Activity-Based Protein Profiling Hot Paper

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A Set of Activity-Based Probes to Visualize Human (Immuno)proteasome Activities

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Abstract: *Proteasomes are therapeutic targets for various cancers and autoimmune diseases. Constitutively expressed proteasomes have three active sites, β1c, β2c, and β5c. Lymphoid tissues also express the immunoproteasome subunits β1i, β2i, and β5i. Rapid and simultaneous measurement of the activity of these catalytic subunits would assist in the discovery of new inhibitors, improve analysis of proteasome inhibitors in clinical trials, and simplify analysis of subunit expression. In this work, we present a cocktail of activity-based probes that enables simultaneous gel-based detection of all six catalytic human proteasome subunits. We used this cocktail to develop specific inhibitors for β1c, β2c, β5c, and β2i, to compare the active-site specificity of clinical proteasome inhibitors, and to demonstrate that many hematologic malignancies predominantly express immunoproteasomes. Furthermore, we show that selective and complete inhibition of β5i and β1i is cytotoxic to primary cells from acute lymphocytic leukemia (ALL) patients.*

Intracellular proteolysis in eukaryotes is mediated predominantly by 26S proteasomes, which consist of 20S proteolytic core particles (CPs) and one or two 19S regulatory complexes. In vertebrates, constitutive 20S proteasome core particles (cCPs) are expressed in all tissues.[1] Proteasome core particles are C_2-symmetric complexes of four stacked rings of seven subunits each. The outer rings contain seven α-subunits, and the inner rings contain seven β-subunits.[2] Catalytic activity resides in the β-rings, with β1c cleaving preferentially after acidic residues (caspase-like), β2c after basic residues (trypsin-like), and β5c after hydrophobic residues (chymotrypsin-like). Lymphoid tissues express IFN-γ inducible immunoproteasome core particles (iCPs), in which β1c, β2c, and β5c are substituted for β1i (LMP2), β2i (MECL-1), and β5i (LMP7), respectively, and the substrate preferences of the iCP subunits differ from their cCP counterparts.[3] For instance, whereas β1c prefers acidic residues at P1, β1i cleaves preferentially after hydrophobic residues at this position. As a consequence, iCPs produce oligopeptides with more hydrophobic and basic C-termini, which can bind to MHCI molecules.[4] The assignment of proteasome catalytic activities can be achieved using synthetic, peptide-based fluorogenic substrates. However, the majority of fluorogenic proteasome substrates do not distinguish between cCP and iCP activities.[5] The ELISA-based ProCISE assay is time-consuming and requires antibodies to all six subunits.[6] Fluorescent, irreversibly binding proteasome probes can be used to assay individual catalytic activities by activity-based protein profiling (ABPP), but the β1c, β1i, β5c, and β5i subunits cannot be resolved on SDS-PAGE when labeled with the same probe.[7] Herein, we describe the development of a set of activity-based probes that allows for simultaneous detection of all catalytic subunits of human cCP and iCP proteasomes in a rapid SDS-PAGE-based assay (Figure 1).

To enable simultaneous resolution of the six proteasome subunits, we equipped the previously developed site-specific inhibitors NC-001,[8] LU-112,[9] and NC-005[10] with three different fluorophores. This approach yielded β1c- and β1i-reactive Cy5-NC-001, β2c- and β2i-reactive BODIPY(FL)-LU-112,[9] and BODIPY(TMR)-NC-005-VS,[11] which modifies the β5c and β5i sites (Figure 1b). We found that complete and optimal modification of the targeted sites is achieved at 100 nM Cy5-NC-001 and BODIPY(TMR)-NC-005-VS and 30 nM BODIPY(FL)-LU-112 (see the Supporting Information, Figure S1; for the inhibition constants, see Figure S2). Treatment of HEK-293 extracts with a cocktail of the three activity-based probes (ABPs) followed by SDS-PAGE and in-gel fluorescence detection yielded three clear bands corresponding to the three cCP activities expressed by HEK-293 cells (Figure 2a). Treatment of lysate from Raji cells, a human B-lymphoblastic cell line expressing both cCP and iCP, with the same ABP cocktail resulted in six well-resolved bands, with the two top bands labeled in green corresponding to β2c and β2i, the two middle bands in blue to β1c and β1i, and the two bottom bands in red to β5c and β5i (Figure 2a). The specific fluorescence signal can be quantified and is directly proportional to the amount of cells/protein present per sample. The detection limit for all six subunits corresponds to 1×10^3 cells per sample (Figure S3). The time-dependent,

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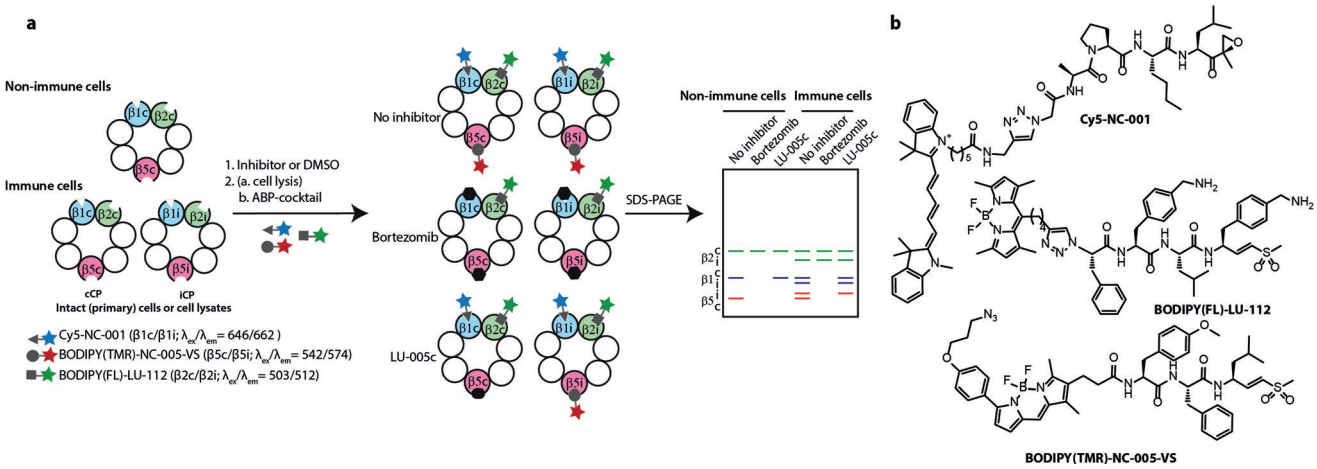

Figure 1. The ABP cocktail enables visualization of the six active human cCP and iCP subunits by SDS-PAGE. a) Schematic representation of competitive ABPP using a proteasome ABP cocktail. b) Structures of Cy5-NC-001, BODIPY(FL)-LU-112, and BODIPY(TMR)-NC-005-VS.

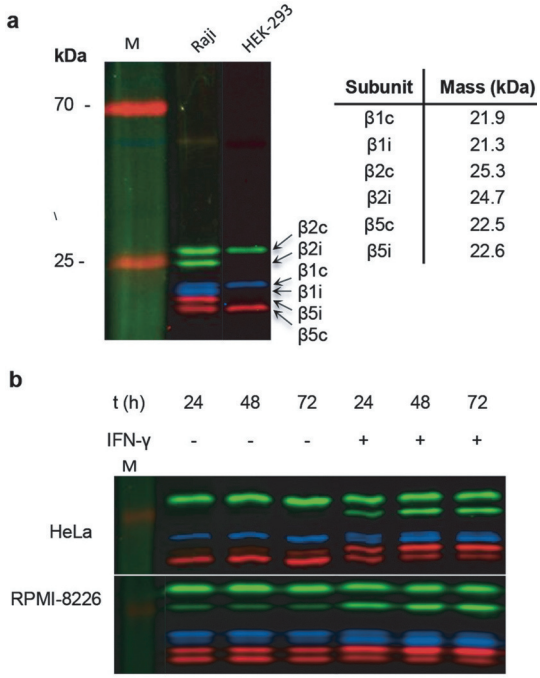

Figure 2. The ABP cocktail enables visualization of the active human cCP and iCP subunits by SDS-PAGE. a) Labeling profiles of Raji and HEK-293 lysates with the ABP probe cocktail. The table shows the molecular weights of the active subunits. b) IFN-γ-treated HeLa cells (very low endogenous iCP) and RPMI-8226 (MM cell line permanently expressing both cCP and iCP) show induction of iCP upon treatment with IFN-γ.

IFN-γ-mediated induction of iCP catalytic subunits can be monitored without the need for proteasome subunit specific antibodies (Figure 2b).[12]

Specific inhibitors of individual subunits are needed to study the role of these subunits in antigen presentation and as drug targets in different diseases.[13] To complement our previously reported subunit-specific inhibitors LU-001i (β1i) and LU-015i (β5i)[14] as well as the in-class broad-spectrum inhibitors NC-001 (β1c/β1i),[10] LU-102 (β2c/β2i),[9] and NC-005 (β5c/β5i),[8] we used our ABP cocktail to discover β1c-, β2c-, β2i-, and β5c-specific inhibitors (Figure 3). LU-001c is a β1c-specific inhibitor, LU-002c is β2c-specific, LU-005c/LU-015c are β5c-specific, and LU-002i is a specific inhibitor of β2i. The subunit specificity of all inhibitors was confirmed in Raji and HEK-293 extracts and in intact RPMI-8226 cells (Figure S4 and Table S1-2 for apparent IC_{50} values). A detailed account of the research that led to the design of these new subunit-specific inhibitors will be published separately (for synthetic procedures, see the Supporting Information). In brief, we recently found that β1c, but not β1i, has strong preference for an acidic residue at P1.[15] Introduction of an aspartate at P1 combined with a proline at P3 (not accepted by β2c/β2i/β5c/β5i) yielded LU-001c, which is not cell-permeable owing to its negative charge at physiological pH. Structure-based design had previously led to the discovery of the β5i-specific inhibitor LU-015i,[14] featuring a large hydrophobic residue at P1 and a small hydrophobic residue at P3. As suggested by X-ray structure analysis,[16] an opposite arrangement of a large hydrophobic residue at P3 and a small one at P1 is preferred by β5c, which led to LU-005c. Although LU-005c was potent in lysates, no inhibition in cells was observed. LU-015c, bearing the same morpholine N-cap as carfilzomib, showed superior intracellular inhibition. X-ray structures of murine cCP and iCP did not reveal any structural differences between the β2c and β2i binding sites.[16] Nevertheless, empirical variation of the size and polarity of the residues at P1–P3 yielded LU-002c (small hydrophobic residue at P2, large basic residue at P1) and LU-002i (large hydrophobic residue at P1—note that the decalin substituent is a mixture of isomers).

Clinical trials of proteasome inhibitors involve pharmacodynamic assessment of proteasome inhibition in blood, where often only combined β5c and β5i activity is measured. At the time of bortezomib development, these β5c and β5i subunits were considered the exclusive targets of antineoplastic agents.[17] However, later studies showed that co-inhibition of other catalytic sites may contribute to the antineoplastic activity of bortezomib and carfilzomib.[7b,18] Therefore, it is important to measure inhibition of all six sites. To demonstrate the applicability of our ABPP assay for

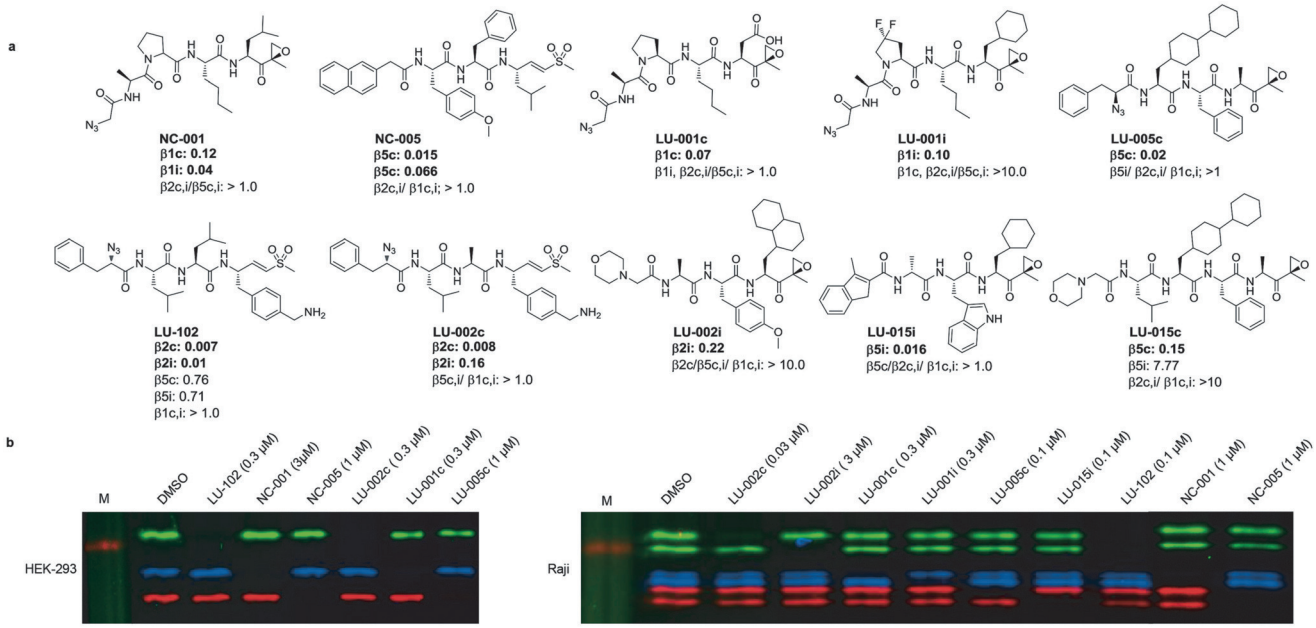

Figure 3. (Newly developed) proteasome subunit specific inhibitors. a) Chemical structures and IC_{50} values (μM) in Raji lysates of (newly developed) proteasome subunit specific inhibitors. b) Selective inhibition of β-subunits in HEK-293 and Raji lysates by subunit-specific proteasome inhibitors assayed by competitive ABPP.

this purpose, we performed side-by-side comparison of proteasome inhibition in RPMI-8226 cells by the clinical drugs carfilzomib and bortezomib, and the clinical candidates oprozomib,[19] delanzomib,[20] and ixazomib[21] (Figure 4a–c, Table S3).[22] We confirmed that all five compounds are potent β5c and β5i inhibitors. As described in the literature, bortezomib co-inhibits β1c/β1i,[23] but β2c and β2i are only partially inhibited at micromolar concentrations. Carfilzomib is rather β5c/β5i-specific at lower concentrations, but blocks all six sites at higher concentrations. Oprozomib, an orally bioavailable analogue of carfilzomib, is more β5c/β5i-selective. Another boronic acid, delanzomib, matches the activity profile of bortezomib except that delanzomib does not inhibit β2c/β2i even at high concentrations. Ixazomib inhibits four sites, β5c, β5i, β1c, and β1i, with about equal potency, but at ten times higher concentrations than bortezomib. A comparison of the recovery of proteasome activity after treatment with the inhibitor for one hour followed by inhibitor washout (Figure 4d,e, Figure S5) confirmed that ixazomib has the fastest off rate.[24] Remarkably, the residence time of the three boronates in the β5c/β5i-active sites is much shorter than in β1c/β1i-active sites.

Information on the relative expression of the constitutive proteasome and the immunoproteasome in primary cells from different hematologic malignancies is limited. Therefore, we compared the expression of active immunoproteasome subunits with their constitutive counterparts in primary cells from ALL, AML, CLL, and MM patients by direct quantification based on gel band intensities (Figure 5a). Expression of immunoproteasomes exceeded expression of constitutive proteasomes in all patient samples. In contrast, MM-derived cell lines (RPMI-8226 and AMO) showed higher expression levels for the constitutive subunits (> 50% β5c). Even more strikingly, in all primary patient samples from lymphoid malignancies (2 × B-ALL, 1 × T-ALL, 2 × CLL), over 90% of the β5 and 75% of the β1 proteasome activity is provided by iCPs. The β2c/β2i ratio was less pronounced, probably reflecting the presence of proteasomes consisting of both constitutive and immunoproteasome subunits, so-called hybrid or intermediate proteasomes.[25] From a drug development perspective, our data strongly indicate that immunoproteasomes should be regarded as a major drug target in MM and lymphoid neoplasms, such as ALL and chronic lymphocytic leukemia (CLL). In support of this, we tested the effect of selective inhibition of β5i activity in primary B- and T-ALL samples. Continuous treatment with LU-015i for 48 hours resulted in dose-dependent cytotoxicity, which however did not fully correlate with β5i inhibition (Figure S6). As we observed β1i co-inhibition at higher concentrations, we reasoned that combined inhibition of β5i and β1i would lead to more efficient cell death. To test this hypothesis, we treated patient cells with the β5i (LU-015i) and/or β1i (LU-001i) inhibitor for one hour, washed out unbound inhibitor to ensure specific inhibition (Figure 5b), and assessed cell viability after 48 hours. We found that in B-ALL-2 and T-ALL cells, specific β5i or β1i inhibition did not lead to any cytotoxicity; however, combined β5i and β1i inhibition led to up to 70% cell death (Figure 5c). In B-ALL-1 cells, combined β5i and β1i inhibition also resulted in significantly higher cytotoxicity than inhibition of only β5i or β1i.

If these findings are confirmed by experiments with additional primary cell samples and animal models, the use of selective inhibitors of the iCP for the treatment of lymphoblastic leukemia and perhaps multiple myeloma may extend the therapeutic window of proteasome inhibitors because low

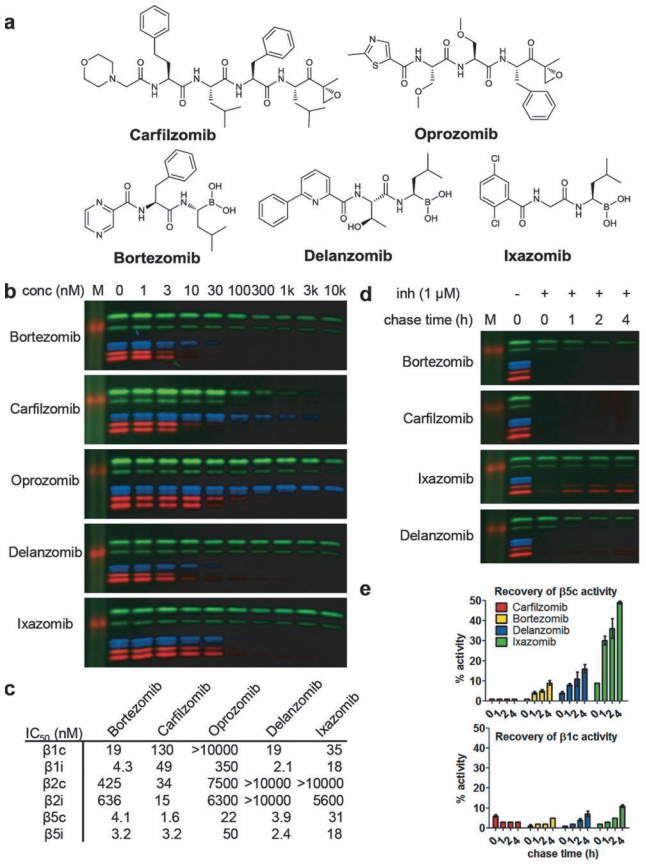

Figure 4. Characterization of proteasome inhibitors used clinically (bortezomib and carfilzomib) and undergoing trials (oprozomib, delanzomib, ixazomib). a) Chemical structures of the inhibitors. b, c) Inhibition profiles (b) and apparent IC_{50} values (c) in living RPMI-8226 cells after treatment for one hour. d, e) Treatment with inhibitors for one hour, followed by washout and chase for 1, 2, or 4 hours shows highest recovery of proteasome activity for ixazomib. Recovery of β5c/β5i is much faster than that of β1c/β1i (e).

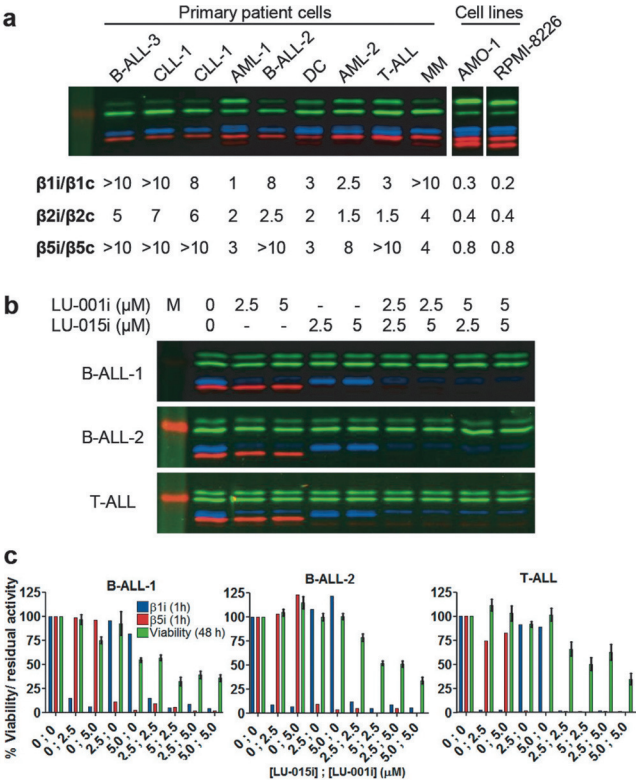

Figure 5. a) Labeling profiles of primary patient cells derived from haematological malignancies, compared to cell lines. Table: ratio between the immuno- and constitutive proteasome subunits. B-ALL: B-cell acute lymphoblastic leukemia; T-ALL: T-cell acute lymphoblastic leukemia; CLL: chronic lymphocytic leukemia; AML: acute myeloid leukemia; DC: acute leukemia of plasmacytoid dendritic cells; MM: multiple myeloma. b) Inhibition profiles of B- and T-ALL cells treated with the indicated concentrations of LU-015i or LU-001i. c) Viability of ALL cells treated with LU-015i and/or LU-001i after pulse treatment for one hour followed by chase for 48 hours compared to β5i and β1i inhibition after one hour.

iCP expression in the majority of tissues should allow administration of higher doses.

The new ABP probe cocktail presented here provides the first means for rapid, antibody-independent measurement of the six catalytically active cCP and iCP subunits, which can be easily adopted to patients. Furthermore, the first comprehensive set of specific inhibitors of all six sites should allow for determining the contribution of individual proteasome subunits to the generation of antigenic peptides. Finally, our panel of ten subunit-specific inhibitors in concert with the activity-based probes provides a good entry point to optimize the therapeutic efficacy of β5-targeting proteasome inhibitors by selected, controlled degrees of co-inhibition of additional active sites of the iCP and/or CP, which may be tailored to a given clinical application or disease state.

Keywords: activity-based protein profiling · anticancer agents · fluorescent probes · inhibitors · proteasome

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