing the immunoproteasome, unlike the current generation of nonspecific proteasome inhibitors, possibly thereby providing an improved therapeutic index. The latter could allow for more chronic dosing that could prove useful not just in cancer, but in treating other disorders such as autoimmune diseases and graft rejection that rely in part on antigen presentation that is processed through the immunoproteasome.

Acknowledgments

References

1. Orlowski RZ, Kuhn DJ. Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res. 2008;14:1649-1657.

2. Ciechanover A. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol. 2005;6:79-87.

3. O'Connor OA, Wright J, Moskowitz C, et al. Phase II clinical experience with the novel proteasome inhibitor bortezomib in patients with indolent non-Hodgkin's lymphoma and mantle cell lymphoma. J Clin Oncol. 2005;23:676-684.

4. Fisher RI, Bernstein SH, Kahl BS, et al. Multicenter phase II study of bortezomib in patients with relapsed or refractory mantle cell lymphoma. J Clin Oncol. 2006;24:4867-4874.

5. Strauss SJ, Maharaj L, Hoare S, et al. Bortezomib therapy in patients with relapsed or refractory lymphoma: potential correlation of in vitro sensitivity and tumor necrosis factor alpha response with clinical activity. J Clin Oncol. 2006; 24:2105-2112.

6. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med. 2003;348:2609-2617.

7. Richardson PG, Sonneveld P, Schuster MW, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med. 2005; 352:2487-2498.

8. Richardson PG, Sonneveld P, Schuster M, et al. Extended follow-up of a phase 3 trial in relapsed multiple myeloma: final time-to-event results of the APEX trial. Blood. 2007;110:3557-3560.

9. Orlowski RZ, Nagler A, Sonneveld P, et al. Randomized phase III study of pegylated liposomal doxorubicin plus bortezomib compared with bortezomib alone in relapsed or refractory multiple myeloma: combination therapy improves time to progression. J Clin Oncol. 2007;25:3892-3901.

10. Kuhn DJ, Chen Q, Voorhees PM, et al. Potent activity of carfilzomib, a novel, irreversible inhibitor of the ubiquitin-proteasome pathway, against preclinical models of multiple myeloma. Blood. 2007;110:3281-3290.

11. Chauhan D, Catley L, Li G, et al. A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from bortezomib. Cancer Cell. 2005;8:407-419.

12. Chauhan D, Hideshima T, Anderson KC. A novel proteasome inhibitor NPI-0052 as an anticancer therapy. Br J Cancer. 2006;95:961-965.

13. Unno M, Mizushima T, Morimoto Y, et al. The structure of the mammalian 20S proteasome at 2.75 A resolution. Structure. 2002;10:609-618.

14. Monaco JJ. A molecular model of MHC class-I-restricted antigen processing. Immunol Today. 1992;13:173-179.

15. Orlowski M, Wilk S. A multicatalytic protease complex from pituitary that forms enkephalin and enkephalin containing peptides. Biochem Biophys Res Commun. 1981;101:814-822.

16. Kloetzel PM. The proteasome and MHC class I antigen processing. Biochim Biophys Acta. 2004; 1695:225-233.

17. Noda C, Tanahashi N, Shimbara N, Hendil KB, Tanaka K. Tissue distribution of constitutive proteasomes, immunoproteasomes, and PA28 in rats. Biochem Biophys Res Commun. 2000;277: 348-354.

18. Jiang H, Monaco JJ. Sequence and expression of mouse proteasome activator PA28 and the related autoantigen Ki. Immunogenetics. 1997;46: 93-98.

19. Eleuteri AM, Angeletti M, Lupidi G, Tacconi R, Bini L, Fioretti E. Isolation and characterization of bovine thymus multicatalytic proteinase complex. Protein Expr Purif. 2000;18:160-168.

20. Rock KL, Goldberg AL. Degradation of cell proteins and the generation of MHC class I-presented peptides. Annu Rev Immunol. 1999;17: 739-779.

21. Gaczynska M, Rock KL, Goldberg AL. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. Nature. 1993;365:264-267.

22. Früh K, Gossen M, Wang K, Bujard H, Peterson PA, Yang Y. Displacement of housekeeping proteasome subunits by MHC-encoded LMPs: a newly discovered mechanism for modulating the multicatalytic proteinase complex. EMBO J. 1994;13:3236-3244.

23. Rivett AJ, Hearn AR. Proteasome function in antigen presentation: immunoproteasome complexes, peptide production, and interactions with viral proteins. Curr Protein Pept Sci. 2004;5:153-161.

24. Teoh CY, Davies KJ. Potential roles of protein oxidation and the immunoproteasome in MHC class I antigen presentation: the 'ProxI' hypothesis. Arch Biochem Biophys. 2004;423:88-96.

25. Strehl B, Seifert U, Kruger E, Heink S, Kuckelkorn U, Kloetzel PM. Interferon-gamma, the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing. Immunol Rev. 2005;207:19-30.

26. Díaz-Hernández M, Hernandez F, Martin-Aparicio E, et al. Neuronal induction of the immunoproteasome in Huntington's disease. J Neurosci. 2003; 23:11653-11661.

27. Mishto M, Bellavista E, Santoro A, et al. Immunoproteasome and LMP2 polymorphism in aged and Alzheimer's disease brains. Neurobiol Aging. 2006;27:54-66.

28. Ethen CM, Hussong SA, Reilly C, Feng X, Olsen TW, Ferrington DA. Transformation of the proteasome with age-related macular degeneration. FEBS Lett. 2007;581:885-890.

29. Fitzpatrick LR, Small JS, Poritz LS, McKenna KJ, Koltun WA. Enhanced intestinal expression of the proteasome subunit low molecular mass polypeptide 2 in patients with inflammatory bowel disease. Dis Colon Rectum. 2007;50:337-348; discussion 348-350.

30. Visekruna A, Joeris T, Seidel D, et al. Proteasome-mediated degradation of IkappaBalpha and processing of p105 in Crohn disease and ulcerative colitis. J Clin Invest. 2006;116:3195-3203.

31. Egerer T, Martinez-Gamboa L, Dankof A, et al. Tissue-specific up-regulation of the proteasome subunit beta5i (LMP7) in Sjogren's syndrome. Arthritis Rheum. 2006;54:1501-1508.

32. Altun M, Galardy PJ, Shringarpure R, et al. Effects of PS-341 on the activity and composition of proteasomes in multiple myeloma cells. Cancer Res. 2005;65:7896-7901.

33. Orlowski M, Cardozo C, Eleuteri AM, Kohanski R, Kam CM, Powers JC. Reactions of [14C]-3,4-dichloroisocoumarin with subunits of pituitary and spleen multicatalytic proteinase complexes (proteasomes). Biochemistry. 1997;36:13946-13953.

34. Cardozo C, Michaud C, Orlowski M. Components of the bovine pituitary multicatalytic proteinase complex (proteasome) cleaving bonds after hydrophobic residues. Biochemistry. 1999;38:9768-9777.

35. Ovaa H. Active-site directed probes to report enzymatic action in the ubiquitin proteasome system. Nat Rev Cancer. 2007;7:613-620.

36. Nam S, Smith DM, Dou QP. Ester bond-containing tea polyphenols potently inhibit proteasome activity in vitro and in vivo. J Biol Chem. 2001; 276:13322-13330.

37. Small GW, Somasundaram S, Moore DT, Shi YY, Orlowski RZ. Repression of mitogen-activated protein kinase (MAPK) phosphatase-1 by anthracyclines contributes to their antiapoptotic activation of p44/42-MAPK. J Pharmacol Exp Ther. 2003;307:861-869.

38. Fadeel B, Orrenius S. Apoptosis: a basic biological phenomenon with wide-ranging implications in human disease. J Intern Med. 2005;258:479-517.

39. Lauricella M, Emanuele S, D'Anneo A, et al. JNK and AP-1 mediate apoptosis induced by bortezomib in HepG2 cells via FasL/caspase-8 and mitochondria-dependent pathways. Apoptosis. 2006;11:607-625.

40. Tsuruta F, Sunayama J, Mori Y, et al. JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. EMBO J. 2004;23:1889-1899.

41. Engelberg D. Stress-activated protein kinases-tumor suppressors or tumor initiators? Semin Cancer Biol. 2004;14:271-282.

42. Goll DE, Thompson VF, Li H, Wei W, Cong J. The calpain system. Physiol Rev. 2003;83:731-801.

43. Huang Z, McGowan EB, Detwiler TC. Ester and amide derivatives of E64c as inhibitors of platelet calpains. J Med Chem. 1992;35:2048-2054.

44. Chen J, Wersinger C, Sidhu A. Chronic stimulation of D1 dopamine receptors in human SK-N-MC neuroblastoma cells induces nitric-oxide synthase activation and cytotoxicity. J Biol Chem. 2003;278:28089-28100.

45. Goldoni M, Vettori MV, Alinovi R, Caglieri A, Ceccatelli S, Mutti A. Models of neurotoxicity: extrapolation of benchmark doses in vitro. Risk Anal. 2003;23:505-514.

46. Cristòfol RM, Gasso S, Vilchez D, Pertusa M,

Rodriguez-Farre E, Sanfeliu C. Neurotoxic effects of trimethyltin and triethyltin on human fetal neuron and astrocyte cultures: a comparative study with rat neuronal cultures and human cell lines. Toxicol Lett. 2004;152:35-46.

47. Sebastià J, Pertusa M, Vilchez D, et al. Carboxyl-terminal fragment of amyloid precursor protein and hydrogen peroxide induce neuronal cell death through different pathways. J Neural Transm. 2006;113:1837-1845.

48. Jagannath S, Richardson PG, Barlogie B, et al. Bortezomib in combination with dexamethasone for the treatment of patients with relapsed and/or refractory multiple myeloma with less than optimal response to bortezomib alone. Haematologica. 2006;91:929-934.

49. Kumar S, Rajkumar SV. Thalidomide and lenalidomide in the treatment of multiple myeloma. Eur J Cancer. 2006;42:1612-1622.

50. Orlowski M. Selective activation of the 20 S proteasome (multicatalytic proteinase complex) by histone H3. Biochemistry. 2001;40:15318-15326.

51. Aki M, Shimbara N, Takashina M, et al. Interferon-gamma induces different subunit organizations and functional diversity of proteasomes. J Biochem (Tokyo). 1994;115:257-269.

52. Leblond J, Hubert-Buron A, Bole-Feysot C, Ducrotte P, Dechelotte P, Coeffier M. Regulation of proteolysis by cytokines in the human intestinal epithelial cell line HCT-8: role of IFNgamma. Biochimie. 2006;88:759-765.

53. Khan S, van den Broek M, Schwarz K, de Giuli R, Diener PA, Groettrup M. Immunoproteasomes largely replace constitutive proteasomes during an antiviral and antibacterial immune response in the liver. J Immunol. 2001;167:6859-6868.

54. Singh S, Awasthi N, Egwuagu CE, Wagner BJ. Immunoproteasome expression in a nonimmune tissue, the ocular lens. Arch Biochem Biophys. 2002;405:147-153.

55. Hemeryck A, Geerts R, Monbaliu J, et al. Tissue distribution and depletion kinetics of bortezomib and bortezomib-related radioactivity in male rats after single and repeated intravenous injection of 14 C-bortezomib. Cancer Chemother Pharmacol. 2007;60:777-787.

56. Delobel P, Leroy O, Hamdane M, Sambo AV, Delacourte A, Buee L. Proteasome inhibition and Tau proteolysis: an unexpected regulation. FEBS Lett. 2005;579:1-5.

57. Młynarczuk-Biały I, Roeckmann H, Kuckelkorn U, et al. Combined effect of proteasome and calpain inhibition on cisplatin-resistant human melanoma cells. Cancer Res. 2006;66:7598-7605.

58. Kisselev AF, Akopian TN, Castillo V, Goldberg AL. Proteasome active sites allosterically regulate each other, suggesting a cyclical bite-chew mechanism for protein breakdown. Mol Cell. 1999;4:395-402.

59. Salzmann U, Kral S, Braun B, et al. Mutational analysis of subunit i beta2 (MECL-1) demonstrates conservation of cleavage specificity between yeast and mammalian proteasomes. FEBS Lett. 1999;454:11-15.

60. Ho YK, Bargagna-Mohan P, Wehenkel M, Mohan R, Kim K-B. LMP2-specific inhibitors: chemical genetic tools for proteasome biology. Chem Biol. 2007;14:419-430.

61. Schmidtke G, Emch S, Groettrup M, Holzhutter HG. Evidence for the existence of a non-catalytic modifier site of peptide hydrolysis by the 20 S proteasome. J Biol Chem. 2000;275:22056-22063.

62. Myung J, Kim KB, Lindsten K, Dantuma NP, Crews CM. Lack of proteasome active site allostery as revealed by subunit-specific inhibitors. Mol Cell. 2001;7:411-420.

63. Kisselev AF, Garcia-Calvo M, Overkleeft HS, et al. The caspase-like sites of proteasomes, their substrate specificity, new inhibitors and substrates, and allosteric interactions with the trypsin-like sites. J Biol Chem. 2003;278:35869-35877.