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Specific Cell-Permeable Inhibitor of Proteasome Trypsin-like Sites Selectively Sensitizes Myeloma Cells to Bortezomib and Carfilzomib

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SUMMARY

Proteasomes degrade the majority of proteins in mammalian cells, are involved in the regulation of multiple physiological functions, and are established targets of anticancer drugs. The proteasome has three types of active sites. Chymotrypsin-like sites are the most important for protein breakdown and have long been considered the only suitable targets for antineoplastic drugs; however, our recent work demonstrated that inhibitors of caspase-like sites sensitize malignant cells to inhibitors of the chymotrypsin-like sites. Here, we describe the development of specific cell-permeable inhibitors and an activity-based probe of the trypsin-like sites. These compounds selectively sensitize multiple myeloma cells to inhibitors of the chymotrypsin-like sites, including antimyeloma agents bortezomib and carfilzomib. Thus, trypsin-like sites are cotargets for anticancers drugs. Together with inhibitors of chymotrypsin- and caspase-like sites developed earlier, we provide the scientific community with a complete set of tools to separately modulate proteasome active sites in living cells.

INTRODUCTION

Proteasomes are proteolytic machines that are responsible for turnover of the majority of proteins in mammalian cells. The proteasome inhibitor bortezomib (Velcade) is being used for treatment of multiple myeloma, and at least five second-generation proteasome inhibitors, carfilzomib (PR-171) (Demo et al., 2007; O'Connor et al., 2009), NPI-0052 (Chauhan et al., 2005), CEP-18770 (Piva et al., 2008), MLN-9708 (Kupperman et al., 2010), and ONX-0912 (PR-047) (Zhou et al., 2009), are in clinical testing.

Proteasomes have three different types of active sites, chymotrypsin-like (β5), trypsin-like (β2), and caspase-like (β1). Cells of the immune system express γ-interferon inducible immunoproteasomes, which have slightly different catalytic subunits, namely the β5i (LMP7), β2i (MECL1), and β1i (LMP2). Of these, the chymotrypsin-like sites (β5 and β5i) have long been considered as the only suitable targets for drug development. Bortezomib and all drugs presently undergoing trials were developed to target these sites (Adams, 2004). However, bortezomib, CEP-18770, and MLN-9708 cotarget the caspase-like sites (Altun et al., 2005; Berkers et al., 2005; Kisselev et al., 2006; Kupperman et al., 2010; Piva et al., 2008), whereas NPI-0052 cotargets trypsin-like and caspase-like sites (Chauhan et al., 2005). This raises the question of whether inhibition of these sites is important for these drugs' antineoplastic activity. Recently, we have demonstrated that, in most multiple myeloma cell lines, cytotoxicity of inhibitors does not correlate with inhibition of the chymotrypsin-like sites but does correlate with loss of specificity and onset of inhibition of the trypsin-like sites (Britton et al., 2009). These data strongly suggest that the trypsin-like sites are important cotargets for antineoplastic agents (Britton et al., 2009). Cell-permeable inhibitors of these sites are needed to test this hypothesis.

Efforts to develop specific inhibitors of the trypsin-like site have met with limited success to date. Most proteasome inhibitors are short N-terminally capped peptides with an electrophilic group at the C terminus. This electrophile interacts, reversibly or irreversibly, with the catalytic N-terminal threonine of the proteasome active site. The peptide moiety of the inhibitor binds to the substrate binding pocket of the active site and is largely responsible for the specificity (Groll and Huber, 2004; Kisselev and Goldberg, 2001), although the specificity may be influenced by the electrophile (Screen et al., 2010). The trypsin-like sites cleave peptide bonds after a basic residue and also prefer basic residues in the P3 position (Groll et al., 2002; Harris et al., 2001; Nazif and Bogyo, 2001). Thus, an ideal inhibitor would have basic residues, preferably arginines, in the P1 and P3 positions. This presents a challenge from the synthetic point of view and would, most likely, render the inhibitor cell-impermeable. In fact, the

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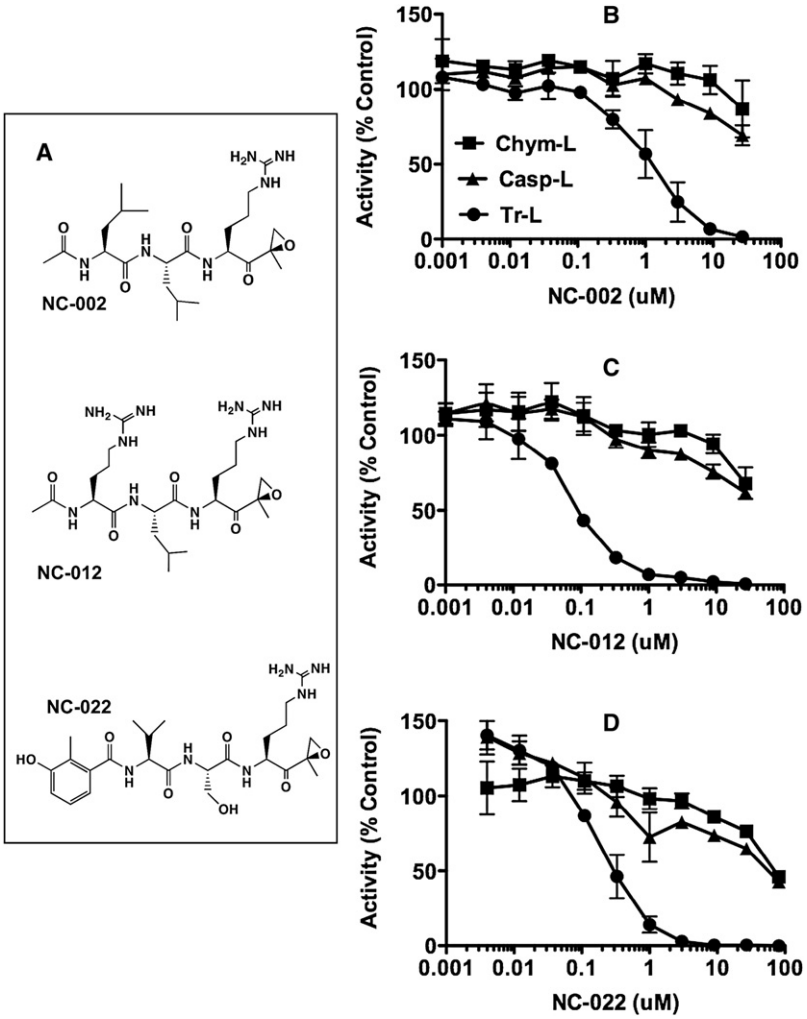


Figure 1. Effect of Inhibitors on Purified 26 Proteasomes

(A) Structures of inhibitors.

(B–D) Inhibition of proteasomes. After 30 min incubation of purified 26S proteasomes with inhibitors, all three activities were measured with fluorogenic substrates. Mock-treated proteasomes served as controls. Results are averages ± SEM of two (B and C) or four (D) independent experiments. Squares, chymotrypsin-like activity; triangles, caspase-like activity; circles, trypsin-like activity.

few β2-specific aldehydes (Loidl et al., 1999) and vinyl sulfones (Groll et al., 2002; Nazif and Bogyo, 2001) are not cell permeable. A cell-permeable peptide vinyl ester (ve) Hmb-VSL-ve, recently reported as specific inhibitor of the trypsin-like sites (Marastoni et al., 2005), did not show any inhibitory activity in our assays (Screen et al., 2010). Thus, at the onset of our work, no cell-permeable, β2-specific inhibitors or activity-based probes were available.

In this work, we describe the development of several cell-permeable peptide epoxyketone inhibitors as well as an active-site probe specific to the trypsin-like proteasome sites. We demonstrate that the most potent of these compounds sensitizes multiple myeloma cells to the specific inhibitors of the chymotrypsin-like sites, to bortezomib, and to the second-generation proteasome inhibitor carfilzomib.

RESULTS

Design and Initial Characterization of Inhibitors

We have designed several peptide epoxyketones to target the trypsin-like site (Figure 1A). Peptide epoxyketones are the most specific of the several structural classes of proteasome inhibitors (Groll and Huber, 2004; Kisselev, 2008; Kisselev and Goldberg, 2001). By forming a stable morpholino adduct with the proteasome catalytic N-terminal threonine, they take specific advantage of the proteasome's unique mechanism for cleaving peptide bonds (Groll et al., 2000). In fact, in more than a decade of research since the discovery of this class of proteasome inhibitors (Meng et al., 1999), no off-target effects of epoxyketones have been found.

Consistent with the nomenclature used in our previous work (Britton et al., 2009) we refer to inhibitors of the trypsin-like sites as NC-0X2, where "NC" stands for the Norris Cotton Cancer Center, "2" indicates that a compound inhibits β2 and β2i sites, and the character in the position marked by "X" changes from compound to compound. The first compound, NC-002 (Ac-LLR-ek), is the epoxyketone derivative of leupeptin. Leupeptin (Ac-Leu-Leu-Arg-al) is a cell-permeable inhibitor of cysteine proteases. In the context of purified proteasome, this peptide aldehyde is a specific inhibitor of the trypsin-like sites (Kisselev et al., 2006; McCormack et al., 1998). Peptide aldehydes inhibit serine, cysteine, and threonine proteases. We reasoned that replacing the aldehyde in leupeptin with a highly proteasome-specific epoxyketone (Groll et al., 2000) to generate Ac-LLR-amc (NC-002) would eliminate reactivity with lysosomal cysteine proteases, retain specificity to the trypsin-like sites, and not alter cell permeability of the compound.

The design of the second compound, NC-012 (Ac-RLR-ek), is based on the sequence of the best substrate of the trypsin-like site (Ac-RLR-amc) we developed earlier (Kisselev and Goldberg, 2005). The third inhibitor, NC-022 (Hmb-VSR-ek) has the same left-handed peptide fragment as the peptide vinyl-ester inhibitor of the trypsin-like sites reported in the literature (Marastoni et al., 2005) that lacked inhibitory activity in our hands (Screen et al., 2010). We chose this fragment because it was optimized to improve specificity toward these sites.

In order to enable the synthesis of the epoxyketone derivatives of arginine, we have modified the established procedure for the synthesis of leucine epoxyketones (Zhou et al., 2009) to allow for proper protection of the guanidine functional group during the procedure (see Supplemental Experimental Procedures available online).

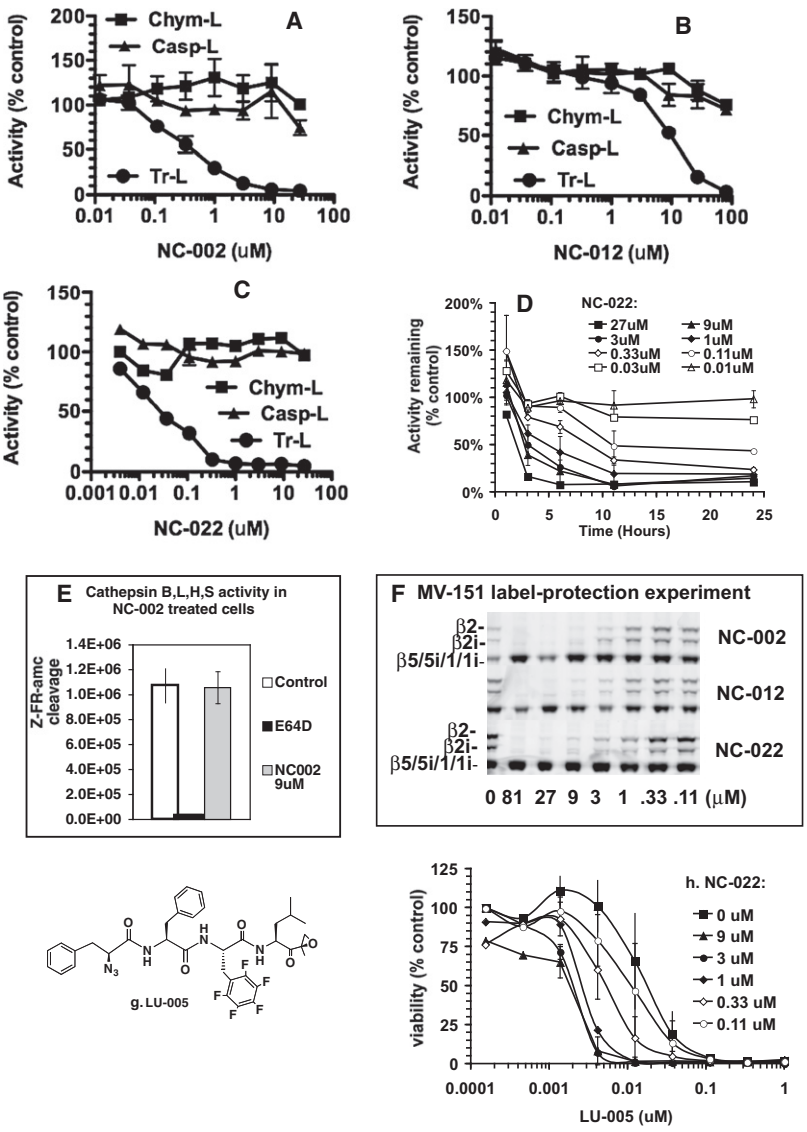


Figure 2. Effect of Compounds on Proteasomes in NCI-H929 Cells

(A–C) After overnight treatment with different concentration of inhibitors, proteasome activities were measured with Proteasome Glo Assay. Squares, chymotrypsin-like activity; triangles, caspase-like activity; circles, trypsin-like activity. Values are averages ± SEM of two (A and C) or three (B) independent experiments.

(D) Kinetics of inactivation of trypsin-like sites by NC-022. Concentrations of NC-022 are indicated on the graph. Similar inactivation rate was observed with NC-002 (Figure S1). Values are averages ± SEM of two independent experiments.

(E) NCI-H929 cells were treated with 9 μM NC-002 or 20 μM E-64d (cell-permeable analog of broad specificity cysteine protease inhibitor E-64) for 6 hr, and cumulative activity of cathepsins B, L, H, and S was measured in organellar extracts (Screen et al., 2010).

(F) Extracts of RPMI-8226 cells (which express more immunoproteasomes than NCI-H929 cells) were incubated with inhibitors for 30 min at concentrations indicated, then with 10 μM fluorescent broad spectrum active site probe MV-151 (Verdoes et al., 2006), followed by fractionation on SDS-PAGE. Gels were scanned on the Typhoon Imager (excitation laser-532 nm; emission filter-560 nm). All three inhibitors block modification of β2 and β2i subunits by MV-151 to a similar extent, indicating similar inhibition of both subunits.

(G) Structure of LU-005. Inhibition of proteasome after 1 hr treatment with LU-005 is presented in Figure S2.

(H) NCI-H929 cells were treated with different concentration of LU-005 for 1 hr followed by continuous treatment with concentrations of NC-022 indicated in the graph. 48 hr after the start of the treatment, viability was measured with the Alamar Blue mitochondrial dye conversion assay. Viability is expressed relative to the control (mock-treated cells). Values are averages ± SEM of two to four independent experiments.

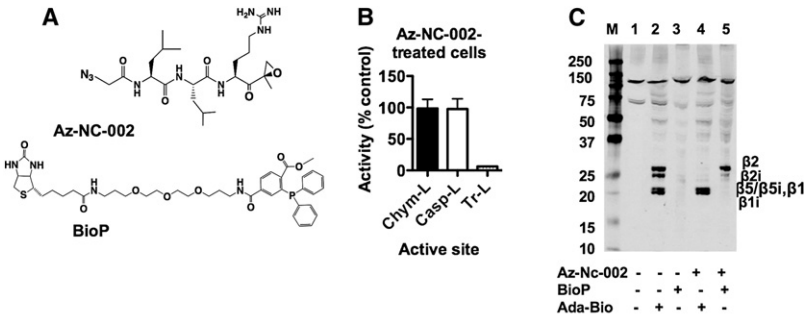
We initially evaluated the proteasome inhibitory potential of our compounds on purified 26S proteasomes from rabbit muscles (Figures 1B–1D). All three are potent and specific inhibitors of the trypsin-like sites. NC-012, as expected for the compound derived from the best substrate, was the most potent and specific in the series.

Next we treated NCI-H929 multiple myeloma (MM) cells with these compounds overnight and determined their proteasome inhibition profile (Figures 2A–2C). NC-002 and NC-022 specifically inhibited trypsin-like activity at submicromolar concentrations, but much higher concentrations of NC-012, the most potent inhibitor of the purified enzyme, were required to achieve inhibition in live cells. We attribute this decrease in potency with live cells to poor cell permeability. For cell-permeable compounds, maximal inhibitory effect was achieved within 6–10 hr after addition of NC-022 (Figure 2D) or NC-002 (Figure S1). Importantly, NC-002, the epoxyketone derivative of the cysteine protease inhibitor leupeptin, does not inhibit lysosomal cysteine proteases (Figure 2E).

Multiple myeloma cells express constitutive proteasomes and immunoproteasomes, and substrates used for the measurement of activity (Figures 2A–2C) are cleaved by both. To determine whether there are any differences in inhibition of constitutive proteasomes or immunoproteasomes by NC-002, NC-012, and NC-022 we used the fluorescent activity-based probe MV-151 (Verdoes et al., 2006) in a label-competition experiment. Extracts of RPMI-8226 MM cells (which express more immunoproteasomes than NCI-H929 cells) were treated first with the NC inhibitors and then with the MV-151 probe. This was followed by fractionation on SDS-PAGE to separate proteasome subunits and by imaging to reveal those subunits labeled by the probe (i.e., unmodified by the inhibitors). All three inhibitors blocked modification of β2 and β2i sites by the probe to a similar extent (Figure 2F). Thus, we conclude that NC-002, NC-012, and NC-022 are equipotent inhibitors of the trypsin-like sites of constitutive and immunoproteasomes.

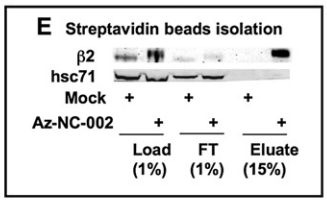
Specific Inhibitors of Trypsin-like Sites Sensitize Cells to Specific Inhibitors of Chymotrypsin-like Sites

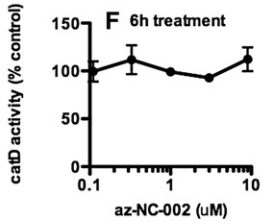
Next, we used our compounds to characterize trypsin-likes sites as targets and cotargets of antineoplastic agents. For this



D MS/MS identification of proteins specifically modified by az-NC-002 and isolated on streptavidin beads

Polypeptide	MW	Exp1	Exp2
	(kDa)	# of peptides	
PSMB7 (beta2)	30	3	6
PSMB10 (beta2i)	29	3	3
Cathepsin D	30	2	4
HSPA8/hsc71	71	2	8
TXNDC5 Thioredoxin domain-cont. protein 5	48	2	3





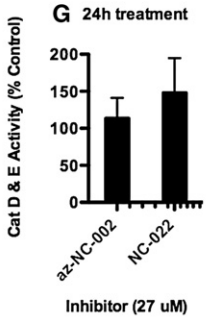


Figure 3. Az-NC-002 Is a β2/β2i-Specific Active Site Probe

(A) Structure of the probe and biotinylated phosphane (BioP).

(B–E) NCI-H929 cells were treated overnight with 9 μM az-NC-002 or mock-treated (control): subsequently, activity of three sites was measured (B), whole cell extracts were prepared, treated with biotinylated phosphane BioP (100 μM) for 1 hr, and divided into two uneven portions. After additional 1 hr treatment with 2 μM biotinylated irreversible inhibitor AdaBio (AdaK(Bio) Ahx_3L_3VS) to mark the migration of all active subunits on the gel (Kessler et al., 2001), a smaller portion of the samples was analyzed by gel electrophoresis and western blot. The membrane was probed with IRDye800-labeled streptavidin (C). A larger portion was used for the isolation of biotinylated polypeptides on Streptavidin beads. Isolated proteins were identified by mass-spectrometry (D). Only proteins reproducibly pulled down from az-NC-002-treated but not from control extracts and identified by at least two high probability peptides in each of the two replicate experiments are presented in the table. Additional data from mass-spectrometric experiment are provided in Table S1. (E) Analysis of the isolation by western blot. Load, sample loaded on Streptavidin-magnetic bead column; FT, unbound fraction; eluate, biotin eluate. Percentages of the total loaded on the gel are indicated in parenthesis.

(F and G) Effect of az-NC-002 and NC-022 on cathepsin D activity in NCI-H929 cells. Cells were treated with inhibitors for 6 hr at concentrations indicated and cathepsin D activity was measured (F). Cells were treated with 27 μM inhibitors for 24 hr and combined activity of cathepsin D and E was measured (G). Mock-treated cells served as control.

Values in (B), (G), and (H) are averages ± SEM of two to three independent experiments.

purpose we used NC-022, the most potent cell-permeable inhibitor. First we tested whether selective inhibition of trypsin-like sites is sufficient to reduce cell viability. We treated NCI-H929 cells with NC-022 for 48 hr and assayed cell viability with Alamar Blue mitochondrial conversion dye. No loss of viability was detected even at concentrations that completely inhibited the trypsin-like sites (data not shown). Thus, targeting trypsin-like sites is not sufficient to induce cytotoxicity in multiple-myeloma cells. (It should be noted that NCI-H929 is the most sensitive to proteasome inhibitors among myeloma cell lines [Britton et al., 2009]).

We next tested whether NC-022 sensitizes myeloma cells to inhibitors of the chymotrypsin-like sites. In the past few years, we have developed several peptide epoxyketone inhibitors of the chymotrypsin-like sites (Britton et al., 2009; Geurink et al., 2010); in these experiments we used the most specific of these, a pentafluorophenylalanine-containing compound referred to as here as LU-005 (Figure 2G; Figure S2) (Geurink et al., 2010) ("LU" stands for Leiden University and "5" indicates that the compound targets β5/β5i active sites). In the first experiment, we determined whether NC-022 sensitizes cells to LU-005, and what concentrations are needed to achieve this sensitization. Consistent with the treatment condition used in our previous work (Britton et al., 2009), where we demonstrated that a specific inhibitor of the caspase-like sites sensitizes myeloma cells to NC-005 (a specific inhibitor of chymotrypsin-like sites), we treated cells with LU-005 for 1 hr and then incubated them in the presence of different concentrations of NC-022 for 48 hr, whereupon an Alamar Blue assay for cell viability was performed. Dramatic dose-dependent sensitization was observed, with the IC_{50} of LU-005 increasing up to 8.5-fold. This maximal sensitization was achieved at 3 μM NC-022 (Figure 2H), which causes 90% inhibition of the trypsin-like sites within 4–6 hr after addition of NC-022 (Figure 2D). NC-002 caused similar sensitization to NC-005 (data not shown). Thus, near-complete inhibition of the trypsin-like sites is needed to achieve maximal sensitization effect.

Development of a β2-Specific Activity-Based Probe

To further confirm that our compounds are specific for the trypsin-like sites and that their biological activity is not due to off-target effects, we have synthesized az-NC-002, an NC-002-derived, activity-based probe (Figure 3A). We have chosen

NC-002 over NC-022 for derivatization because it was easier to introduce an azido group into this molecule. Addition of the azido group does not alter the specificity of the inhibitor (Figure 3B). Polypeptides modified by this probe were visualized on western blot after treating extracts of probe-treated NCI-H929 cells with azido-reactive biotinylated phosphane (BioP) in a Staudinger-Bertozzi ligation (Ovaa et al., 2003). One major az-NC-002-specific streptavidin-reactive band was detected (Figure 3C, lane 5). This matches the size of the band of the β2 subunit, which harbors the catalytic threonines of the trypsin-like sites. A weaker band of slightly lower mobility, matching the mobility of β2i band, was also detected. Corroborating that these bands are of proteasomal subunits, az-NC-002 treatment prevented subsequent modification of β2 and β2i subunits by another proteasome-specific probe (Ada-K[Bio]-Ahx$_3$L$_3$VS, lane 4). (A number of endogenously biotinylated proteins in the 70–100 kDa region were also detected and can serve as a loading control.)

To further confirm that the probe covalently modifies β2 and β2i subunits, we denatured the proteasome after BioP modification, isolated biotinylated polypeptides on streptavidin beads, and identified bound polypeptides by mass spectrometry after on-beads trypsin digestion. Peptides derived from β2 and β2i subunits were present in the samples isolated from extracts of the probe-treated cells but not from extracts of the control cells (Figure 3D; Table S1). No peptides derived from other catalytic subunits were detected. Thus, we conclude that az-NC-002 is a trypsin-like site-specific activity-based probe.

Surprisingly, several other polypeptides were also reproducibly identified as specific az-NC-002 targets. These include the aspartic protease cathepsin D (29 kDa), molecular chaperone hsc71 (71 kDa), and thioredoxin domain-containing protein TXNDC5 (48 kDa, Figure 3D). Of these, lysosomal aspartic protease cathepsin D (Benes et al., 2008) was of greatest concern to us. It has the same molecular weight as the β2 subunit, so some of the streptavidin-reactive material in the β2-band (Figure 3C) may be cathepsin D. To determine the significance of this potential off-target effect, we measured inhibition of cathepsin D by az-NC-002 but could not detect any significant inhibition (Figures 3F and 3G). We conclude that this probe either reacts with cathepsin D outside of the active site or inhibits a small fraction of the enzyme, detectable in the mass spectrometry experiment but not in the activity assay. Similarly, NC-022 did not inhibit cathepsin D even at concentrations as high as 27 μM (Figure 3G). Thus, chemical modification of cathepsin D is unlikely to contribute to the biological effects of the NC compounds.

There are no major streptavidin-reactive az-NC-002-specific bands in the 45–50 kDa and ~70 kDa region of the gel, where two other targets of az-NC-002, TXNDC5 and hsc71, migrate (Figure 3C). Probe modification of these proteins is responsible for one of the background bands in lane 5 on Figure 3C. We used western blot to determine which fraction of cellular hsc71 binds to streptavidin beads in extracts of az-NC-002-treated cells. Under conditions when most of β2-antibody reactive material was detected in streptavidin-bound fraction, the majority of hsc71-antibody reactive material was detected in the streptavidin-unbound fractions (Figure 3E). Thus, az-NC-022 modifies a small fraction of hsc71 and is therefore very unlikely to affect the overall protein-folding capacity of the cell.

Trypsin-like Sites Are Better Cotargets of Antimyeloma Agents than Caspase-like Sites

In the next set of experiments (Figure 4), we tested whether NC-022 sensitizes other MM cells to LU-005 and whether it is a more potent sensitizer than a specific inhibitor of caspase-like sites NC-001 (Britton et al., 2009). We have chosen four additional myeloma cell lines, MM1.R, RPMI-8226, KMS-18, and KMS-12-BM, for these experiments. These cell lines vary up to 40-fold in their sensitivity to bortezomib and NC-005 (Britton et al., 2009). In all experiments, NC-022 was used at a concentration that inhibited trypsin-like activity by more than 90% after 6 hr incubation.

In all MM cell lines, NC-022 reduced the IC_{50} for LU-005 by 4- to 10-fold. In three (MM1.R, RPMI-8226, KMS-18), NC-022 caused similar sensitization as NC-001 (Figures 4C–4E). In two others (NCI-H929 and KMS-12-BM, Figures 4A and 4B), NC-022 was a more potent sensitizer than NC-001. Thus, the trypsin-like sites are important cotargets of antineoplastic drugs in multiple myeloma cells; in fact, they are better cotargets than the caspase-like sites.

To confirm that LU-005 functions as a specific inhibitor of the chymotrypsin-like sites and to determine whether sensitization occurs upon clinically achievable inhibition of the chymotrypsin-like sites, we measured inhibition of all sites at the end of 1 hr treatment with LU-005 (Table 1). In patients treated with bortezomib, inhibition of the chymotrypsin-like sites that can be achieved at maximal tolerated doses does not exceed 70% (Hamilton et al., 2005); in patients treated with carfilzomib, it approaches 90% (O'Connor et al., 2009). As can be seen from Table 1, in all but the KMS-18 cell line, sensitization by NC-022 is observed upon clinically achievable 50%–80% inhibition of the chymotrypsin-like sites. Thus, sensitization of myeloma cells to specific inhibitors of the chymotrypsin-like sites by NC-022 is of potential clinical significance.

Effects of Combined Inhibition of Caspase-like and Trypsin-like Sites on Myeloma Cells

Due to the lack of effective, selective, and cell-permeable inhibitors of the trypsin-like sites, the effects of combined inhibition of the trypsin-like and caspase-like sites (in the absence of inhibition of the chymotrypsin-like sites) on growth and viability of mammalian cells could not be studied hitherto. We observed that continuous exposure to a mixture of NC-022 and NC-001 (at concentrations at which caspase-like and trypsin-like sites are both blocked by more than 90%) reduced cell viability by 20%–50% (Figure 4F). Proteasome inhibitors block cell proliferation and induce apoptosis. This moderate decrease could be a consequence of inhibition of cell proliferation without cell death. To determine whether apoptosis occurs, we measured caspase activation in the NCI-H929 and MM1.R cell lines. We found that treatment with a combination of NC-001 and NC-002, in contrast to LU-005 treatment, did not cause any significant increase in caspase activity (Figure 4G). Therefore, we conclude that the moderate decrease in viability in cells cotreated with NC-001 and NC-002 is not due to apoptosis and most likely reflects inhibition of cell proliferation. We would like to emphasize that this is the first example of a biological effect on mammalian cells due to inhibition of the caspase-like and trypsin-like sites in the absence of inhibition of the chymotrypsin-like sites.

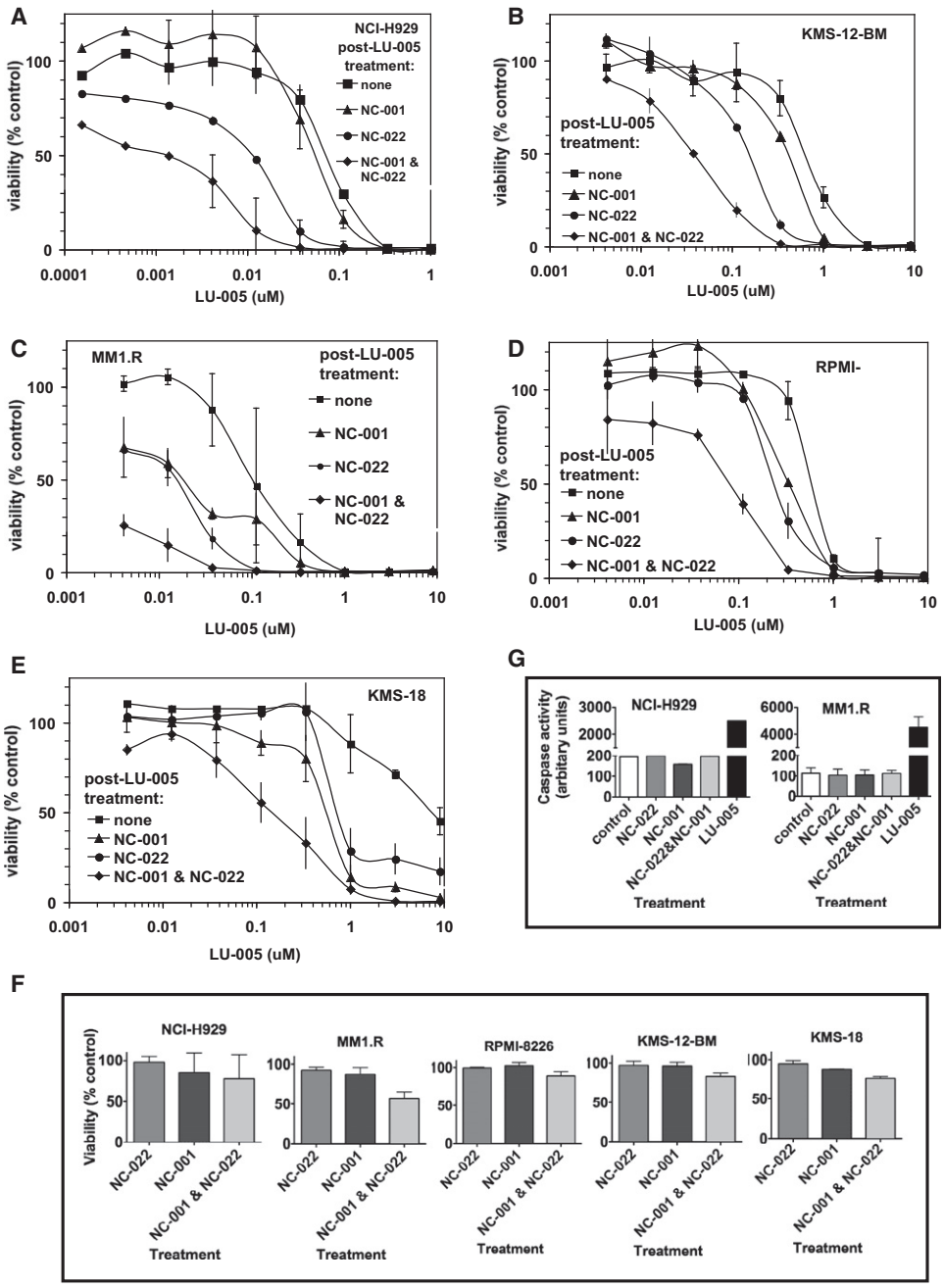


Figure 4. Comparative Analysis of Sensitization of Different Myeloma Cells to Specific Inhibitor of the Chymotrypsin-like Sites (LU-005) by NC-022 and Specific Inhibitor of Caspase-like Sites (NC-001)

(A–E) Sensitization of various MM cells to LU-005. Multiple cells were treated with LU-005 for 1 hr and then divided into five aliquots. Four aliquots were cultured further in the presence of either NC-022 (circles), or 2 μM NC-001 (triangles), or both (diamonds), or in the absence of inhibitors (squares). Concentration of NC-022 (27 μM in RPMI-8226, 9 μM in KMS-12-BM, 3 μM in all other cell lines) was adjusted to inhibit the trypsin-like activity by 90% within first 3–6 hr of incubation. The fifth aliquot was used to measure proteasome inhibition, which is presented in Table 1.

(F) After 48 hr treatment with 2 μM NC-001 and/or NC-022 (same concentrations as in A and B), cell viability was measured with Alamar Blue.

(G) Effect of NC-001 and NC-022 on apoptosis in NCI-H929 and MM1.R cells. After 9 hr incubation with inhibitors, caspase-3/7 activity was measured. Values on all graphs are averages ± SE of two to three independent experiments.

We next studied the effects of the mixture of NC-001 and NC-022 on MM cells sensitivity to LU-005. As in the previous experiments, cell were treated with LU-005 for 1 hr and then cultured with a NC-001/NC-022 mixture after removal of LU-005. The mixture of NC-001 and NC-022 appeared to be a much stronger sensitizer than NC-022 alone (Figures 4A–4E). Notably, there was always a concentration of LU-005 at which a mixture of NC-001 and NC-002 caused a dramatic loss of

Table 1. NC-022 and, More Dramatically, a Mixture of NC-022 and NC-001 Sensitize Multiple Myeloma Cells to LU-005 upon Clinically Achievable Proteasome Inhibition

		Viability (% Control)			Inhibition of Active Sites (% Control)		
Cell line	LU-005 (μM)	-NC-022	+NC-022	+NC-002 & NC-001	β5	β2	β1
NCI-H929	0.11	30 ± 2	2 ± 1	0.65 ± 0.25	86 ± 3	11 ± 4	6 ± 8
	0.043	80 ± 8	10 ± 2	1.5 ± 0.5	72 ± 8	6 ± 4	15 ± 20
	0.011	94 ± 11	48 ± 3	10 ± 4	48 ± 5	−6 ± 1	5.5 ± 4.5
	0.004	100 ± 13	69 ± 0	36 ± 14	22 ± 11	−13 ± 7	−7 ± 10
	0.0014	97 ± 9	77 ± 2	50 ± 17	22 ± 4	−9 ± 2	−14 ± 15
MM1.R	0.11	47 ± 42	1.7 ± 0.2	1.2 ± 1.2	76 ± 3	23 ± 4	27 ± 5
	0.043	88 ± 20	18.5 ± 5.5	2.8 ± 1.5	54 ± 12	5 ± 11	6 ± 12
	0.011	106 ± 4	57 ± 10	15 ± 9	31 ± 10	5 ± 1	3 = 16
	0.004	102 ± 4	66 ± 1	26 ± 6	17 ± 4	6.5 ± 0.5	6 ± 8
RPMI-8226	1.0	10 ± 10	6 ± 3	1 ± 0	82 ± 16	22 ± 19	37 ± 4
	0.33	94 ± 1	31 ± 9	4.6 ± 0.7	62 ± 32	13 ± 11	37 ± 15
	0.11	108 ± 4	95 ± 2	39 ± 5	49 ± 30	3 ± 5	23 ± 2
	0.037	109 ± 2	104 ± 5	76 ± 3	45 ± 26	1 ± 17	16 ± 7
	0.012	109 ± 1	108 ± 4	82 ± 11	16 ± 8	−21 ± 4	−34 ± 5
KMS-12-BM	1.0	27 ± 6	1.5 ± 0.1	1 ± 0	95 ± 2	29 ± 7	11 ± 16
	0.33	80 ± 9	12 ± 2	2 ± 0	81 ± 3	3 ± 18	6 ± 12
	0.11	94 ± 16	65 ± 2	20 ± 4	56 ± 9	4.7 ± 1.1	3 ± 16
	0.037	89 ± 8	90 ± 5	50 ± 2	48 ± 9	−12 ± 1	−1 ± 5
	0.012	101 ± 5	104 ± 9	79 ± 6	16 ± 8	−21 ± 3.5	−34 ± 5
KMS-18	9.0	45 ± 8	17.5 ± 7.5	0.8 ± 0.2	97 ± 1	62 ± 5	42 ± 2
	3.0	72 ± 2	24 ± 8	1 ± 0.1	94 ± 4	50 ± 14	39 ± 12
	1.0	88 ± 16	29 ± 12	8 ± 4	85 ± 6	38 ± 5	32 ± 4
	0.33	108 ± 0	106 ± 16	33 ± 20	69 ± 8	24 ± 6	29 ± 7
	0.11	108 ± 1	106 ± 4	56 ± 16	47 ± 4	12 ± 16	21 ± 16
	0.037	108 ± 1	104 ± 3	79 ± 14	14	−13 ± 13	0 ± 8

Results of proteasome inhibition and viability measurements form experiment shown in Figure 4. Cells were treated with LU-005 for 1 hr, followed by measurement of peptidase activities; a fraction of the cells was cultured in the presence of NC-022, of NC-022 and NC-001, or of none of the inhibitors for 48 hr, whereupon cell viability was measured. Viability values are the same as in the graphs in Figure 4. Values are averages ± SEM of two (activity) or three (viability) independent measurements. Negative values indicate activation. See caption of Figure 4 for further experimental details.

cell viability as compared to the effect of LU-005 as a single agent (i.e., from 80%–100% to 10%–20%, Figures 4A–E). At this concentration, LU-005 inhibited chymotrypsin-like sites by a clinically achievable 50–85% (Table 1). A mixture also sensitized cells at much lower concentrations of LU-005 (i.e., upon much smaller inhibition of chymotrypsin-like sites, Table 1) than either NC-001 or NC-002 alone.

NC-022 Specifically Sensitizes Myeloma Cells to Bortezomib and Carfilzomib

To further strengthen the clinical relevance of our observations, we tested whether NC-022 sensitizes MM cells to the FDA-approved proteasome inhibitor bortezomib and to carfilzomib, a second-generation peptide epoxyketone proteasome inhibitor undergoing phase II–III clinical trials (Demo et al., 2007; O'Connor et al., 2009). We used two cell lines in these experiments, one of the most bortezomib-sensitive (NCI-H929) and one of the most bortezomib-resistant (KMS-12-BM) (Britton et al., 2009). Both cell lines were sensitized to the two agents (Figure 5). In NCI-H929 cells, sensitization to both compounds occurred upon clinically achievable proteasome inhibition (Table 2). In KMS-12-BM cells, sensitization to bortezomib, although more dramatic than in NC-H929 cells, was observed above clinically achievable inhibition of the chymotrypsin-like sites. Sensitization to carfilzomib was observed at clinically achievable levels.

To assess whether coinhibition of trypsin-like sites increases toxicity to normal cells, we tested whether NC-022 increases toxicity of bortezomib and carfilzomib to peripheral blood mononuclear cells (PBMNCs). NC-022 did not sensitize cells from any of the three donors to either of two agents (Figure 5; Figure S3). This lack of sensitization is surprising because NC-001 sensitizes PBMNCs to inhibitors of the chymotrypsin-likes sites (Britton et al., 2009). Thus, NC-022 selectively sensitizes malignant MM cells to bortezomib and carfilzomib.

DISCUSSION

Site-specific, cell-permeable inhibitors of the proteasome's trypsin-like sites have long been missing from the otherwise impressive palette of reagents available to study the role of the proteasome and its active sites in different aspects of cellular function. The compounds described herein fill this void.

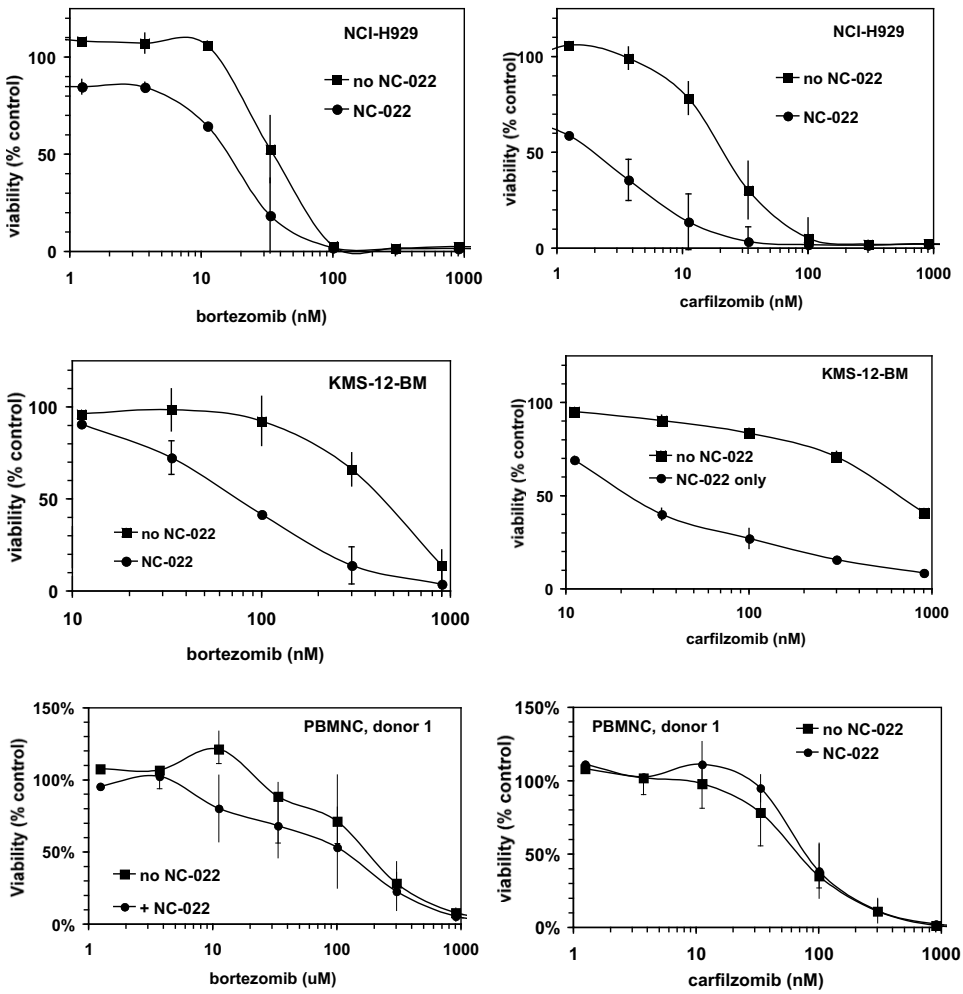


Figure 5. NC-022 Specifically Sensitizes Multiple Myeloma Cells to Bortezomib and Carfilzomib

NCI-H929, KMS-12-BM, or PBMNC cells were treated with bortezomib or carfilzomib for 1 hr and then with NC-022 for 48 hr after which their viability was measured. NC-022 (3 μM in NCI-H929 and 9 μM in KMS-12-BM) was used at concentrations that inhibit trypsin-like activity by more than 90%. Proteasome activity was measured immediately after 1 hr bortezomib and carfilzomib treatment and 5 hr after the start of NC-022 treatment, and is presented in Table 2. Results of treatments of PBMNC from two additional donors are presented in Figure S3. Results are means ± SEM of two to three independent experiments. Squares, post-bortezomib/carfilzomib culturing in the absence of NC-022; circles, culturing in the presence of NC-022.

The significance of this work is two-fold. First, it describes the development of (1) cell-permeable specific inhibitors of the trypsin-like sites of the proteasome and (2) an active site probe derived from these inhibitors. Second, we use one of these compounds, NC-022, to demonstrate that these sites are cotargets of antineoplastic drugs in multiple myeloma. Trypsin-like sites appear to be better cotargets than caspase-like sites for two reasons. First, in two out of five cell lines tested, NC-002 caused better sensitization to chymotrypsin site-specific inhibitor than NC-001, while in three others sensitization was similar (Figure 4). Second, NC-022 selectively sensitized MM cells to carfilzomib and bortezomib (Figure 5); sensitization by NC-001 was not selective (Britton et al., 2009). It remains to be determined whether NC-022 could be developed into a drug to be used in combination with bortezomib and carfilzomib or whether development of newer agents that inhibit chymotrypsin- and trypsin-like sites with equal potency would be a better approach to translate the results of this work into novel treatments for patients.

The importance of this work goes beyond oncology. Several years ago, we found that the ability of leupeptin (used as specific inhibitor of the trypsin-like sites of purified proteasomes) to block degradation of model substrates depends on the content of basic residues in a substrate (Kisselev et al., 2006). Using inhibitors developed in this work as well as proteomic approaches, we can now ask whether basic proteins will be selectively stabilized upon treatment of cells with β2-specific proteasome inhibitors.

Proteasomes are involved in a variety of biological processes (e.g., inflammation and immune response). One immediate application of these compounds would be to study the role of trypsin-like sites in the generation of MHC class I epitopes. Although it is well established that these peptides or their precursors are generated by proteasomes, the role of individual active

Table 2. NC-022 Sensitizes Multiple Myeloma Cells to Bortezomib and Carfilzomib upon Clinically Achievable Proteasome Inhibition

				Inhibition of Active Sites (% control)					
				Chymotrypsin-like Sites			Trypsin-like Sites		
				Time after Start of Experiment			Time after Start of Experiment		
	Inhibitor	Viability (% Control)		1 hr[a]	6 hr	6 hr	1 hr	6 hr	6 hr
Cell Line	(nM)	- NC-022	+NC-022		-NC-022	+NC-022		-NC-022	+NC-022
NCI-H929	**Bortezomib**								
	100	2.9 ± 1.5	1.9 ± 1.5	96 ± 2	86 ± 1	93 ± 2	36 ± 17	45 ± 2	96 ± 1
	33	53 ± 17	19 ± 19	89 ± 2	85 ± 4	48 ± 35	26 ± 15	31 ± 0	94 ± 1
	11	106 ± 2	65 ± 1	64 ± 7	41 ± 3.5	34 ± 29	8 ± 11	14 ± 2	93 ± 2
	3.7	107 ± 5	85 ± 3	9 ± 7	13 ± 1	14 ± 13	0 ± 7	6 ± 4	93 ± 2
KMS-12-BM	900	14 ± 12	3.8 ± 2.5	93 ± 5	84 ± 16	88 ± 5	28 ± 5	26 ± 19	93 ± 1
	300	66 ± 9	14 ± 11	93 ± 3	76 ± 18	83 ± 6	22 ± 12	24 ± 18	91 ± 2
	100	92 ± 9	42 ± 10	90 ± 6	64 ± 23	75 ± 6	19 ± 3	15 ± 11	92 ± 1
	33.3	98 ± 13	72 ± 1	82 ± 8	56 ± 24	62 ± 7	10 ± 11	8 ± 15	92 ± 0
NCI-H929	**Carfilzomib**								
	33	30 ± 11	4 ± 2	75 ± 17	95 ± 7	71 ± 23	17 ± 8	46 ± 24	94 ± 3
	11	78 ± 15	14 ± 8	39 ± 35	89 ± 11	52 ± 32	0 ± 14	39 ± 36	93 ± 2
	3.7	99 ± 9	36 ± 15	14 ± 43	65.5	41 ± 29	−7 ± 18	3	93 ± 3
KMS-12-BM	900	41 ± 7	9 ± 1	98 ± 3	97 ± 0	99 ± 4	75 ± 14	72 ± 2	94 ± 3
	300	71 ± 2	15 ± 1	97 ± 3	97 ± 1	95 ± 4	49 ± 18	69 ± 2	93 ± 2
	100	83.5 ± 3	27 ± 5	95 ± 3	97 ± 1	90 ± 3	18 ± 8	62 ± 2	94 ± 2
	33	91 ± 3	40 ± 3	90 ± 3	97 ± 1	84 ± 6	0 ± 11	45 ± 4	91 ± 2
	11	95 ± 3	69 ± 2	75 ± 6	67 ± 17	63 ± 13	−3 ± 10	5 ± 25	91 ± 2

Results of proteasome inhibition and viability measurements from experiment shown in Figure 5. Viability values are the same as in the graphs on Figure 5. Values are averages ± SE of two (activity) or three (viability) independent measurements. Negative values indicate activation. Caspase-like activity is presented in Table S2. See legend to Figure 5 for further experimental details.

[a] Cells were treated with bortezomib and carfilzomib for 1 hr, and activity was measured immediately after washout of the drug. NC-022 was added, and 5 hr after NC-022 addition proteasome activity was measured again. Note that trypsin-like activity was inhibited by >90% but inhibition of the chymotrypsin-like activity was not altered by NC-022 treatment. Viability was measured 48 hr after the start of the experiment.

sites in the excision of specific epitopes is not known (Groettrup and Schmidtke, 1999; Rock et al., 2002). Specific activity of the trypsin-like sites of immunoproteasomes (β2i) is several-fold higher than that of their counterparts in the constitutive particles (β2) (Cascio et al., 2001). Some MHC class I ligands have basic residues at the C terminus (Rammensee et al., 1995). The C-termini of these specific peptides may be generated by cleavages at the trypsin-like sites. Because of a lack of specific inhibitors of these sites, this hypothesis could not previously be tested; it can be tested now using the reagents developed in this work.

SIGNIFICANCE

The cell-permeable inhibitors of trypsin-like sites reported here fill the largest remaining void in the impressive palette of proteasome inhibitors available to biologists. This study completes the development of site-specific inhibitors and activity based probes of proteasome different active sites, at least for the constitutive proteasome. These active sites can now be downregulated individually to the desired extent in living cells. Inhibitors developed in this study will find wide use to study the role of trypsin-like sites in protein degradation, MHC class I antigen presentation and other biological processes, and, as demonstrated in this study for multiple myeloma, to determine whether these sites can be targeted for the treatment of other cancers or different diseases.

EXPERIMENTAL PROCEDURES

Synthesis of Inhibitors

Synthesis of NC-002, NC-012, NC-022, and az-NC-002 and analytical data for compounds are described in the Supplementary Materials section. Bortezomib was purchased from LC laboratories. Carfilzomib was synthesized as described (Britton et al., 2009; Demo et al., 2007; Zhou et al., 2009). BioP was synthesized as described (Verdoes et al., 2008).

Proteasome Purification and Assays

26S proteasomes were purified from rabbit muscle as described (Screen et al., 2010). To determine inhibition of purified proteasomes, they were incubated with inhibitors for 30 min at 37°C followed by assay of activity with fluorogenic substrates Suc-LLVY-amc (chymotrypsin-like site), Ac-RLR-amc (trypsin-like site), and Ac-nLPnLD-amc (caspase-like site). See Geurink et al. (2010) for detailed description of the procedure. 12% Novex Bis-Tris gels (Invitrogen) with MOPS running buffer were used for electrophoretic separation of catalytic subunits modified by active-site probes. Inhibition of active sites inside cells was assayed using luminescent ProteasomeGlo assay (Promega) (Moravec et al., 2009) as described in our previous study (Britton et al., 2009). Results of the cell-based ProteasomeGlo assay are undistinguishable from the activity measurement with fluorogenic substrates in extracts, in which background cleavage by non-proteasomal proteases is accounted for by subtracting

activity left in extract after treatment with high concentrations of highly specific proteasome inhibitor epoxomicin (Britton et al., 2009) (see Figure S4B for data and Supplemental Experimental Procedures for details of the assay with fluorogenic substrates). Cathepsin B, H, L, S activity was measured with pan-cathepsin substrate Z-FR-amc (Kirschke and Wiederanders, 1994) in extracts of cytosol-less cells at pH 6.0 as described in the previous study (Screen et al., 2010). Cathepsin D activity was measured in cytosol-less extracts using SensoLyte®520 Cathepsin D Fluorometric Assay Kit (AnaSpec). Combined cathepsin D and E activity was measured using the same kit, in which cathepsin D substrate provided with the kit was replaced with 7-Methoxycoumarin-GKPILFFRLK(Dnp)-r-NH_2 (where "r" stand for D-Arg) internally quenched fluorogenic substrate of cathepsin D and E. In this case, we used pH 3.0 assay buffer provided with the kit was used for cell extraction. All activity observed using both procedures was inhibited by more than 98% by specific inhibitor of aspartic proteases pepstatin A.

Cell Culture

All cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. Viability of multiple myeloma cells was measured with Alamar Blue mitochondrial dye conversion assay. Viability of PBMNC was measured using Cell Titer-Glo luminescent cell viability assay (Promega), which is based on quantification of ATP present in the cells. Caspase-3/7 activity was measured using ApoONE 3/7 homogeneous assay (Promega). (This assay uses Ac-DEVD-Rhodamine110 cell permeable fluorogenic substrate.)

Isolation and Identification of Polypeptides Modified by az-NC-002

Cells were treated with the activity-based site probe overnight and lyzed with 50 mM Tris-HCl, 10% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5% CHAPS, 1 mM ATP. After 1 hr treatment with 100 μM BioP proteins were denatured with 1% SDS, followed by affinity purification of biotinylated polypeptides on Streptavidin coated magnetic beads. After on-beads trypsin digestion, samples were analyzed by LC-MS/MS. See Florea et al. (2010) for the detailed description of the procedure. IRDye 800 CW-conjugated Streptavidin was purchased from Rockland, hsc71 antibodies from Abcam (Cat # 19136), and β2 antibodies from Abgent (Cat #AP2914b).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables and can be found with this article online at doi:10.1016/j.chembiol.2011.02.015.

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