Selective substrates and activity-based probes for imaging of the human 20S proteasome in cells and blood samples

Wioletta Rut[1], Marcin Poręba[1,2], Paulina Kasperkiewicz[1,2], Scott J. Snipas[2], Marcin Drąg[1]

[1]Department of Bioorganic Chemistry, Faculty of Chemistry, Wroclaw University of Science and Technology, Wyb.Wyspianskiego 27, 50-370 Wroclaw, Poland

[2]Program in Cell Death and Survival Networks, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA92037, USA

Corresponding: wioletta.rut@pwr.edu.pl, marcin.drag@pwr.edu.pl

Abstract

The proteasome is an enzyme complex critical for maintaining protein homeostasis. Perturbed proteasome function leads to pathologies including cancer, autoimmune and neurodegenerative disease. Therefore, the proteasome constitutes an excellent molecular target for pharmaceutical development. The catalytic 20S proteasome core possesses caspase-like (β-1), trypsin-like (β-2) and chymotrypsin-like (β-5) activities, each with different specificity. Here, using the HyCoSuL approach we designed and synthesized novel and selective fluorogenic substrates for each of these three proteasome activities and applied them to assess inhibition of proteasome subunits by MG-132 and a clinically-used inhibitor bortezomib. Our results confirm the utility of designed substrates in biochemical assays. Furthermore, selective peptide sequences obtained in this manner were used to construct fluorophore-labeled activity-based probes and then utilized to detect each proteasome subunit simultaneously in lysates of HEK-293F cells and red blood cells. Overall, we describe a simple and rapid method useful to measure proteasome activity in whole human blood samples that could enable early diagnosis of pathological states associated with aberrantly upregulated proteasome activity.

Keywords: HyCoSuL, fluorogenic substrates, specific activity-based probes, proteasome β-subunits

In eukaryotic cells, intracellular protein homeostasis is maintained by the Ubiquitin-Proteasome System (UPS) and by the lysosomal degradation pathway. The UPS is responsible for specific degradation of short-lived cytosolic and nuclear proteins and thus tightly controls processes as diverse as apoptosis, cell cycle regulation, DNA repair, gene transcription, signal transduction, and the inflammatory response [1, 2]. The primary component of the UPS is a multi-catalytic enzyme complex called the 26S proteasome, which is composed of a 20S proteolytic core (constitutively expressed in all eukaryotic cells) and one (or two) 19S regulatory particles [3]. The 20S proteasome can also be associated with another regulatory subunit, the 11S/PA28, which stimulates its activity [4]. The catalytic core is a 28-subunit barrel-shaped structure organized as four stacked rings of seven subunits. The two outer rings consist of seven α-subunits, while the two inner rings contain seven β-subunits. The α-rings function structurally, whereas the β-subunits mediate 20S proteasome proteolytic activity. All three β-subunits (β-1, β-2, and β-5) possess different primary substrate specificities. The β-1 subunit exhibits caspase-like activity and cleaves peptide bonds after acidic amino acids. β-2 hydrolyzes substrates C-terminal to positively charged residues, displaying trypsin-like activity, and the β-5 subunit exhibits chymotrypsin-like activity and cuts preferentially after hydrophobic residues [5]. These three distinct active sites make the proteasome an excellent biological machinery for protein degradation. Whereas, under inflammatory conditions these three catalytic β-subunits can be replaced by inducible subunits: β-1i, β-2i and β-5i resulting in the formation of a new proteolytic complex called immunoproteasome [6].

Site-directed mutagenesis analysis of the yeast proteasome has revealed that chymotrypsin-like activity of proteasome has the greatest impact on proteolysis [7, 8], an observation that has promoted development of numerous synthetic inhibitors of the β-5 subunit [9]. Three (Bortezomib, Carfilzomib, Ixazomib) have been approved as drugs to treat multiple myeloma therapy and other malignancies [10-12]. Up to now, few studies have addressed inhibition of β-1 and β-2 subunits, although Kisselev and co-workers demonstrated significantly reduced protein degradation when chymotrypsin-like plus either caspase-like or trypsin-like sites were blocked simultaneously [13]. Further studies using site-specific inhibitors revealed that the therapeutic efficacy of proteasome inhibitors against cancer is enhanced by co-inhibition of caspase-like and trypsin-like activity [14, 15], suggesting that all three subunits could serve as targets in tumor therapy.

Activity based probes (ABPs) are chemical tools useful to investigate proteasome subunit inhibition. Proteasome ABPs are composed of (1) a C-terminal electrophile "warhead" (either vinyl sulfone (VS) or epoxyketone), (2) a targeting peptide sequence, (3) a linker, and (4) a

reporter group, often biotin or a fluorophore. Most ABPs are derived from sequences of broad-spectrum proteasome inhibitors and react to all proteasome active sites [16, 17]. However, recently several subunit-selective ABPs have been shown to detect immuno- and constitutive proteasome [18]. ABPs have been used to compare expression of immune- and constitutive proteasome in primary patient cells from hematological malignancies [18].

Here, we identify peptide sequences potentially useful to design selective substrates and ABPs for each subunit of human 20S proteasome. To date, proteasome substrate preferences have been determined using only natural amino acids, making it more difficult to identify specific substrates for each subunit [19-21]. However, incorporation of unnatural amino acids into a peptide sequence is essential to construct subunit-selective probes due to overlapping substrate specificity of the 20S proteasome [17, 18, 22, 23]. Thus, we determined the extended substrate specificity profile of proteasome catalytic subunits using P1 diverse hybrid combinatorial substrate libraries (HyCoSuL), which are comprised >100 non-proteinogenic amino acids in each position. Next, we analyzed the obtained substrate specificity profiles to identify a peptidic scaffold selective for each proteasome subunit and then designed and synthesized a set of specific fluorogenic substrates recognized only by one proteasome subunit, a result previously unreported. We then utilized those peptidic motifs to synthesize subunit-selective ABPs. Finally, to detect all proteasome subunits simultaneously we applied three different fluorophores in ABP structure. ABP selectivity was confirmed using purified samples of human 20S proteasome, HEK-293F cell lysates and red blood cell lysates.

Results and Discussion

20S proteasome substrate specificity in the P1 position

We began by determining proteasome substrate preference in the S1 pocket. We screened a defined library, Ac-Ala-Arg-Leu-P1-ACC (where P1=139 amino acids), enabling analysis of substrate specificity of each catalytic β-subunit of the proteasome. Analysis of Ac-Ala-Arg-Leu-P1-ACC revealed that hydrolysis C-terminal to the basic amino acid arginine represents a predominant activity of human proteasome (Figure 1). Arginine derivatives such as *L*-hArg, *L*-Arg(Me) and *L*-Phe(guan) were also recognized but with four times lower efficiency than *L*-Arg (Figure 1). Cleavage after hydrophobic residues (*L*-Leu, *L*-Ala, *L*-2-Abu, *L*-Nva, *L*-2Aoc, and *L*-Tyr(Bzl)) was moderate, and acidic amino acids were almost not recognized. These results indicate overall that amino acids in P4-P2 positions are critical for proteasome activity

and selecting of preferred residues in S4-S2 pockets can lead to obtain of selective substrate for each β-subunit [24].

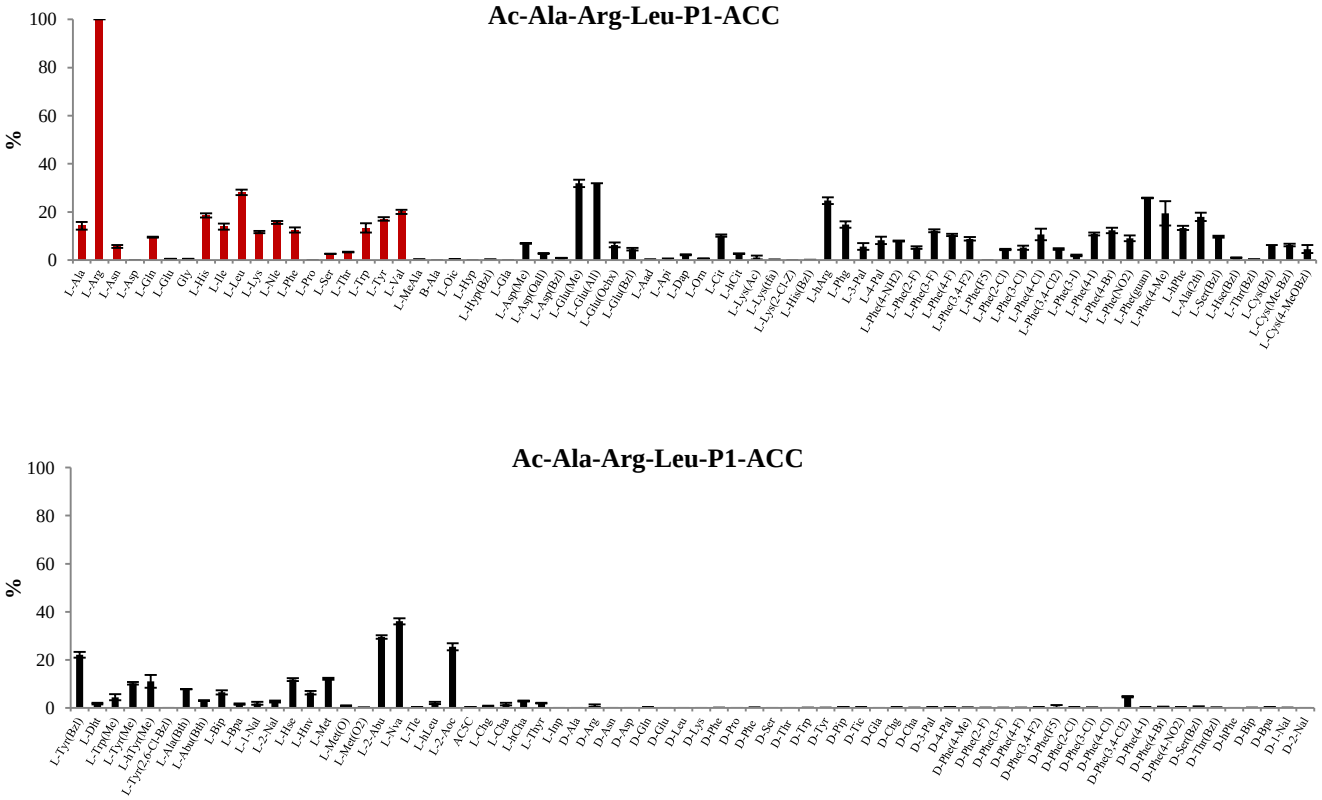


Figure 1. Proteasome substrate preferences in the P1 position. X-axis represents 19 natural (red) and 120 unnatural (black) amino acids; Y-axis represents the average relative activity adjusted to a percentage of the best amino acid.

P4-P2 substrate preferences

To extensively profile β-subunit substrate specificities, we employed three previously described HyCoSuLs with acidic *L*-Asp (for the β1-subunit), basic *L*-Arg (for the β2-subunit) and aliphatic *L*-Ala (for the β5-subunit) in the P1 position. These libraries are comprised of over 100 unnatural and 19 natural amino acids in P4-P2 positions (Figure 2) [25-27]. For analysis of β5-subunit we selected alanine since, based on the P1 library screening results, this aliphatic amino acid was recognized by proteasome.

P2 position analysis. We found that caspase-like subunit shows preference in the P2 position for hydrophobic amino acids such as *L*-Trp, *L*-Nle, *L*-Phe, *L*-hLeu, *L*-2-Aoc, *L*-Lys(2-Cl-Z), and *L*-hCha (see Supplemental Information for a list of unnatural amino acid abbreviations). Tyrosine and its derivatives (*L*-hTyr, *L*-hTyr(Me)) were also well recognized. The most

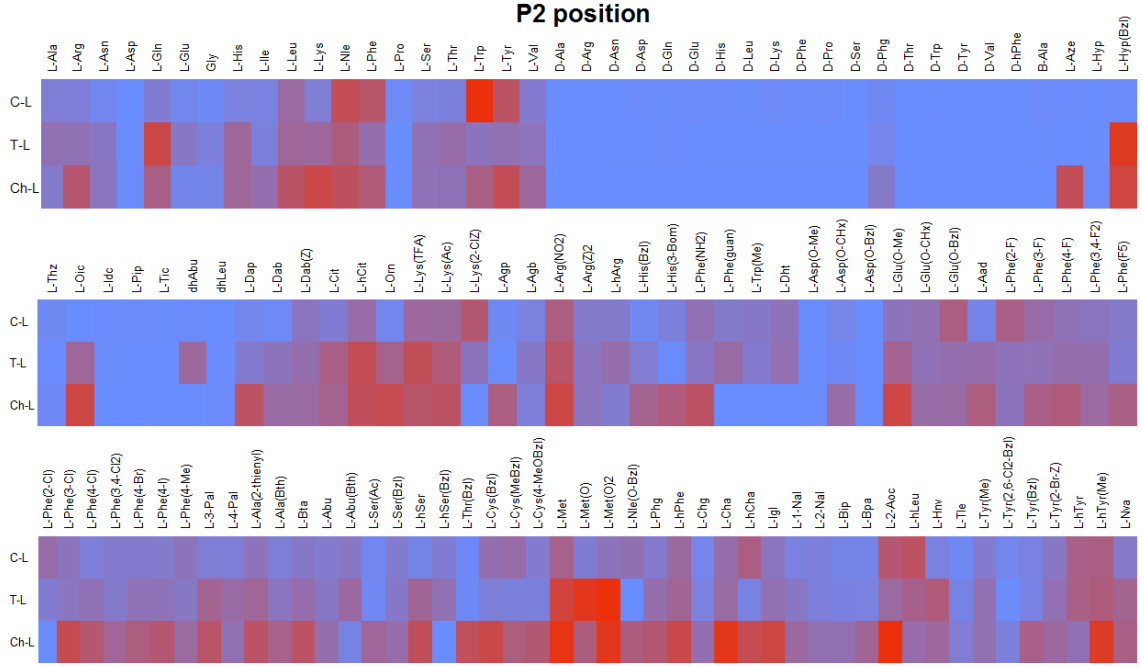
preferred amino acids in the S2 pocket in terms of trypsin-like subunit were *L*-Met(O)$_2$, *L*-Met(O), *L*-Met, *L*-Hyp(Bzl) and *L*-Gln. Chymotrypsin-like site recognized basic and hydrophobic amino acids almost at the same level, the best of them were *L*-2-Aoc, *L*-Met, *L*-Met(O)$_2$, *L*-Cha, *L*-Hyp(Bzl), *L*-Oic, *L*-Orn, *L*-Dap, *L*-Lys, and *L*-Arg. The three catalytic β-subunits did not prefer *D*-amino acids or proline in the P2 position.

P3 position analysis. Caspase-like subunit displayed narrow specificity recognizing few hydrophobic residues such as *L*-Pro, *L*-2-Abu, *L*-Nva, *L*-Leu, *L*-Nle, and *L*-Ala. Trypsin-like site recognized most amino acids except acidic residues, *D*-amino acids, proline and its derivatives. The most preferred amino acids were *L*-2-Abu, *L*-hSer, *L*-Met, *L*-hArg, *L*-Phe(guan), *L*-Val, *L*-Arg, and *L*-Thr. In the P3 position, small and large hydrophobic residues, such as *L*-2-Nal, *L*-hPhe, *L*-Phe(4-I), *L*-Phe(4-Cl), *L*-Chg, *L*-Cha, *L*-2-Aoc, and *L*-Phe(NH_2), were recognized by chymotrypsin-like subunit.

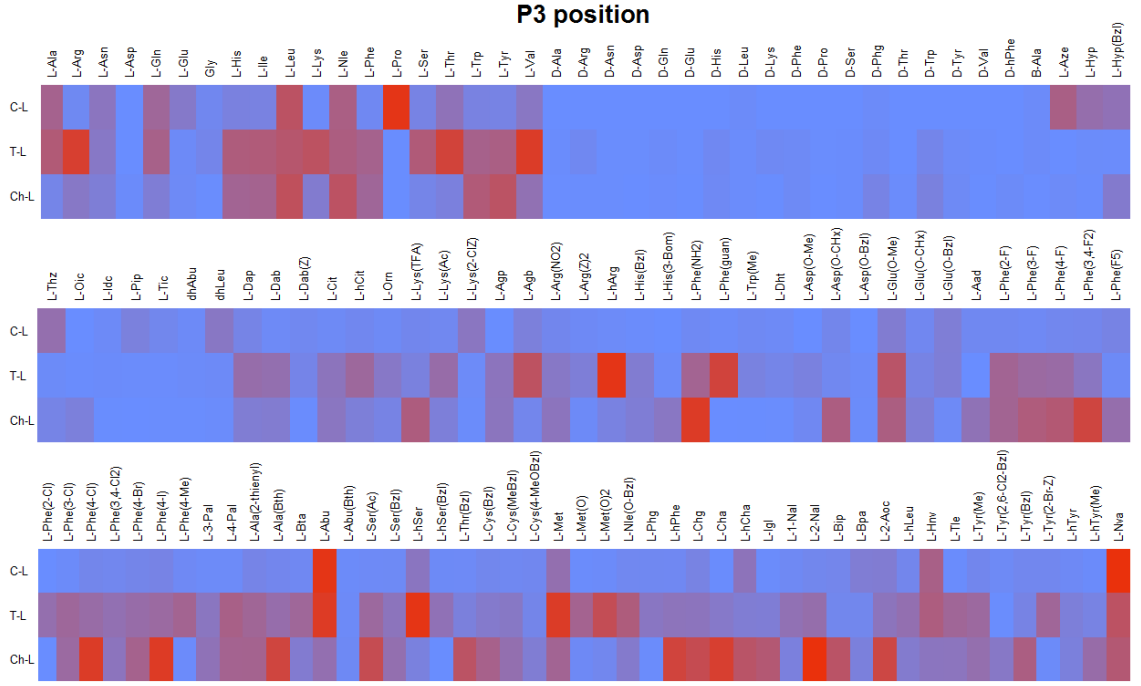
P4 position analysis. The subunit with caspase-like activity exhibited narrow substrate specificity in the S4 pocket. The most preferred amino acids were positively charged residues such as *L*-hArg, *L*-Phe(guan), *L*-Arg, *L*-Lys. *L*-Oic and *L*-Lys(Tfa) were 2-times worse recognized (50% compared to the best) and the rest of amino acids were weakly recognized (<40%).Trypsin-like site possessed broad substrate preferences in the P4 position, recognizing even *D*-amino acids. Chymotrypsin-like subunit preferred a large number of hydrophobic amino acids in S4 pocket.

HyCoSuL analysis allowed identification of key amino acids to serve as a scaffold in order to develop specific substrates for each proteasome subunit. A substrate for caspase-like activity should contain (1) proline in position P3 and (2) positively-charged residues in P4. A substrate for trypsin-like activity should contain (1) methionine, its derivatives either basic amino acids in the P3 position and (2) *D*-amino acids and β-Ala in P4 position. Finally, a substrate for chymotrypsin-like activity should possess (1) bulky hydrophobic amino acids at P3 and (2) aliphatic or aromatic residues at P4. Amino acids required at other positions were not as clearly defined, requiring additional sequence optimization.

P2 position



P3 position



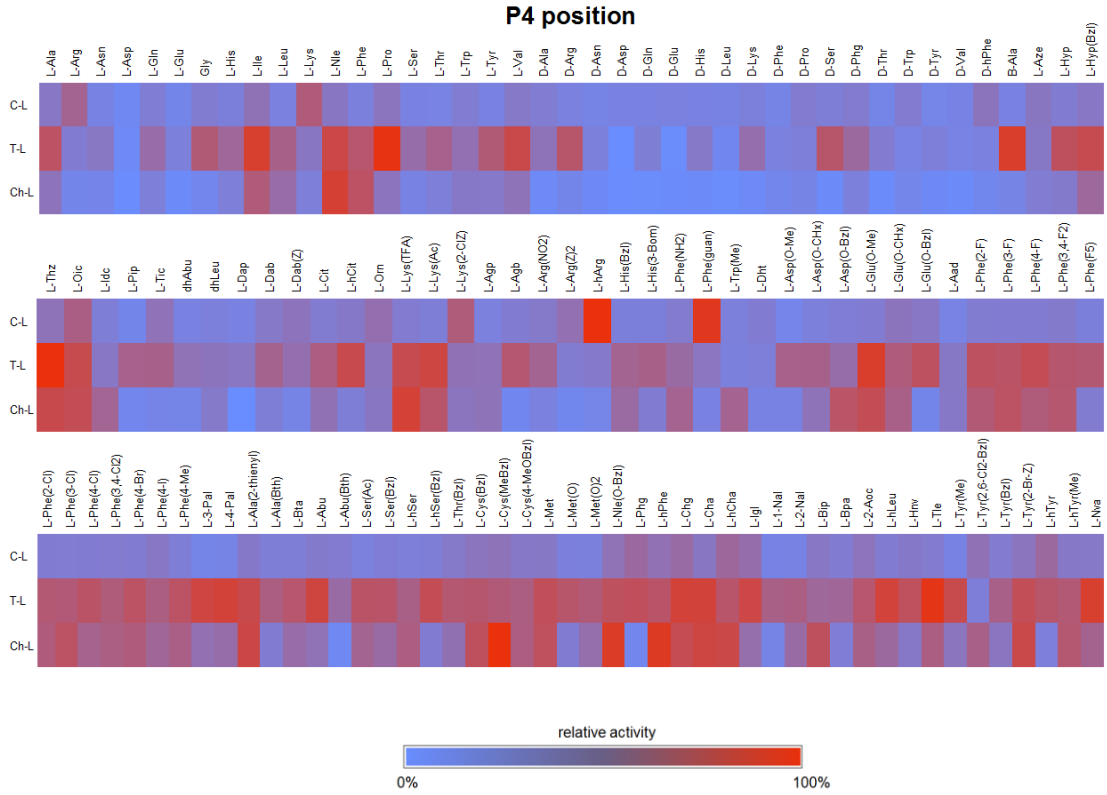


Figure 2. Proteasome substrate specificity profiles presented as heat maps (C-L, caspase-like activity; T-L, trypsin-like activity; Ch-L, chymotrypsin-like activity).

Design of subunit-selective substrates

To develop proteasome subunit-selective ABPs, we first designed selective ACC-labeled substrates. We analyzed HyCoSuL substrate specificity profiles in the P4-P2 positions for each proteasome activity and selected amino acids preferred only by one of the three β-subunits at a particular position or, at least, residues poorly recognized by the other two β-subunits. We then synthesized several substrates and measured their hydrolysis rate relevant to each proteasome activity. Due to lack of subunit-specific inhibitors of proteasome activity we could not inhibit two proteasome β-subunits and assess substrate hydrolysis by the third. Furthermore, it is known that inhibition of one site can modify activity of the two other sites in the proteolytic core [28, 29]. To confirm substrate specificity for the targeted β-subunit, we synthesized substrates containing the same amino acids in the P4-P2 positions (selected for one activity) but with three different residues (*L*-Arg, *L*-Asp and *L*-Ala) in the P1 position and then measured hydrolysis rate at two different substrate concentrations (100 μM and 5 μM) (Table 1).

Table 1. Substrate selectivity toward proteasome β-subunits.

Target subunit	Substrate sequence	RFU/s/nM					
		[S] = 5 µM			[S] = 100 µM		
		P1=Asp	P1=Arg	P1=Ala	P1=Asp	P1=Arg	P1=Ala
β-1	WRP1 Ac-hArg-Pro-Phe(2F)-P1-ACC	9.8	0	0.08	26.75	0.15	0.56
	WRP2 Ac-hArg-Pro-Abu-P1-ACC	4.0	0	0	16.13	0	0
β-2	WRP3 Ac-βAla-Met(O)$_2$-Thr-P1-ACC	0	2.5	0.04	0.11	15.08	0.19
	WRP4 Ac-*D*Arg-hSer-Thr-P1-ACC	0.23	8.50	0.04	0.35	33.75	0.40
	WRP5 Ac-βAla-hSer-Thr-P1-ACC	0.04	2.53	0.03	0.37	12.38	0.27
	WRP6 Ac-*D*Arg-Met(O)$_2$-Thr-P1-ACC	0	0.80	0	0	1.76	0
β-5	WRP7 Ac-Phe-Thr(Bzl)-His(3-Bom)-P1-ACC	0.06	0.19	2.17	0.22	0.44	4.60
	WRP8 Ac-Nle-Tyr(Bzl)-Dap-P1-ACC	0.06	0.15	1.36	0.23	0.77	5.58

At low substrate concentrations, peptide sequences WRP1 and WRP2 were specifically recognized only by β-1 site and WRP3 and WRP4 by β-2 subunits, whereas at high substrate concentrations, WRP2 was recognized only by caspase-like subunit, and WRP1, WRP3 and WRP4 displayed low off-target effects (see Table 1). The β-5 subunit-specific peptide sequence (WRP7) was weakly recognized by the β-2 subunit at both substrate concentrations and very poorly recognized by the β-1 subunit. Once we identified peptide sequences for chymotrypsin-like activity, we incorporated small aliphatic amino acids (*L*-2-Abu, *L*-Nva, or *L*-Leu) into the P1 position, and used these three amino acids, as they were better recognized than alanine (Figure S1 Supporting Information). We then selected two most selective substrates for β-1 subunit, two for β-2 and one for β-5 and determine their kinetic parameters (see Table 2).

Table 2. Kinetic parameters for subunit-specific substrates.

Substrate	Proteasome activity	K_M, µM	k_{cat}, s^{-1}	k_{cat}/K_M, $M^{-1}s^{-1}$
Ac-hArg-Pro-Phe(2F)-Asp-ACC, WRP23	C-L	15.88 ± 1.04	1.844 ± 0.130	116 105 ± 1 271
Ac-hArg-Pro-Abu-Asp-ACC, WRP58	C-L	43.16 ± 1.54	1.343 ± 0.098	31 127 ± 1 285
Ac-βAla-Met(O)$_2$-Thr-Arg-ACC, WRP65	T-L	50.99 ± 2.99	2.233 ± 0.075	43 790 ± 3 233
Ac-*D*Arg-hSer-Thr-Arg-ACC, WRP89	T-L	9.43 ± 0.82	2.837 ± 0.217	301 005 ± 28 471
Ac-Phe-Thr(Bzl)-His(3-Bom)-Leu-ACC, WRP119	Ch-L	ND	ND	20 609 ± 1 392

ND-not determined because of low substrate solubility in assay conditions

(C-L, caspase-like activity; T-L, trypsin-like activity; Ch-L, chymotrypsin-like activity)

To date, changes in proteasome activity are measured using commercially available substrates (Suc-LLVY-AMC, Bz-VGR-AMC, or Z-Leu-Leu-Glu-AMC) [6, 30, 31], for which subunit specificity has never been precisely analyzed. To confirm the selectivity of obtained substrates,

we utilized them to measure proteasome inhibition in HEK-293F lysates following treatment of cell lysates with1 nM to 10 µM bortezomib and detection of residual proteasome activity (Figure 3). Kinetic studies revealed complete inhibition of chymotrypsin-like activity at 88 nM bortezomib. Further increases in bortezomib concentration led to full inhibition of caspase-like (444 nM) and trypsin-like (>5 µM) activities. These results were confirmed by analysis with subunit-selective ABPs (see "Detection of the 20S proteasome in cell lysates" below). Moreover, we observed an increase in trypsin-like activity relative to controls when chymotrypsin-like activity was inhibited by 60%. These results are consistent with those of Priestman and coworkers [29].

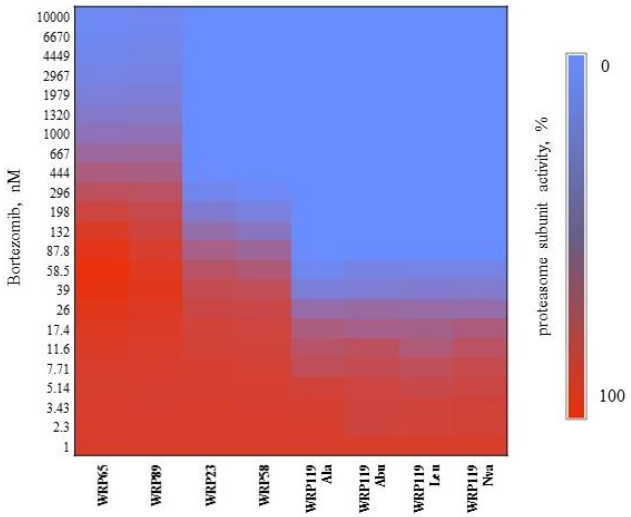


Figure 3. Inhibition of proteasome β-subunits by bortezomib presented as a heat map. Bortezomib was incubated at various concentrations (ranging from 1 nM to 10 µM) with pre-warmed HEK-293F lysates for 30 min at 37°C, and then the appropriate substrate (for β-1 (WRP23, WRP58), β-2 (WRP65, WRP89), and β-5 subunit (WRP119 Ala, WRP119 Abu, WRP119 Leu, WRP119 Nva); [S]=100 µM) was added and hydrolysis measured for a 15-30 minute period.

Subunit-selective ABPs

We then utilized our subunit-selective peptide sequences to synthesize tagged ABPs. Probes included a N-terminal biotin tag, a 6-aminohexanoic acid (6-ahx) linker, the subunit-specific peptide sequence, and vinyl sulfone as an irreversible reactive group. Kinetic analysis using purified human 20S proteasome revealed that Biotin-WRP23-VS (Biotin-6-ahx-WRP23-vinylsulfone) and Biotin-WRP58-VS are specific for β-1 and do not inhibit β-2 and β-5 subunits, even at high (50 µM) probe concentration (see Figure S2 in Supporting Information). We observed 50% β-1 inhibition at 25 nM Biotin-WRP23-VS and 100 nM Biotin-WRP58-VS,

results mirroring kinetic analysis of WRP23 and WRP58 substrates. WRP23 is recognized ~4-times better (k_{cat}/K_M) than WRP58 by caspase-like subunit. Relevant to trypsin-like activity, both specific ABPs, Biotin-WRP65-VS and Biotin-WRP89-VS, slightly inhibit β-1 subunits at high probe concentration (≥ 25 μM, Figure S2). Relevant to chymotrypsin-like activity, as noted above, we had synthesized four ABPs with different amino acids (*L*-2-Abu, *L*-Nva, *L*-Leu, and *L*-hPhe) at the P1 position. Kinetic analysis revealed that Biotin-WRP119-Abu-VS and Biotin-WRP119-Nva-VS probes are more potent toward the β-5 subunit, but less selective than Biotin-WRP119-Leu-VS and Biotin-WRP119-hPhe-VS probes (Figure S2).

Detection of the 20S proteasome in cell lysates

To determine whether we could detect individual proteasome β-subunit activity in cell lysates, we analyzed HEK-293F cells, since they express only the constitutive 20S proteasome (not the immunoproteasome) [18]. HEK-293F cell lysates were prepared and incubated with selected biotin-labeled probes at concentrations ranging from 33 to 1000 nM. We also performed time course analysis by incubating cells lysates with probes for various time and then analyzed the samples by Western blotting (see Figures S3 and S4 in Supporting Information). We observed specific β-1 subunit labeling at 100 nM probe after a 5-minute incubation. Biotin-WRP65-VS and Biotin-WRP89-VS inhibited trypsin-like activity in a concentration-dependent manner. Optimal detection of the β-2 subunit was achieved at 100 nM probe after a 30-minute incubation. Relevant to β-5, we observed significant labeling of chymotrypsin-like activity at 33 nM using all four probes tested, but, among them, probes containing *L*-Abu and *L*-Nva at P1 also inhibited the β-2 subunit at 333 nM. Optimal labeling of the β-5 subunit was achieved after a 5-minute incubation with 100 nM probe. Treatment of HEK-293F cell lysates with the biotinylated probe cocktail (100 nM of each probe, 30-min incubation) led to the appearance of three clear bands representing each of the three catalytic proteasome subunits (Figure 4).

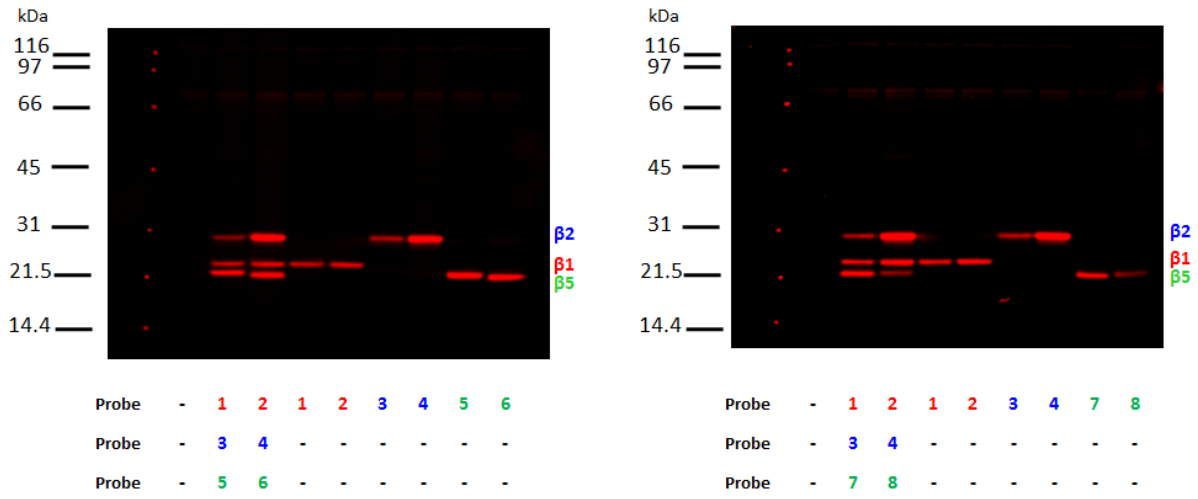


Figure 4. Proteasome detection in HEK-293F cell lysates using a Biotin-labeled probe cocktail containing 1-Biotin-WRP58-VS, 2-Biotin-WRP23-VS, 3-Biotin-WRP89-VS, 4-Biotin-WRP65-VS, 5-Biotin-WRP119-Abu-VS,6-Biotin-WRP119-Nva-VS, 7-Biotin-WRP119-Leu-VS, and 8-Biotin-WRP119-hPhe-VS. The concentration of all probes was 100 nM.

To detect proteasome subunits simultaneously we exchanged the biotin tag for three different fluorophores: Cy5-WRP23-VS (red), Cy7-WRP119-Leu-VS (green), BODIPY-WRP89-VS (blue) (Figure 5A). Analysis showed that the optimal probe concentration required to detect β-subunits in lysates following a 30-minute incubation period was as follows: Cy5-WRP23-VS, 500 nM; BODIPY-WRP89-VS, 100 nM; and Cy7-WRP119-Leu-VS, 25 nM. Comparable to analysis of biotinylated probes, we incubated HEK-293F lysates with a cocktail of fluorescent probes and detected the three catalytic β-subunits simultaneously via Western blotting (Figure 5B). Likewise, probes with Leu and Asp in the P1 position labeled also an unknown protein between 45 and 66 kDa. To identify this band, we performed a pulldown experiment with one of the Cy5-labeled probe followed by proteomic analysis. The LC-MS/MS analysis identified 132 putative proteins, however none of them appear to be a proteolytic enzyme. (Table S1, Supporting Information). We then performed a similar experiment using red blood cell lysates and observed selective labeling of proteasome β-subunits by fluorescent probes (note that erythrocytes express only the constitutive 20S proteasome [32]) (Figure 5C). Due to hemoglobin absorption properties (maximum absorption at 420 and 580 nm [33]), we detected background when we tested Cy5 and BODIPY fluorophores. Nonetheless, we presented a simple and rapid method useful to measure proteasome activity in blood samples that could enable early diagnosis of pathological states in which the proteasome is overexpressed.

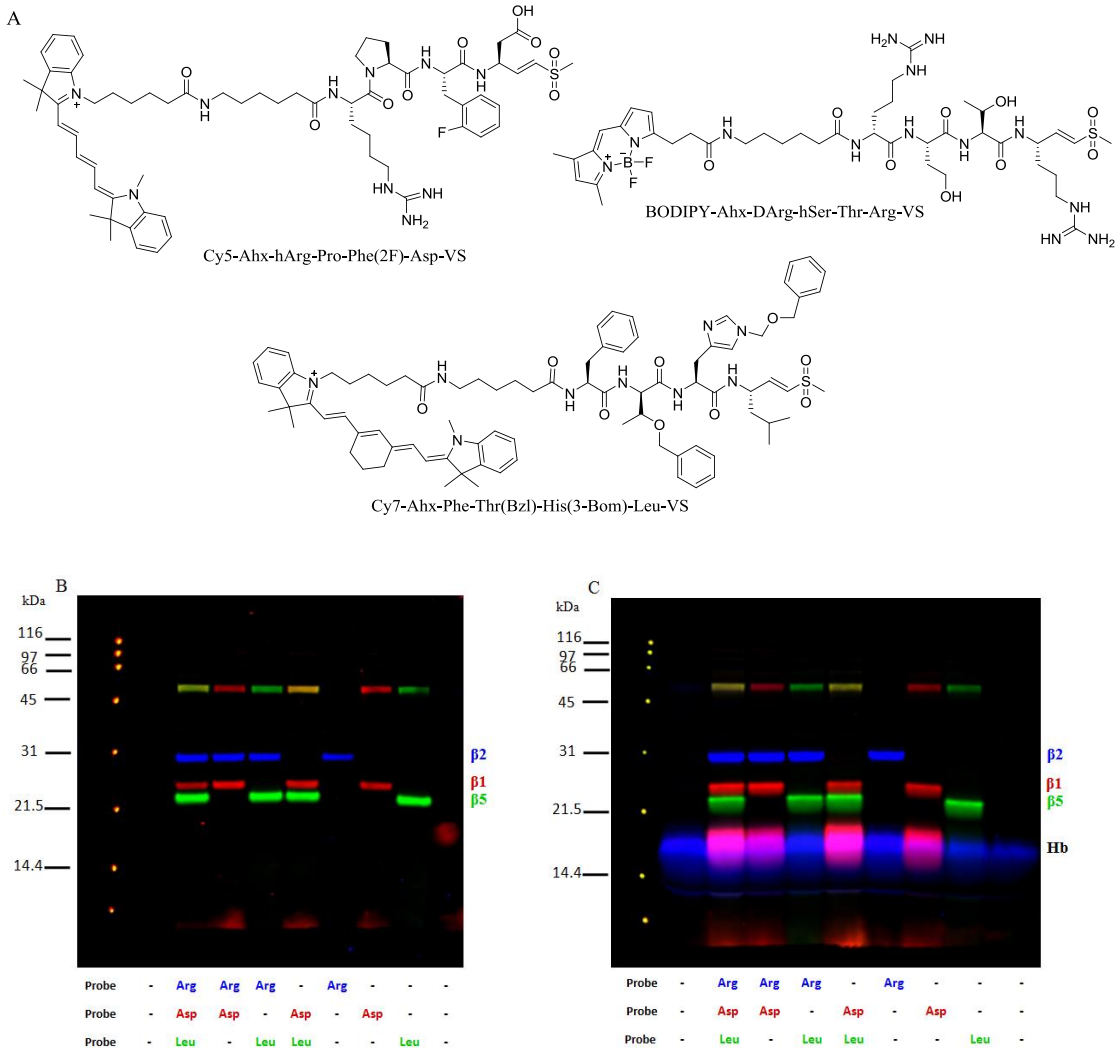


Figure 5. Simultaneous labeling of proteasome subunits using an ABP cocktail containing three different fluorophores (**A)** Structure of ABPs used in analyses of HEK-293F cell lysates (**B**), and red blood cell lysates (**C**). Probe concentrations in **B** were Cy5-WRP23-VS, 500 nM; Cy7-WRP119-Leu-VS, 25 nM; and BODIPY-WRP89-VS, 100 nM. In **C**, concentrations were Cy5-WRP23-VS, 3.3 µM; Cy7-WRP119-Leu-VS, 33 nM; and BODIPY-WRP89-VS, 333 nM. Arg, Asp and Leu indicate amino acids in the probe P1 position.

Currently, three proteasome inhibitors are approved as therapies for hematological malignancies. All target the human β-5 subunit but at high concentrations also inhibit β-1 and β-2. Further studies show that co-inhibition of β-1 and β-2 may enhance antitumor activity of β-5-targeting proteasome inhibitors [14, 15]. Therefore our subunit-specific ABPs could be useful to assess inhibition of all three catalytic proteasome sites. To test this hypothesis, we created proteasome inhibition profiles using different concentrations of bortezomib and MG-132 ranging from 3 nM to 10 µM (Figure 6). We observed complete β-5 inhibition at 100 nM

bortezomib or MG-132. Furthermore, bortezomib fully inhibited caspase-like activity at 300 nM and trypsin-like activity at 3 µM. MG-132 inhibited β-1 subunit activity at 1µM and β-2 activity at 3 µM.

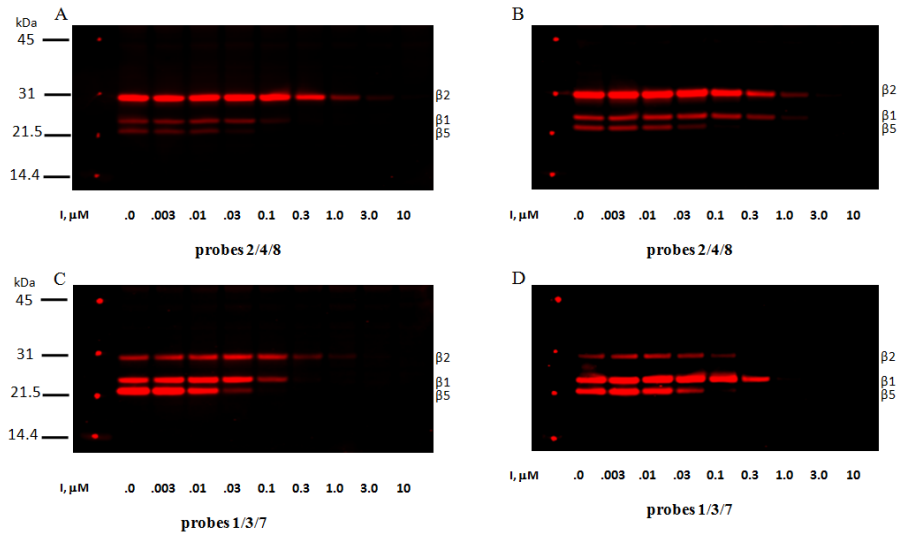


Figure 6. Profile of proteasome inhibition by bortezomib (A, C) and MG-132 (B, D) using an ABP cocktail containing probes 1-Biotin-WRP58-VS, 2-Biotin-WRP23-VS, 3-WRP89-VS, 4-WRP65-VS, 5-Biotin-WRP119-Abu-VS, 6-Biotin-WRP119-Nva-VS, 7-Biotin-WRP119-Leu-VS, and 8-Biotin-WRP119-hPhe-VS. Each probe was used at 100 nM. HEK-293F lysates were incubated 30 min with inhibitor followed by incubation with the probe for an additional 30 minutes.

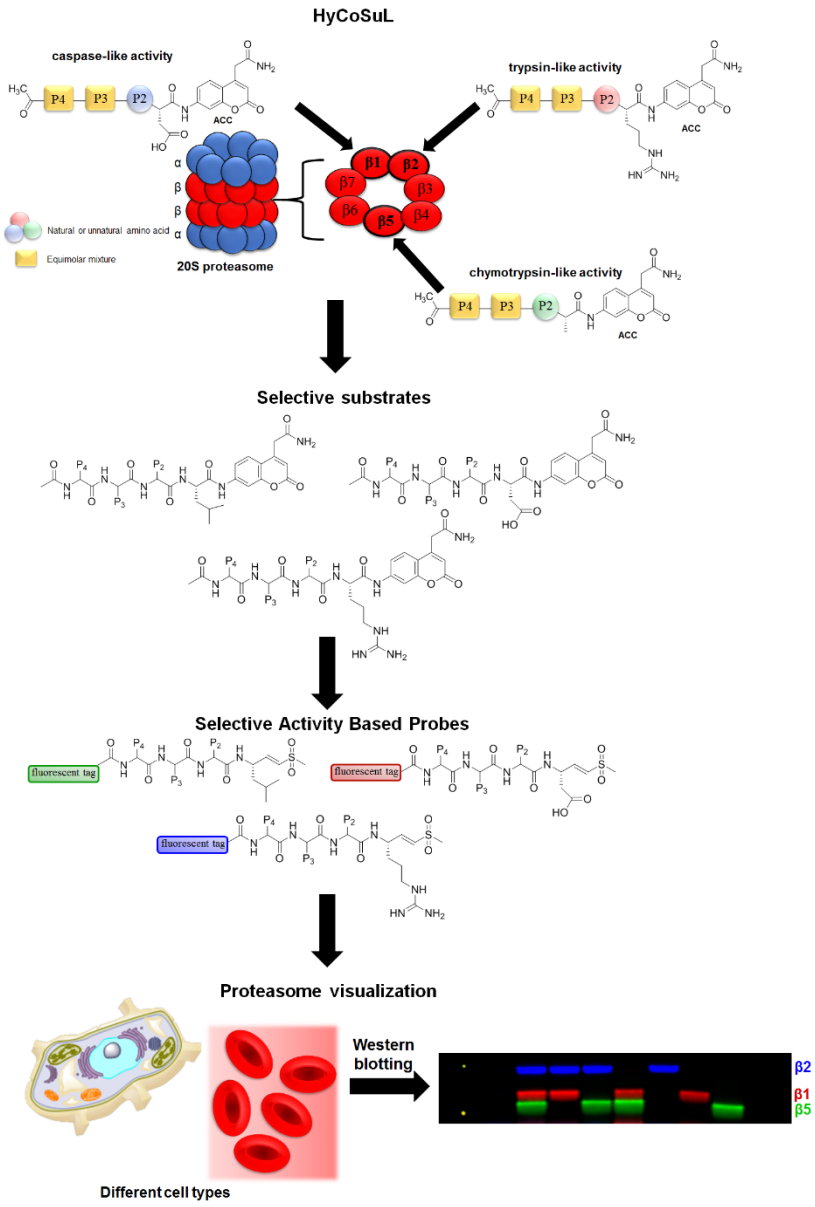


Figure 7. Use of the HyCoSuL approach to identify subunit-selective substrates and ABPs.

Conclusions

As noted, peptide-based proteasome inhibitors used to treat malignancies target chymotrypsin-like activity of the β-5 subunit [34]. However, Kisselev and co-workers showed that inhibition of caspase-like and/or trypsin-like activity sensitizes myeloma cells to β-5 inhibitors like bortezomib and carfilzomib [14, 15], suggesting that β-1 and β-2 subunits could also be targeted. Therefore, new subunit-selective chemical tools are needed to assess relative contributions of each proteasome activity in various cellular contexts. To date, subunit-selective substrates for each 20S proteasome activity have not been described. Tools described here fill this gap.

So far, proteasome subunit substrate specificity has been determined using combinatorial libraries of natural amino acids [19], limiting discovery of subunit-selective peptide sequences. Thus, we applied the Hybrid Combinatorial Substrate Library approach using libraries composed of natural and unnatural amino acids to identify subunit-selective fluorogenic substrates and activity-based probes for each catalytic subunit. An important issue in analysis of proteasome activity is to ensure that activity is in fact inhibited. Different methods are used to assess this, the most convenient being use of fluorogenic substrates. Since the proteasome exhibits numerous catalytic activities, it is important to utilize selective substrates to assess inhibition of each subunit separately. To confirm the utility of our analysis we measured attenuation of proteasome β-subunit activity by bortezomib and MG-132 in HEK-293F cell lysates. In further studies, the identified peptide sequences were applied to synthesis of ABPs. ABPs contained vinyl sulfone as a reactive group, given its easy synthesis and ability to covalently modify active site threonine side chain to create a vinyl sulfone moiety suitable for ABP synthesis [31]. Initially, we tagged the probe with biotin and confirmed its selectivity using HEK-293F cell lysates. For chymotrypsin-like activity, we synthesized four ABPs with different amino acids at P1, since this activity prefers aliphatic and aromatic residues. In this case, probes containing Abu and Nva were more potent than probes with Leu and hPhe but less selective; thus we selected leucine in P1 position for further studies. We then exchanged the biotin-tag for a fluorophore to enable simultaneous detection of catalytic subunits. The fluorophores utilized (BODIPY, Cy5 and Cy7) exhibit minimal spectral overlap. Using HEK-293F and red blood cell lysates, we demonstrated subunit-selectivity of identified ABPs. We employed these cell types as they express only the constitutive 20S proteasome (not the immunoproteasome). Proteasome detection in red blood cells using our probes is the first step to development of a simple blood test to determine proteasome activity in patients with hematological malignances and could serve as a diagnostic in conditions in which proteasome activity is upregulated. Matondo and coworkers report that sensitivity of human acute myeloid leukemia (AML) cell lines to proteasome inhibitors is positively correlated with proteasome content [35]. This finding suggests that proteasome levels in AML cells may predict responses to proteasome inhibitors. A biological blood test to determine proteasome activity in cancer samples would facilitate choice of appropriate therapy.

In summary, subunit-selective compounds (substrates and ABPs) identified here could be useful to investigate the 20S proteasome in biological samples. Selective substrates could be used to evaluate therapeutic efficacy of β-5 proteasome inhibitors by controlling concentration-

dependent co-inhibition of the two remaining β-sites. Furthermore, tools described here could be utilized to define the contribution of each catalytic proteasome activity in normal and pathological states, and in terms of the latter, could enable application of an effective treatment, such as inhibition of particular β-subunit.

Material and Methods

Reagents

All chemical reagents were from commercial suppliers and used without further purification. Reagents used for the solid-phase peptide synthesis are as follows Rink amide RA resin (particle size 200-300 mesh, loading 0.74 mmol/g), all Fmoc-amino acids, HBTU (*O*-benzotriazole-*N*,*N*,*N*`,*N*`-tetramethyl-uronium-hexafluoro-phosphate), HATU (2-(1-H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluranium hexafluorophosphatemethanaminium), piperidine, DICI (diisopropylcarbodiimide) and TFA (trifluoroacetic acid) were purchased from Iris Bioteh GmbH (Marktredwitz, Germany); HOBt anhydrous (*N*-hydroxybenzotriazole) was from Creosauls; 2,4,6-collidine (2,4,6-trimethylpyridine), HPLC pure acetonitrile, and TIPS (triisopropylsilane) were from Sigma-Aldrich (Poznan, Poland); and DIPEA (*N*,*N*-diisopropylethylamie) was from VWR International (Gdansk, Poland). DMF (*N*,*N*`-dimethylformamide), DCM (dichloromethane), MeOH (methanol), Et_2O (diethyl ether), AcOH (acetic acid), and P_2O_5 (phosphorus pentoxide) were from Avantor (Gliwice, Poland). Individual substrates and ABPs were purified by HPLC on a Waters M600 solvent delivery module with a Waters M2489 detector system using a semi-preparative Waters Spherisorb S10ODS2 column. Solvent composition was as follows: phase A (water/0.1% TFA) and phase B (acetonitrile/0.1% TFA). The purity of each substrate and ABPs was confirmed with an analytical HPLC system using a Waters Spherisorb S5ODS2 column. The molecular weight of each substrate and ABP was confirmed by high-resolution mass spectrometry on a High Resolution Mass Spectrometer WATERS LCT premier XE with Electrospray Ionization (ESI) and Time of Flight (TOF) module. The human 20S proteasome was purchased from Boston Biochem (Cambridge, MA, USA).

Synthesis of libraries

The defined libraryAc-Ala-Arg-Leu-P1-ACC and combinatorial libraries P1-Arg, P1-Asp, and P1-Ala were synthesized on solid support (Rink Amide Resin) as described [25, 26, 36, 37]. Synthesis of the fluorogenic leaving group, ACC (7-amino-4-carbamoylmethylcoumarin),was carried out based on an efficient large-scale method described by Maly et al.[38].

Subunit-specific substrates synthesis

Synthesis of individual tetrapeptide fluorogenic substrates was performed on a solid support according to the Solid Phase Peptide Synthesis (SPPS) method as described [38] using a semiautomatic robot for parallel synthesis FlexChem® Model 202 Ambient Rotator with FlexChem® Peptide Synthesis System. In the first step, the fluorescent tag Fmoc-ACC-OH was attached to Rink Amide Resin using HOBt and DICI as coupling reagents (2.5eq of each reagent). After Fmoc-group de-protection using 20% piperidine in DMF (three cycles: 5, 5, 25 minutes),one of the three amino acids in the P1 position (2.5eq Fmoc-*L*-Arg(Pbf)-OH, Fmoc-*L*-Asp(tBu)-OH, and Fmoc-*L*-Ala-OH) was pre-activated with 2.5eq HATU and 2.5eq 2,4,6-collidine in DMF and added to 1eq H_2N-ACC-resin (100 mg). After 12 hours, the mixture was filtered and washed three times with DMF, and then 1.5eq of Fmoc-amino acids in the P1 position with coupling reagents were added to the resin and gently agitated 12 hours. The Fmoc-group was then removed, and a solution of 2.5eq Fmoc-P2-OH, 2.5eq HOBt, and 2.5eq DICI in DMF was added to H_2N-P1-ACC-resin and agitated 2.5 hours. The Fmoc-protecting group was then hydrolyzed as described above. A ninhydrin test was performed after every amino acid coupling and Fmoc removal. Coupling of amino acids in the P3 and P4 positions was carried out in a manner comparable to the P2 position. The free N-terminus was acetylated using 5eq HBTU, 5eq AcOH, and 5eq DIPEA in DMF. After 1h agitation the solution was removed and the resin was washed five times with DMF, three times with DCM, and three times with MeOH and then dried over P_2O_5. Finally, substrates were hydrolyzed from the resin with a mixture of TFA/TIPS/H_2O (% v/v/v/ 95:2.5:2.5), precipitated in Et_2O, purified by HPLC, and lyophilized. ACC-labeled substrate purity was confirmed by analytical HPLC. Each obtained compound was dissolved in DMSO at a final concentration of 10 mM and stored at -80°C until use.

Library and substrate screening

For kinetic studies, ACC release in the presence of enzyme was monitored continuously for 30 minutes at the appropriate wavelength (λ_{ex}= 355nm, λ_{em}= 460 nm). Assays were performed using a spectrofluorometer (Molecular Devices Spectramax Gemini XPS) in 384-well plates containing substrate plus the 20S proteasome, which had been incubated 15-minute 37°C in assay buffer (0.5 mM EDTA, 20 mM HEPES, pH=7.5[39]). Assay conditions were 1 µl of substrate in DMSO and 49 µl of enzyme. The library concentration in each well was 4 µM of Ac-ARL-P1-ACC, 50 µM of the P1=Arg and P1=Asp libraries, 25 µM of P1=Ala library. The

enzyme concentration was 2 nM for defined library screening and 6 nM for combinatorial libraries. Kinetic experiments were repeated three times and results reported as an average with standard deviation. The linear portion of each progress curve was utilized to determine substrate hydrolysis rate. Based on the RFU/s value (Relative Fluorescence Unit per second), substrate specificity profiles were established, with highest values from each library position set to 100% and adjusting other results accordingly. Individual substrate hydrolysis was measured in the same assay conditions; substrate concentration was 5 and 100 µM, and the 20S proteasome concentration was 4 nM.

Determination of kinetic parameters for subunit-specific substrates

Kinetic parameters of ACC-labeled substrates were assayed in 96-well plates. Wells contained 80 µl 20S proteasome (1.5-3 nM) and 20 µl substrate at eight different concentrations (0.3 to 200 µM). Assay buffer was the same described above for library screening. Hydrolysis was measured for 30 minutes at the appropriate wavelength (λ_{ex}= 355nm, λ_{em}= 460 nm). Experiments were carried out three times and kinetic results reported as averages with standard deviation.

ABP synthesis

Obtained subunit-selective peptide motifs were utilized to design covalent and irreversible ABPs. Two biotin-labeled ABPs were synthesized for caspase-like activity, two for trypsin-like activity and four for chymotrypsin-like activity. The vinyl sulfone reactive group was synthesized based on published methods [40, 41]. Fmoc-AA-OH (3 mmol) was dissolved in 3 ml DMF. To this were added 3.3 mmol HOBt and 3.3 mmol DCC and the mixture stirred 30 minutes at room temperature. Dicyclohexylurea was then removed by filtration, and 3.6 mmol N,O-dimethylhydroxylamine hydrochloride, 3.6 mmol DIPEA and 3 ml of DMF were added. The reaction mixture was stirred at room temperature for 2 hours and then diluted with ethyl acetate and extracted with saturated aqueous $NaHCO_3$, 5% of citric acid and brine. The organic layer was dried over $MgSO_4$ and evaporated. The crude product was used without further purification (yield > 95% by HPLC). Obtained Weinreb amide was dissolved in anhydrous THF (5 ml), put under an argon atmosphere and cooled to 0°C. LiAlH4 (4.5 mmol, 1M solution in THF) was added, and then the reaction was stirred 25 minutes and quenched with 6 ml 5% $KHSO_4$ solution. The organic layer was extracted with ethyl acetate, washed twice with 5% $KHSO_4$ and once brine, dried over $MgSO_4$, and concentrated under reduce pressure. NaH (3,6 mmol) was added to a cold solution of diethyl methylsulfonylmethylphosphonate (3 mmol) in

anhydrous THF (6 ml) under an argon atmosphere. After 30 minutes, freshly obtained aldehyde in THF (6 ml) was added, and the mixture was stirred for 1 h. HPLC analysis then undertaken and indicated complete conversion of the starting compound. The reaction was quenched by adding 5% $KHSO_4$ (12 ml) and diluted with ethyl acetate. The organic layer was washed with 5% $KHSO_4$ and brine, dried over $MgSO_4$, and concentrated. The crude Fmoc-AA-VS was purified by column chromatography on silica gel (Hex:EtOAc 2:1). Biotin-Ahx-P4-P3-P2-OH fragments were synthesized using 2-chlorotrityl chloride resin. In the first step, Fmoc-P2-OH (2.5 eq) was dissolved in anhydrous DCM, pre-activated with DIPEA (3 eq) and added to the cartridge with resin (1 eq). After 3h the mixture was filtered, washed 3 times with DCM and 3 times with DMF, and the Fmoc-group was removed using 20% piperidine in DMF. Amino acids in P3 and P4 positions and Fmoc-Ahx-OH were attached to the resin according to standard solid phase peptide synthesis protocol [42].The biotin tag was coupled to H_2N-Ahx-P4-P3-P2-resin using 2.5 eq HBTU and 2.5 eq DIPEA as coupling reagents in a DMF:DMSO mixture (1:1, v/v). After 3 h, the resin was washed 3 times with DMF, 3 times with DCM, and 3 times with MeOH and dried over P_2O_5. Finally, the peptide was hydrolyzed from the resin with a mixture of DCM/TFE/AcOH (v/v/v, 8:1:1). The solution was filtered and concentrated. Obtained crude peptide was dissolved in ACN:H_2O (v/v, 3:1), lyophilized and then used without further purification (purity > 95%). Fmoc-AA-VS was treated with the mixture of diethylamine: ACN (1:1, v/v) for 1h and volatiles were then removed under reduced pressure. Biotin-Ahx-P4-P3-P2-OH (1.2 eq) was pre-activated with HATU (1,2eq) and 2,4,6-collidine (3 eq) in DMF and added to a small flask containing 1 eq of H_2N-AA-VS. The reaction was agitated for 2 hours and the product was purified on HPLC. Finally, Biotin-Ahx-P4-P3-P2-P1-VS was added to a mixture of TFA/DCM/TIPS (% v/v/v/ 70:27:3) to remove side chain amino acid protecting groups. After 30 minutes, solvents were evaporated and ABPs were purified on HPLC.

ABPs with fluorescent tags were obtained following procedures similar to those used for biotinylated probes, but Boc-Ahx-OH was used instead of Fmoc-Ahx-OH. H_2N-Ahx-P4-P3-P2-P1-VS (1.2 eq) was dissolved in DMF and DIPEA (3 eq) and fluorophore (1 eq) was added. The reaction mixture was stirred 1 h and, and the probe was purified on HPLC.

Determination of proteasome inhibition by biotinylated ABPs

To assess proteasome inhibition by biotinylated ABPs, purified 20S proteasome (2-4 nM) was incubated with various concentrations (0-50 µM) of ABPs at 37°C for 30 minutes before

proteasome activity measurement. Estimation of β-subunit activity was undertaken using specific substrates (WRP23, WRP89, and WRP119). ACC liberation was monitored for 30 minutes at the following wavelengths: 355 nm excitation, 460 nm emission.

Proteasome activity in HEK-293F lysates (specific substrates)

Lysates were prepared by hypotonic lysis of FreeStyle HEK-293 cells (Life Technologies, Grand Island, NY, USA) as described[43]. Lysate protein concentration was determined using the Bradford assay (12 mg/ml), and 200 µl aliquots of lysates were stored at -80°C until use. For substrate activity assays, lysates were thawed, diluted 20x in HEB buffer, and pre-warmed at 37°C for 10min. Then, 99 µl of lysates were mixed with 1 µl (20mM in DMSO, final concentration 200 µM) of ACC-labeled substrates, and substrate hydrolysis was measured over a 15-30 minute period. The linear portion of the kinetic curve was used for analysis. All fluorescence assays were performed using a CLARIOstar spectrofluorimeter (BMG LABTECH) with the 355nm (excitation) and 460 nm (emission) setup (fluorescence gain: 650). In other experiments, Bortezomib (MedKoo Bioscience) and biotin-labeled vinyl-sulfone probes were incubated at various concentrations with pre-warmed HEK-293F lysates for 30min at 37°C, and then the appropriate substrate (for β-1, β-2, and β-5 subunits; [S]=100 µM) was added and hydrolysis measured for a 15-30 minute period. The linear portion of the kinetic curve was used for analysis.

Proteasome labeling in HEK-293F lysates

Proteasome labeling was performed at 37°C. Probe concentration and incubation time varied with analysis; however, in each experiment, lysates were diluted 5x in HEB buffer (final protein concentration: 2.4 mg/ml), and the total lysate volume was 80 µl. Once labeling was complete, 80 µl of 2xSDS/DTT was added and the sample boiled for 5min at 95°C, sonicated (Branson Sonifier 250, Duty Cycle 50, Output Control 5, 4 pulses), spun down (3min, 4500g), and run on 12% Bis-Tris Plus 10-well gels (Invitrogen) at 30 µl sample/well and 36 µg protein/well. In experiments with 1 probe, electrophoresis was run at 200V for 30min. In experiments with all three probes, electrophoresis was run for 15min at 80V followed by 75 min at 130V to optimize subunit separation. Proteins were then transferred to nitrocellulose membranes (0.2 µm, Bio-Rad) for 60min at 10V. Ponceau staining verified equal loading. Membranes were blocked with 2% BSA in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBS-T) for 60min at room temperature. **Biotin-labeled probes** were then detected with fluorescent streptavidin IRDye®

680RD (1:10 000, LI-COR, red) and proteasome subunits were detected by subsequent mouse anti-human monoclonal IgG_1 antibodies (20S Proteasome β-1 (D-9) 1:2000, 20S Proteasome β-2 (MCP165) 1:2000, and 20S Proteasome β-5 (A-10) 1:2000, all from Santa Cruz Biotechnology) and fluorescent donkey anti-mouse IRDye® 800CW (1:10 000, LI-COR, green) in TBS-T. Primary antibodies were incubated with the membrane for 1 hour at room temperature. The secondary antibody (green) and fluorescent streptavidin (red) were then incubated with the membrane for 30min at room temperature. Fluorescence was scanned at 700nm (red) and 800nm (green) using an Odyssey fluorescence imaging system (LI-COR), and blots were analyzed using Image Studio software.

Fluorescent-labeled probes. Three fluorescent probes were used: BODIPY-WRP89-VS for the β-2 subunit, Cy5-WRP23-VS for β-1, and Cy7-WRP119-Leu-VS for β-5. For dual-color labeling of the probe/proteasome complex we used following fluorescent secondary antibodies: (1) donkey anti-mouse IRDye® 680LT (1:10 000, LI-COR, red) in combination with BODPIY-WRP89-VS and Cy7-WRP119-Leu-VS probes, and (2) donkey anti-mouse IRDye® 800CW (1:10 000, LI-COR, green) in combination with the Cy5-WRP23-VS probe. Red and green channels were screened as described above, and BODIPY fluorescence was scanned on a FUJI scanner (laser 473nm, channel LPB, voltage 400V). Blots were analyzed using Image Studio either ImageJ software.

Proteasome labeling by ABPs in red blood cell lysates

Red blood cells (RBCs) were isolated from whole blood (human) using Polymorphprep™ (AXIS-SHIELD PoC AS, Norway) according to the manufacturer. In brief, 20 ml of whole blood was carefully layered over 20 ml of Polymorphprep™ in a 50 ml conical centrifuge tube and centrifuged at 500 x g for 30 min at room temperature to obtain 8 ml of RBCs. Cells were washed once with 20 ml of Hepes-buffered saline (0.85% (w/v) NaCl and 10 mM Hepes-NaOH, pH 7.4) and centrifuged at 500 x g for 15 min. Then, 5 ml RBCs were diluted in 10 ml of 1 x RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA, pH 7.3) and incubated for 15 min at room temperature. Before labeling RBCs were diluted three times in Hepes-buffered saline (0.85% (w/v) NaCl. Lysates were centrifuged at 10,000×g for 5 min, and the supernatant was collected, aliquoted and stored at -80°C until use. Note that the highest proteasome activity was observed in freshly prepared lysates. RBC lysates were then diluted 33x in HEB buffer (total dilution, 100x) and incubated with fluorescent probes in a reaction volume of 80 µl. The sample was then combined with 80 µl 2xSDS/DTT, boiled, sonicated,

and run on a gel. Electrophoresis, immunoblotting, and fluorescence scanning were conducted as for HEK-293F lysates.

Immunoprecipitation

To pre-clear HEK-293F lysates, 200 µl of A/G protein beads (ImmunoPure® immobilized, Pierce) were washed four times with HEB buffer (1ml per wash) and mixed with 200 µl lysate and 200 µl HEB buffer. The mixture was agitated 1 hour at room temperature. Then beads were centrifuged (500 × g, 5 min, room temperature), and the supernatant/lysate (400 µl) was transferred to a separate vial, to which 4 µl of the 1mM Cy5-Leu-VS probe was added, followed by a 1-hour incubation at 37°C. Next, 50 µl Cy5 antibody (mouse monoclonal IgG, sc-166896, Santa Cruz Biotech) was added to the lysate, and the mixture was agitated for 1 hour at 37°C. Then 200 µl of prewashed (4 times with PBS) A/G beads was added to the lysate, followed by a 2-hour incubation at 37°C. The mixture was then centrifuged (500 × g, 5 min, room temperature) and the supernatant discarded. Remaining beads were washed with PBS and re-centrifuged. After 3 cycles of centrifugation/washing, the last supernatant was removed and 80 µl 2xSDS/DTT was added to the resin, followed by boiling the sample for 5 min. Beads were then centrifuged (10,000×g 1 min) and 35 µl of supernatant was loaded on a 4-12% Tris-Bis10-well gel, followed by SDS-PAGE analysis. Electrophoresis was conducted for 30min at 200V, and the gel was scanned at 700 nm to detect Cy5-labeled bands (LI-COR). After scanning, gels were stained with Instant Blue (Expedeon).

Sample preparation for LC-MS/MS analysis.

Coomassie-stained gels were de-stained and cysteine disulfide bonds were reduced with TCEP (Thermo Fisher Scientific) and then alkylated with iodoacetamide (Sigma), prior to overnight digestion with Trypsin/Lys-C mix endoproteinase (Promega). Digested peptides were extracted from gel pieces with acetonitrile and dried in a SpeedVac. Digested peptides were then desalted on a C18 column, using an automated liquid handling system (Bravo AssayMap from Agilent Technologies).

Dried samples were reconstituted with 2% acetonitrile, 0.1% formic acid and analyzed by LC-MS/MS using a Proxeon EASY nanoLC system (Thermo Fisher Scientific) coupled to an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). Peptides were separated using an analytical C_{18} Acclaim PepMap column (0.075 x 250 mm, 2µm particles (Thermo Scientific))and a 60-min linear gradient of 2-28% solvent B at a flow rate of 300 nl/min. The

mass spectrometer was operated in positive data-dependent acquisition mode. MS1 spectra were measured with a resolution of 60,000, an AGC target of 1e6, and a mass range from 350 to 1400 m/z. Up to 10 MS2 spectra per duty cycle were triggered, fragmented by collision-induced dissociation, and acquired in the ion trap with an AGC target of 1e4, an isolation window of 2.0 m/z, and a normalized collision energy of 35. Dynamic exclusion was enabled with a duration of 20 sec.

Data analysis

All mass spectra from were analyzed with MaxQuant software version 1.5.5.1. MS/MS spectra were searched against the *Homo sapiens* Uniprot protein sequence database (version January 2016) and GPM cRAP sequences (commonly known protein contaminants). Precursor mass tolerance was set to 20ppm and 4.5ppm for the first search, where initial mass recalibration was completed, and for the main search, respectively. Product ions were searched with a mass tolerance 0.5 Da. The maximum precursor ion charge state used for searching was 7. Carbamidomethylation of cysteines was searched as a fixed modification, while oxidation of methionines and acetylation of the protein N-terminus were searched as variable modifications. The enzyme was set to trypsin in a specific mode and a maximum of two missed cleavages was allowed for searching. The target-decoy-based false discovery rate (FDR) filter for spectrum and protein identification was set to 1%.

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