Indian Journal of Experimental Biology
Vol. 64, May 2026, pp. 394-399
DOI: 10.56042/ijeb.v64i05.24289

Effective method for studying β5 subunit activity of immunoproteasome *in vitro*

Guo Jinxing[1], Hu Jiahang[1] & Fu Dan[2]*

[1]HongQi Medicine, [2]College of Radiation and Imaging Science, Mudanjiang Medical University, Tongxiang Road 3, Mudanjiang 157011, China

Received 28 September 2025; revised 9 January 2026

The immunoproteasome and its dysfunction are implicated in multiple diseases. In multiple myeloma, immunoproteasomes promote cancer cell survival, making them an important therapeutic target for antagonist development. Here, we present a straightforward method for detecting β5i (the β5 subunit of the immunoproteasome) cellular activity *in vitro*. This cell-based approach utilizes a specific β5i substrate (Ac-Ala-Asn-Trp-AMC), which is cleaved by immunoproteasomes and releases a fluorescent signal with an emission peak at 460 nm. After multiple optimizations, we found that adding an equal volume of substrate solution to 30 µL cell lysate, incubating for 10 minutes at 37°C, and measuring fluorescence at 460 nm yielded IC_{50} values for ONX-0914 (a selective inhibitor of low-molecular mass polypeptide-7) and bortezomib that are consistent with published data, with repeatable and stable results across different cell lines. Additionally, comparison with the β5c commercial kit (Promega, G8661), which is compatible with the β5c substrate, demonstrated excellent sensitivity and accuracy. In summary, this protocol facilitates the screening and determination of subunit specificity for novel immunoproteasome inhibitors.

Keywords: Immunoproteasomes,Bortezomib, ONX-0914 (selective inhibitor of low-molecular mass polypeptide-7), β5i (β5 subunit of immunoproteasome)

The proteasome is the primary system responsible for protein degradation in cells, with approximately 60–80% of cellular proteins relying on the proteasome pathway to maintain homeostasis[1].The proteasome is a highly complex molecular machine built from 20S core particles[2],each composed of 28 subunits stacked in four homologous rings to form a hollow cylindrical structure[3].The two inner rings each contain seven β subunits, which are flanked by two outer rings composed of seven α subunits each[4,5].The proteolytic chamber is formed by three catalytically active subunits: β1(caspase-like activities), β2(trypsin-like activities), and β5(chymotrypsin-like activities)[6]. In the mammalian system, the constitutive proteasome is ubiquitous[7],whereas the immunoproteasome is predominantly expressed in monocytes and lymphocytes[8,9]. Each proteasome type presents distinct cleavage preferences based on its proteolytic β subunits and generates specific peptide substrates for the antigen-presenting primary histocompatibility complex class I (MHC-I)[10]. The β1i and β5i subunits of the immunoproteasome have distinct chemical environments in their active sites, which influence the generation of specific MHC-I epitopes[11]. The β1i subunit has a hydrophobic active site, whereas the β5i subunit has a more hydrophilic one, and these differences shape the properties of the peptide fragments[12].

Accumulating evidence indicates that the immunoproteasome is involved in the pathogenesis of neurodegenerative diseases, autoimmune disorders[13], cardiovascular diseases[14], and certain cancers[15].Selective inhibition of the β5i subunit has demonstrated clinical benefits in conditions such as arthritis and colorectal carcinoma[16].In parallel, the constitutive proteasome has served as a drug target, with three FDA-approved inhibitors including bortezomib[17]. However, to accelerate β5i inhibitor discovery and assessment, there is a pressing need to improve and diversify current assay strategies and to establish a robust, reliable method for β5i inhibitor development. We reviewed the commonly used approaches for detecting β5i activity *invitro*, such as purification and assay of endogenous immunoproteasomes, which require careful validation to exclude contamination from constitutive proteasomes[17,18].The use of stable green fluorescent protein fusion reporters is widespread for monitoring

*Correspondence:
Phone: +86-138-4539-3635
E-mail: fudansunming101@gmail.com

proteasome function in cells;however, these reporters only indirectly reflect immunoproteasome activity and can be affected by changes in protein translation[19]. In cell lysate-based assays, cells are treated with inhibitors prior to incubation with the 4,4-difluoro-4-bora-3a, 4a-diaza-s-indacene cyanophenyl substituents (BODIPY-NC-005), followed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and measurement of the β5i band intensity[20]. These protocols are labor-intensive and costly.We aim to develop a more streamlined assay that measures the enzymatic activity of the β5i subunit using the fluorescent substrate Ac-ANW-AMC (Ac-Ala-Asn-Trp-AMC), enabling efficient evaluation of potential β5i inhibitor candidates or lead compounds.

Reagents and Materials

Raji and Jurkat cells (ATCC, American Type Culture Collection) were cultured in RPMI-1640 medium (Thermofisher, Catalog number 11875093) with 10% FBS (Fetal Bovine Serum), (Thermofisher, Catalog number A5670701,) and 1% penicillin-streptomycin (Thermofisher, Catalog number 15140122). Bortezomib (Catalog number s1013) and ONX-0914 (Catalog number s7172) were ordered from Selleckchem company, and Ac-ANW-AMCs(Catalog number s-320) was ordered from R&D company, Digitonin (Catalog number D141) and NP-40 (Nonidet P-40 substitute) (Catalog number 492016) from Sigma company, and the proteasome-GLO™ kit (Catalog number G8661, Promega) was usedfor this assay.The protease inhibitor cocktail from ThermoFisher (Catalog number 78429).Measuring fluorescent intensity with Biotek Synergy H1 hybrid reader. We use GraphPad Prism 8.0 to do the analysis and statistics.

Cell culture

Raji and Jurkat cells were cultured in RPMI-1640 medium added into 10% FBS and 1% penicillin-streptomycin, THP-1 cells were cultured in RPMI-1640 complete medium with 2-mercaptoethanol (Gibco catalog number 21985023) to a final concentration of 0.05 mM, incubating the culture at 37°C in a suitable incubator with 5% CO2. Do not allow the cell overgrowth and subculture when cell numbersreach outten million with Corning® T-75 flasks (Catalog #431464).

Cell viability assay

Prepare 48-well plates (Corning, Catalog# CLS356505) by seeding mammalian cells in the desired culture medium. Optimize cell density and medium volume according to specific experimental requirements. Set up control wells containing only medium (no cells) to determine background luminescence. Add test compounds to the experimental wells and incubate for the specified duration. Prior to luminescence measurement, equilibrate the plate at room temperature (23°C) for approximately 30 minutes. Add CellTiter-Glo® 2.0 Reagent in a volume equal to the culture medium present in each well (for example, add 100 µL reagent to 100 µL medium). Mix the plate contents on an orbital shaker for 2 minutes to ensure thorough cell lysis. Incubate the plate at room temperature for 10 minutes to stabilize the luminescent signal, then measure luminescence using the Synergy H1 hybrid reader.

The final process of the β5i activity assay

Step 1. Prepare cell lysis buffer and substrate solution

Cell lysis buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, 1 mM DTT (Dithiothreitol), 0.01% NP-40, 1 mM digitonin, and protease inhibitor cocktail.

Ac-ANW-AMC Working solution: Freshly making 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP, 80 µM Ac-ANW-AMC, and 1 mM DTT.

Step 2. Prepare cell lysate

1. Seed 1×10^5 cells into a 48-well plate. Add different concentrations of inhibitors to duplicate wells and incubate for 6 hours at 37°C.

2. Harvest cells and centrifuge at 2,000 rpm for 5 minutes. Remove the medium, wash the pellet with 1 mL PBS, and further remove residual PBS.

3. Resuspend the pellet in 100-µL lysis buffer, pipette 20 times, incubate on ice for 30 minutes, and centrifuge at 13,000 rpm for 10 minutes.

Step 3. Perform the assay

Load 30 µL cell lysate into a white 96-well plate and mix with 30 µL substrate solution. Incubate at 37°C for 10 minutes. Set up the microplate reader: 37°C, 10-minute delay, dynamic assay, Ex 380 nm and Em 460 nm.

Results and Discussi

The basic mechanismofdetecting β5i subunit activity *in vitro*

Raji and Jurkat cell lines are frequently used for immunoproteasome purification and β5i inhibitor screening. Bortezomib, which has an IC50 of 3.3 nM against β5i[17], is approved for multiple myeloma[21], but its clinical application to solid tumors is limited by adverse effects and long-term drug resistance[22]. The development of immunoproteasome-selective

Table 1 — Cell numbers versus cell lysate buffer

Cell Number	Buffer 1(slope and R^2)	Buffer 2(slope and R^2)	Buffer 3(slope and R^2)
200000	306; R^2=0.90	243; R^2=0.95	260; R^2=0.92
100000	302; R^2=0.87	271; R^2=0.93	233; R^2=0.90
50000	140; R^2=0.90	110; R^2=0.87	162; R^2=0.90
25000	98; R^2=0.86	90; R^2=0.95	129; R^2=0.93

Table 2 — Reaction slopes with 1 mM digitonin

Cell Number	Digitonin (slope and R^2)
100000	338.7; R^2=0.97
200000	326.5; R^2=0.99
300000	393.5; R^2=0.99

inhibitors is therefore critical. ONX-0914 shows greater activity toward β5i (IC50 5.7 nM), and its derivative KZR616 (Zetomipzomib), with 80-fold β5i selectivity, is in clinical trials for systemic lupus erythematosus[23,24]. To validate our approach, we compiled published data to optimize the protocol and compared it to a commercial β5c kit, confirming that both β5i and β5c can be detected from the same cell lysate. This assay uses a specific immunoproteasome substrate and an optimized permeabilization buffer to retain enzymatic activity. The Ac-ANW-AMC peptide substrate is cleaved by immunoproteasomes, generating a fluorescent signal at 460 nm[25].

To optimize the assay, we first determined the ideal cell number and lysis buffer by comparing reaction rates using three different buffers. As shown in Table 1, 100,000 cells are sufficient for the assay, and Buffer 1 produced the highest immunoproteasome activity compared to Buffer 2 and Buffer 3. Increasing digitonin to 1 mM further improved efficiency, suggesting that complete cell lysis enhances immunoproteasome release and substrate binding (Table 2). We confirmed that the protease inhibitor cocktail is not required for this assay. A 100 µL cell lysis buffer is effective, and there was no significant difference observed between 60 µL and 80 µL reaction volumes. Therefore, we recommend a 60 µL reaction system to reduce costs (Table 3). Higher substrate concentrations resulted in steeper reaction slopes, although nonspecific recognition by other proteasome subunits may occur. A final substrate concentration of 40 µM provided high productivity (Table 4). Following the optimized protocol, we tested ONX-0914 inhibition in Raji cells. The IC_{50} was 2.78 µM, higher than published data (5 nM). For β5c, the IC_{50} measured using Proteasome-Glo kits was 5-fold higher than published data (Fig 1a and 1b). Due to the toxicity of ONX-0914, cells were incubated with the inhibitor for 6 hours; overnight incubation resulted in cell death (Fig 1c).

Table 3 — Cocktail inhibitors effect on reaction

Group	AMC Mean V	Y intercept	R^2
60 µL - cocktail	435578	13803	0.99
60 µL+ cocktail	449314	12492	0.99
80 µL - cocktail	710395	20251	0.99
80 µL+ cocktail	754204	19705	0.99

Table 4 — AMC substrate concentration in different reaction

Group	AMC Mean V	Y Intercept	R^2
60 µL+40µM AMC	419305	11667	0.99
60 µL+20µM AMC	226543	5786	0.99
60 µL+10µM AMC	84772	2239	0.99
80 µL+40µM AMC	638560	16779	0.99
80 µL+20µM AMC	308321	8086	0.99
80 µL+10µM AMC	120974	2966	0.99

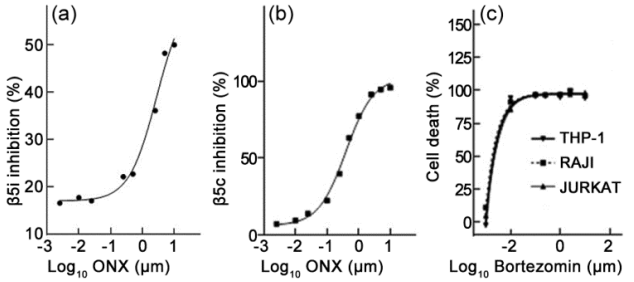

Fig. 1 — The basic protocol of detection β5i subunit activity (a) and (b): IC_{50} values for β5i and β5c were 2.78 µM and 0.39 µM, respectively. (c): Prolonged incubation with bortezomib caused cell death.

Optimization of Detergents in Cell Lysis Buffer

To achieve complete cell lysis, we optimized the lysis buffer by combining 1 mM digitonin with 0.01% NP-40. Although digitonin alone produced larger debris after centrifugation, the addition of NP-40 resolved this problem. We also included 1 mM DTT to preserve immunoproteasome activity. These modifications reduced the IC_{50} of β5c to 72 nM (Fig 2a), though the IC_{50} of β5i remained above the ideal value (Fig 2b). Lowering the substrate concentration to 10 µM did not alter the final IC_{50} but decreasing the cell lysate volume delayed the maximum reaction rate (Fig 2c). Additionally, incubating the reaction buffer (with 80 µM Ac-ANW-AMC) and cell lysate at room temperature for 30 minutes improved the results, as DMSO becomes insoluble at lower temperatures (Fig 2d). Adjusting this step brought the IC_{50} of β5i closer to 34 nM.

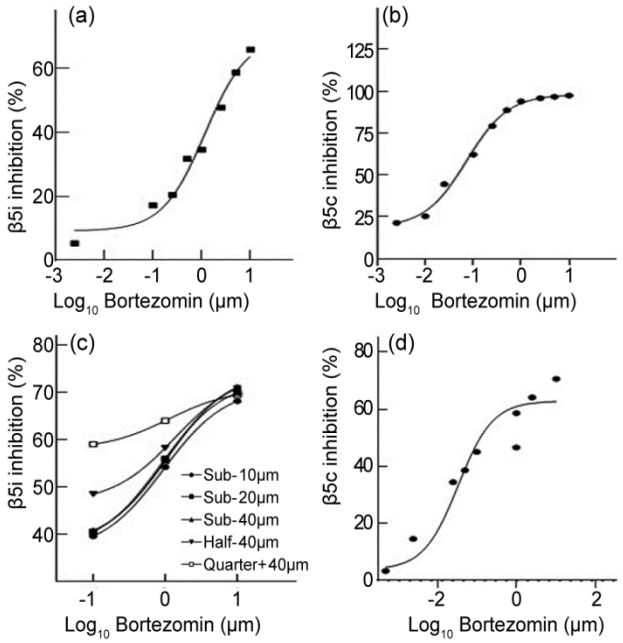

Fig. 2 — Optimization of detergents in cell Lysis buffers (a): The IC_{50} of bortezomib is shown in digitonin plus NP-40 buffer. (b): IC_{50} of bortezomib in the optimized lysis buffer. (c): Effect of substrate concentration on reaction curves. (d): Room temperature incubation improves reaction efficiency.

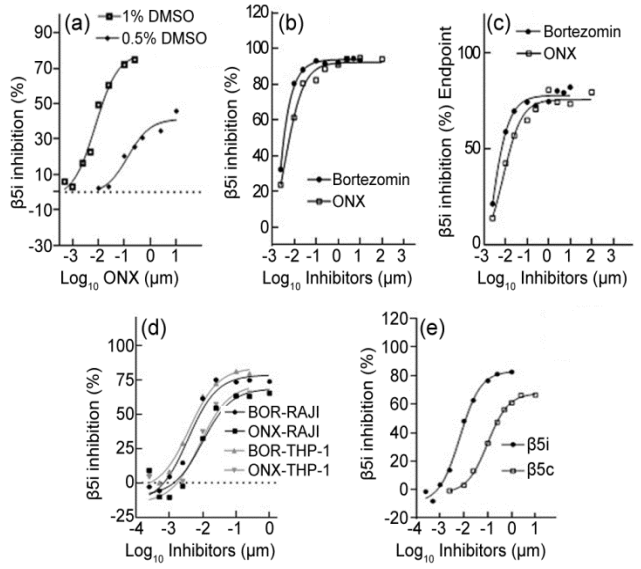

Fig. 3 — Correction the key step in the protocol; (a): Higher DMSO inhibits the efficiency of immunoproteasome cleavage; (b) and (c): Comparable IC_{50} values from kinetic and endpoint analyses; (d): Consistent IC_{50} values of β5i inhibitors across cell lines; (e): Compatibility of cell lysate with the β5c substrate.

Effect of DMSO concentration

We assessed the impact of DMSO concentration on assay efficiency. Lower DMSO levels improved the kinetic curve, yielding an IC_{50} of 7.6 nM, which closely matches published data. In contrast, a higher DMSO concentration (1%) elevated the IC_{50} to 136 nM, indicating inhibition of immunoproteasome activity (Fig 3a). After optimizing the protocol, we obtained consistent IC_{50} values for both bortezomib (2.5 nM) and ONX-0914 (3.5 nM), with linear kinetic curves and similar endpoint values (Fig 3b and 3c). To evaluate assay stability and repeatability, we compared the IC_{50} values for both inhibitors across two cell lines; Fig 3d demonstrates that the results were comparable and reproducible. These findings indicate that the final protocol is suitable for studying functional immunoproteasomes. Additionally, the β5i assay was compatible with the Proteasome-Glo® Kit, enabling simultaneous β5c assays using the same Raji cell lysate (Fig 3e). Thus, two different assays can be performed with a single cell lysate preparation.

Discussion

Manipulating the catalytic activity of immunoproteasome subunits has become increasingly important for developing selective inhibitors and treatments for inflammatory diseases[26,27]. While cell-based assays have been developed, their use has been limited by the availability of specific substrates and assay sensitivity[28].The protocol presented here can be applied to a variety of cell lines and enables detection of both β5i and β5c subunit activity from the same cell lysate, offering a better understanding of the immunoproteasome's role in disease progression and facilitating the identification of small-molecule interactors. This user-friendly assay features a stable, linear kinetic rate and endpoint values suitable for statistical analysis, with low background noise and an excellent signal-to-noise ratio.

Proteasome activity is commonly monitored using peptide substrates such as tripeptides or tetrapeptides conjugated to 7-amino-4-methylcoumarin (AMC), which exhibits weak fluorescence when linked to a peptide but emits a strong signal around 440 nm upon cleavage by the proteasome[29]. However, commercially available AMC peptides are poorly permeable, which limits their application in cell-based assays. In this protocol, using digitonin alone can result in incomplete lysis and unstable fluorescence signals. By combining digitonin with low concentrations of NP-40, efficient nuclear disassembly and cell lysis are achieved. Additionally, since Ac-ANW-AMC is dissolved in DMSO, it is crucial to minimize the DMSO concentration in the reaction to avoid immunoproteasome inhibition. To

ensure consistent results, we equilibrated both the substrate stock and working solutions at room temperature before beginning the assay.

A similar strategy was used to develop the immunoproteasome-selective activity-based probe TBZ-1[30]. Although such probes are widely used in vitro, a common problem is that they do not necessarily mimic endogenous protein proteolysis and often lack strict immunoproteasome specificity. Other researchers have developed BODIPY-functionalized activity-based probes to address these limitations, but these probes are often not commercially available, are synthetically challenging, or do not readily permit fluorophore exchange[31]. We aim to further improve the selectivity profile of chemical substrates to monitor additional immunoproteasome subunits such as β1i and β2i in the future. Excitingly, our current results show that this protocol is sensitive enough to detect reductions in β5i activity in various cell lines treated with different inhibitors, demonstrating its potential in drug discovery efforts targeting the immunoproteasome.

Conclusions

Beyond studies focused on constitutive proteasomes, there is an urgent need for a reliable immunoproteasome detection assay suitable for both research and industrial applications. In this study, we demonstrated that our cell-based protocol can effectively detect β5i activity in the desired cell type following inhibitor treatment, highlighting its utility in drug discovery. The protocol, developed from prior experience and optimized for β5i subunit activity, is compatible with Proteasome-Glo® kits. This compatibility enables two assays using distinct proteasome substrates in parallel, reducing assay costs. With this method, we obtained IC_{50} values for bortezomib (2.5 nM) and ONX-0914 (3.5 nM) that match published data and support the investigation of immunoproteasome β5i function. Overall, this cell-based assay is especially well suited for rapid, large-scale screening of β5i-selective inhibitors. We are also exploring whether this approach can be extended to assess β1i and β2i inhibitors.

Conflict of interest

All authors declare that there is no conflict of interest.

References

1 Cromm PM & Crews CM. The Proteasome in Modern Drug Discovery: Second Life of a Highly Valuable Drug Target. *ACS Cent Sci*, 3 (2017) 830.

2 Kish-Trier E & Hill CP. Structural biology of the proteasome. *Annu Rev Biophys*, 42 (2013) 29.

3 Jung T, Catalgol B & Grune T. The proteasomal system. *Mol Aspects Med*, 30 (2009) 191.

4 Groll M, Ditzel L, Lowe J, Stock D, Bochtler M & Bartunik HD, Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature*, 386 (1997) 463.

5 Lowe J, Stock D, Jap B, Zwickl P, Baumeister W & Huber R. Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 A resolution. *Science*, 268 (1995) 533.

6 Murata S, Sasaki K, Kishimoto T, Niwa S, Hayashi H & Takahama Y. Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science*, 316 (2007) 1349.

7 Kunjappu MJ & Hochstrasser M. Assembly of the 20S proteasome. *Biochim Biophys Acta*, 1834 (2014) 2.

8 Groettrup M, Kirk CJ & Basler M. Proteasomes in immune cells: more than peptide producers. *Nat Rev Immunol*, 10 (2010) 73.

9 Murata S, Takahama Y & Tanaka K. Thymoproteasome: probable role in generating positively selecting peptides. *Curr Opin Immunol*, 20 (2008) 192.

10 Basler M, Kirk CJ & Groettrup M. The immunoproteasome in antigen processing and other immunological functions. *Curr Opin Immunol*, 25(2013) 74.

11 Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW & Lauer C. A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med*, 15 (2009) 781.

12 Koerner J, Brunner T & Groettrup M. Inhibition and deficiency of the immunoproteasome subunit LMP7 suppress the development and progression of colorectal carcinoma in mice. *Oncotarget*, 8 (2017) 50873.

13 Sabarinathan R. Immunoproteasome as a biomarker for immunotherapy. *Nat Rev Cancer*, 25 (2025) 6.

14 Nie Y, Ma Z, Zhang B, Sun M, Zhang D, Li HH & Song X. The role of the immunoproteasome in cardiovascular disease. *Pharmacol Res*, 204 (2024) 107215.

15 Mancuso F, Di Chio C, Di Matteo F, Smaldone G, Iraci N & Giofre SV. Recent Advances in the Development of Immunoproteasome Inhibitors as Anti-Cancer Agents: The Past 5 Years. *Molecules*, 30 (2025) 755.

16 Sogbein O, Paul P, Umar M, Chaari A, Batuman V & Upadhyay R. Bortezomib in cancer therapy: Mechanisms, side effects, and future proteasome inhibitors. *Life Sci*, 358 (2024) 123125.

17 Groll M, Berkers CR, Ploegh HL & Ovaa H. Crystal structure of the boronic acid-based proteasome inhibitor bortezomib in complex with the yeast 20S proteasome. *Structure*, 14 (2006) 451.

18 Loy CA & Trader DJ. Synthesis and Application of a Caged Bioluminescent Probe for the Immunoproteasome. *Curr Protoc*, 4 (2024) e70057.

19 Berkers CR, Verdoes M, Lichtman E, Fiebiger E, Kessler BM & Anderson KC. Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib. *Nat Methods*, 2 (2005) 357.

20 Mundt S, Engelhardt B, Christopher KJ, Groettrup M & Basler M. Inhibition and deficiency of the immunoproteasome subunit LMP7 attenuates LCMV-induced meningitis. *Eur J Immunol*, 46 (2016) 104.

21 Moreau P, Richardson PG, Cavo M, Orlowski RZ, San Miguel RZ & Palumbo A. Proteasome inhibitors in multiple myeloma: 10 years later. *Blood*, 120 (2012**)** 947.

22 Cengiz Seval G & Beksac M. The safety of bortezomib for the treatment of multiple myeloma. *Expert Opin Drug Saf*, 17 (2018**)** 953.

23 Bruin G, Huber EM, Xin BT, Van Rooden EJ, Al-Ayed K & Kim KB. Structure-based design of beta1i or beta5i specific inhibitors of human immunoproteasomes. *J Med Chem*, 57 (2014**)** 6197.

24 Johnson WB, Lowe E, Anderl JL, Fan A & Muchamuel T. Required Immunoproteasome Subunit Inhibition Profile for Anti-Inflammatory Efficacy and Clinical Candidate KZR-616 ((2 S,3 R)- N-((S)-3-(Cyclopent-1-en-1-yl)-1-((R)-2-methyloxiran-2-yl)-1-oxopropan-2-yl)-3-hydroxy-3-(4-methoxyphenyl)-2-((S)-2-(2-morpholinoacetamido) propanamido) propenamide). *J Med Chem*, 61 (2018**)** 11127.

25 Kim S, Park SH, Choi WH & Lee MJ. Evaluation of Immunoproteasome-Specific Proteolytic Activity Using Fluorogenic Peptide Substrates. *Immune Netw*, 22 (2022) e28.

26 Thibaudeau TA & Smith DM. A Practical Review of Proteasome Pharmacology. *Pharmacol Rev*, 71(2019**)** 170.

27 Muli CS, Loy CA & Trader DJ. Immunoproteasome as a Target for Prodrugs. *J Med Chem*, 68 (2025) 6507.

28 Burov A, Funikov S, Vagapova E, Dalina A, Rezvykh A, Shyrokova E, Lebedev T, Grigorieva E, Popenko V, Leonova O, Spasskaya D, Spirin P, Prassolov V, Karpov V & Morozov A. A Cell-Based Platform for the Investigation of Immunoproteasome Subunit beta5i Expression and Biology of beta5i-Containing Proteasomes. *Cells*, 10 (2021) 3049.

29 Linn CP, Mize PD, Hoke RA, Quante JM & Pitner JB. Synthesis of serine-AMC-carbamate: a fluorogenic tryptophanase substrate. *Anal Biochem,* 200 (1992) 400.

30 Halder S, Loy CA & Trader DJ. Synthesis and Application of a Versatile Immunoproteasome Activity Probe. *Chembiochem*, 25 (2024) e202400571.

31 Kim M, Serwa RA, Samluk L, Suppanz I, Kodron A, Stepkowski TM, Elancheliyan P, Tsegaye B, Oeljeklaus S, Wasilewski M, Warscheid B & Chacinska A. Immunoproteasome-specific subunit PSMB9 induction is required to regulate cellular proteostasis upon mitochondrial dysfunction. *Nat Commun*, 14 (2023) 4092.