An effective method studies the β5 subunit activity of immunoproteasome in *vitro*

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Method Article

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Abstract

The immunoproteasome has been found to connect multiple diseases, such as multiple myeloma and autoimmune disorders, and these antagonists of immunoproteasomes urgently need to be developed into drugs to relieve the suffering of patients. We summarized previous experiences and finally provided a simple and sensitive approach to detect β5i (the β5 subunit of the immunoproteasome) activity for research or industrial purposes. The results show that the published IC50 of ONX-0914 or bortezomib is repeatable with our assay. We also detected β5i activity in different cell lines, and the IC50 is acceptable and stable to be verified by many tests. It is worth noting that the combination of digitonin and a lower concentration of NP-40 will maintain higher immunoproteasome activity and completely lyse the cell membrane. Meanwhile, a lower concentration of DMSO is required to improve the kinetic process and reach the maximum rate of reaction. Compared to the β5c assay, the β5i commercial kit is limited to being selected. We prefer to make a highly efficient kit for the β5i assay to meet the academic or clinical needs of researchers, and it could be extensively used due to the cheaper cost and stability in the application.

Introduction

The proteasome is a predominant system responsible for protein degradation, and approximately 80% of cellular proteins use the proteasome pathway to maintain the homeostasis of regulation. [1] The proteasome is an overly complex molecular structure consisting of 20S core particles. [2, 3] The 20S comprises 28 subunits that are stacked in four homologous rings, forming a hollow cylindrical structure. The two inner rings each contain seven β subunits, which are enclosed by the two outer rings assembled from seven α subunits. [4, 5] The proteolytic chamber is formed by the β ring, which harbors the three catalytical active subunits β1, β2, and β5, which exhibit caspase-like activity, trypsin-like activity, and chymotrypsin-like activity, respectively. In the mammalian system, the constitutive proteasome (CP) has been identified in all tissues, whereas the immunoproteasome (ICP) predominantly appears in monocytes and lymphocytes. [7, 8, 9] Each of these two proteasomes harbors a unique set of catalytic β-subunits resulting in different cleavage preferences and specifically generates substrates for the antigen-presenting major histocompatibility complex-1 in the immune system. [10, 11, 12]

The immunoproteasome has been associated with the development of neurodegenerative diseases, autoimmune disorders, inflammation, and certain types of cancer. The particular inhibition of the β5i subunit has gained clinical benefit in the treatment of arthritis and colorectal carcinoma. [13, 14] Correspondingly, the constitutive proteasome has been developed into a drug with three FDA (Food and Drug Administration)-approved products on the market, such as bortezomib. [15, 16] To improve the process of β5i inhibitor identification and evaluation, it is urgent to optimize the current assay and determine an effective and stable method to facilitate the development of β5i inhibitors. We recapitulated most of the regular methods used to detect β5i activity in vitro, for example, endogenous immunoproteasome purification and assays, and it is necessary to confirm contamination by constitutive proteasomes. The stable GFP-fusion reporter is widely used to monitor proteasome activity in cell culture,

which indirectly responds to the activity of immunoproteasomes and cannot rule out interference due to protein translation[17]. Based on the cell lysate and competition assay, the cells were exposed to the inhibitor for 1 hour at 37°C before incubation with BODIPY-NC-005 and then subjected to SDS-PAGE. Finally, Cy3 signaling was detected in a wet gel to measure the fluorescent densitometry, but more time and expensive experiments would be required[18]. Here, we will modify the assay based on the enzyme activity of the β5i subunit targeting its fluorescent substrate Ac-ANW-AMC and judge the efficacy of potential candidates or leader compounds of β5i inhibitors.

Bortezomib was approved for the treatment of multiple myeloma and mantle cell lymphoma. Its severe toxicities and adverse effects limit its clinical application on solid tumors; another major problem is its drug resistance, which urgently needs to be solved. Bortezomib is a nonselective protease inhibitor and exerts stronger inhibitory activity by forming a reversible covalent boron oxygen bond with the free hydroxyl of the Thr residue[19]. Bortezomib exhibits activity against β5i with an IC_{50} value of 3.3 nM[20]. Compared to the indiscriminate inhibition of nonselective inhibitors, the development of immunoproteasome-selective inhibitors is urgently needed. ONX-0914 shows an improved activity toward β5i (IC_{50} 5.7 nM) [21], KZR616, a derived inhibitor from ONX-0914, and exhibited 80-fold selectivity against β5i under clinical trials for the treatment of systemic lupus erythematosus[22]. To determine the reliability of the approach, we will adopt these published data to optimize our protocol, and we will also combine the β5c commercial kit to further confirm whether this strategy of experiments is feasible to detect both β5i and β5c from the same cell lysate.

Materials And Reagents

Raji and THP-1 cells were cultured in RPMI-1640 medium with 10% FBS and 1% penicillin-streptomycin. When the cell concentration reached $8x10^5$ cells/mL, the cell concentration did not exceed 1×10^6 cells/mL, and the medium was renewed every 2 days. Bortezomib (s1013) and ONX-0914 (s7172) were ordered from Selleckchem, and Ac-ANW-AMCs were ordered from R&D (s-320). Digitonin (D141) and NP-40 (492016) were purchased from Sigma, and the proteasome-GLO™ kit (G8661) was purchased from Promega. A BioTek Synergy H1 Hybrid Multi-Mode Reader was used to perform all assays. We performed the analysis and statistics with GraphPad Prism 8.0 version.

The basic process of detecting β5i subunit activity in cultured cells

Raji and Jurkat cell lines are usually used to purify immunoproteasomes and screen β5i inhibitors in vitro. Bortezomib is the first FDA-approved proteasome inhibitor for the treatment of certain multiple myeloma, and ONX-0914 is the first selective β5i inhibitor as an ideal positive control. The cell-based reagents contain a specific immunoproteasome substrate in the reaction buffer and an optimized cell permeabilization buffer that retains the high activity of immunoproteasomes. The peptide substrate Ac-ANW-AMC is cleaved by immunoproteasomes and will generate fluorescent signaling detected by the microplate reader at an emission of 460 nM. We first determined the cell number and cell lysis buffer that showed the best activity to reach the highest reaction rate, so we tried to utilize three different lysis

buffers to compare their slopes of reaction. Figure 1a indicates that one hundred thousand cells are good enough to make the reaction, and the immunoproteasome exhibits the best activity in the presence of buffer 1, including digitonin. Increasing digitonin to 1 mM dramatically improved the efficiency under these conditions, suggesting that completely lysed cells contribute to the release of more immunoproteasomes recognizing its substrate (Fig. 1b). We further confirm that the protease cocktail is dispensable for the reaction in a shorter period; one million cells versus 100 μL cell lysis buffer are working well, there is no difference between the 60 μL and 80 μL reaction systems, and other benefits can save the cost of experiments with the 60 μL reaction system (Fig. 1c). Indeed, more substrates will lift the steep slope than the lower concentration, a potential danger factor for substrates that might be nonspecifically recognized by other proteases. The final concentration of 40 μM substrate presented productive outcomes in our hands (Fig. 1d). Following the current protocol, we measured the inhibition of ONX-0914 in Raji cells, and the IC_{50} of 2.78 μM was higher than the published data (5 nM). In addition to the β5i assay, we could not obtain the ideal IC_{50} of β5c if the cell lysis buffer of the proteasome-GLO kits was changed to 1 mM digitonin buffer. The IC_{50} of 352 nM is 5-fold higher than the published data (Fig. 1e). According to the toxicity of inhibitors, these inhibitors were added and incubated for 6 hours with cultured cells; otherwise, the cell proliferation was reduced. Figure 1g.

Optimization of detergents in cell lysis buffer

We tried to combine two different detergents in cell lysis buffer to give rise to the potency of lysed cells. Digitonin (1 mM) easily resulted in some larger debris in the cell pellets after centrifugation, and 0.01% NP-40 was added to the buffer and reversed this observation. We also used 1 mM DTT to maintain the stable activity of the immunoproteasome. These changes reduced the IC_{50} of β5c to 72 nM (Fig. 2b), but the IC_{50} of β5i was still higher than the ideal value (Fig. 2a). Using this lysis buffer, the lower substrate does not affect the final IC50 except for the relative kinetic rate; however, decreasing the amount of the cell lysate leads to the delayed maximum rate (Fig. 3c). We also found a trick of this assay. The reaction buffer with 80 μM Ac-ANW-AMC mixed with the same amount of cell lysate was held for 30 minutes at room temperature before use. We wondered whether DMSO becomes insoluble at lower temperatures (Fig. 2d). If the key step was correct, the IC_{50} of β5i was 34 nM.

High doses of DMSO in resolving substrates limit the maximum rate

As mentioned above, we assume that DMSO tolerance is a variable factor in determining the kinetic rate. If so, we measured the IC_{50} of ONX-0914 with different DMSO concentrations. Figure 3a shows that lower concentrations of DMSO are useful to improve the kinetic process. The IC_{50} of 7.6 nM is close to the published data; conversely, the final 1% DMSO of the reaction will inhibit the activity of enzymes and increase the IC_{50} to 136 nM. After modification of the current protocol, we obtained perfect IC50 values of bortezomib and ONX-0914 that reached approximately 2.5 nM and 3.5 nM, respectively (Fig. 3b). The kinetic rate was in the manner of lineage, and we still obtained a similar result to the value of the endpoint to calculate IC_{50} (Fig. 3c). To determine whether this assay is stable to run in different cell lines,

we also compared the IC50 in both Raji cells and THP-1 cells. Figure 3d shows the comparable IC_{50} of both inhibitors exhibited in different cells. Now, we believe that the current version is acceptable to study functional immunoproteasomes in the future. We also detected the compatibility of the β5i assay with proteasome-GLO kits. Raji cell lysate was used to measure β5i activity, which also works in the β5c assay. The IC_{50} of ONX-0914 was 78 nM with the substrate of β5c, so we could finish two different assays with the same lysis buffer (Fig. 3d).

The final protocol of the β5i assay kit

1. Cell lysis buffer and β5i substrate solution

Cell lysis buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM MgCL2, 5 mM ATP, 1 mM DTT, 0.01% NP-40, 1 mM digitonin and protease cocktails. Ac-ANW-AMC working solution: 50mM Tris, pH 7.5, 150mM NaCl, 5mM MgCL2, 5mM ATP, 80uM Ac-ANW-AMC and 1mM DTT, freshly making before using, 30μL substrate solution mixed with 30μL cell lysate.

2. Preparation of cell lysates in the β5i assay

2.1 A total of 1×10^5 Raji cells were seeded into 48-well plates, and different doses of inhibitors were added to duplicate wells and incubated for 6 hours with cells at 37°C.

2.2 The cells were harvested and centrifuged at 10000 rpm for 2 min, the medium was removed, the cell pellet was washed once with 1 mL PBS, and the remaining PBS was removed.

2.3 One hundred microliters of cell lysis buffer was used to resuspend the cell pellet, which was pipetted up and down 20 times, left on ice for 30 minutes, and then centrifuged at 13000 rpm for 10 minutes.

2.4 Loading 30uL cell lysate into 96 wells white plate and mixed well with another 30uL substrate working solution, incubation 5 to 10min at 37 degrees, start setting up the microplate reader with the following program during incubation.

2.5 Step 1: Set up the temperature at 37 degrees; Step 2: delay 1 min; Step 3: select the dynamic assay, total time 30 min, 30 cycles; Step 4: insert fluorescent detection with Ex 380 and Em 460.

Tips1: Leave the Ac-ANW-AMC working solution at room temperature before loading.

Tips 2: The AC-ANW-AMC stock solution was made into 8 mM DMSO so that the final DMSO concentration was 0.5% during the reaction period.

2.6 The cell lysate is compatible with proteasome-GLO kits (G8661)

Loading 30uL cell lysate and then adding 30uL Suc-LLVY-Glo™ Substrate working solution, incubation 5 to 10min at room temperature, Measure the luminescence of each sample with a microplate luminometer as directed by the manufacturer(G8661).

2.7 Blank control: substrate working solution + cell lysis buffer (without cells) and vehicle control used DMSO.

Conclusions

Compared to constitutive proteasome functional assays, feasible detection assays of immunoproteasomes are urgently needed for research or industrial purposes. Based on previous experiences, we modified somewhere and released the current version that applies to β5i subunit activity. Intriguingly, this protocol is compatible with proteasome-GLO kits (G8661), so we can obtain two IC_{50} values of the same inhibitor with special substrates, which would benefit from the cost of experiments and improve the efficiency of evaluation. Environmental factors that affect the rate of the reaction will also affect the intensity of the signal output and the stability of the fluorescent signal. For consistent results, the substrate stock and working solution were equilibrated to a constant temperature before performing the assay. Digitonin binding to lower concentrations of NP-40 is potent for disassembly of the nucleus, and Digitonin alone easily results in incompletely lysed cells and unstable fluorescence signaling. If chemicals are dissolved with DMSO, the lower proportion in the final reaction must be reduced; up to 1% DMSO dramatically inhibits the enzyme interplaying with Ac-ANW-AMC and results in major effects on the signaling output. Our assay is simple to hold the process; the kinetic rate is in the manner of a lineage with a stable slope, and the endpoint value of each cycle can be used in statistics. This assay exhibits low background and excellent signal-to-noise ratios. Meanwhile, we also consider whether the major parts of the assay could match the evaluation of β1i and β2i inhibitors accompanied by their specific substrates.

Declarations

Author Contributions

Hengjuan Lv conducted most of the experiments, analyzed the results, and wrote the paper with Dan Fu. Hui Yue assisted in finishing the statistics of the data. Dan Fu prepared the manuscript, conceived the idea for the project, and designed the experiments.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

CP, constitutive proteasome; β5i, the β5 subunit of immunoproteasome; ICP, immune-proteasome; MHC-1, antigen-presenting major histocompatibility complex-1; FDA, Food and Drug Administration; IC50, the half maximal inhibitory concentration.

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Figures

Figure 1

The basic process of β5i subunit activity detection.

Figure 1-a, Optimization of the cell lysis buffer and cell numbers of the reaction in vitro. The slope is on behalf of kinetic rare. Figure 1-b, The defined cell numbers result in distinct slopes in digitonin buffer. Figure 1-c, the optimized volume of reaction makes no difference during the reaction. Figure 1-d, the concentration of AMC substrate robustly influences the process of reaction. Figure 1-e, 1-f, the IC50 of β5i or β5c is showing 2.78uM and 0.39uM respectively, which does not catch the published data. Figure 1-g, Longer incubation inhibitors with cells reduced cell proliferation.

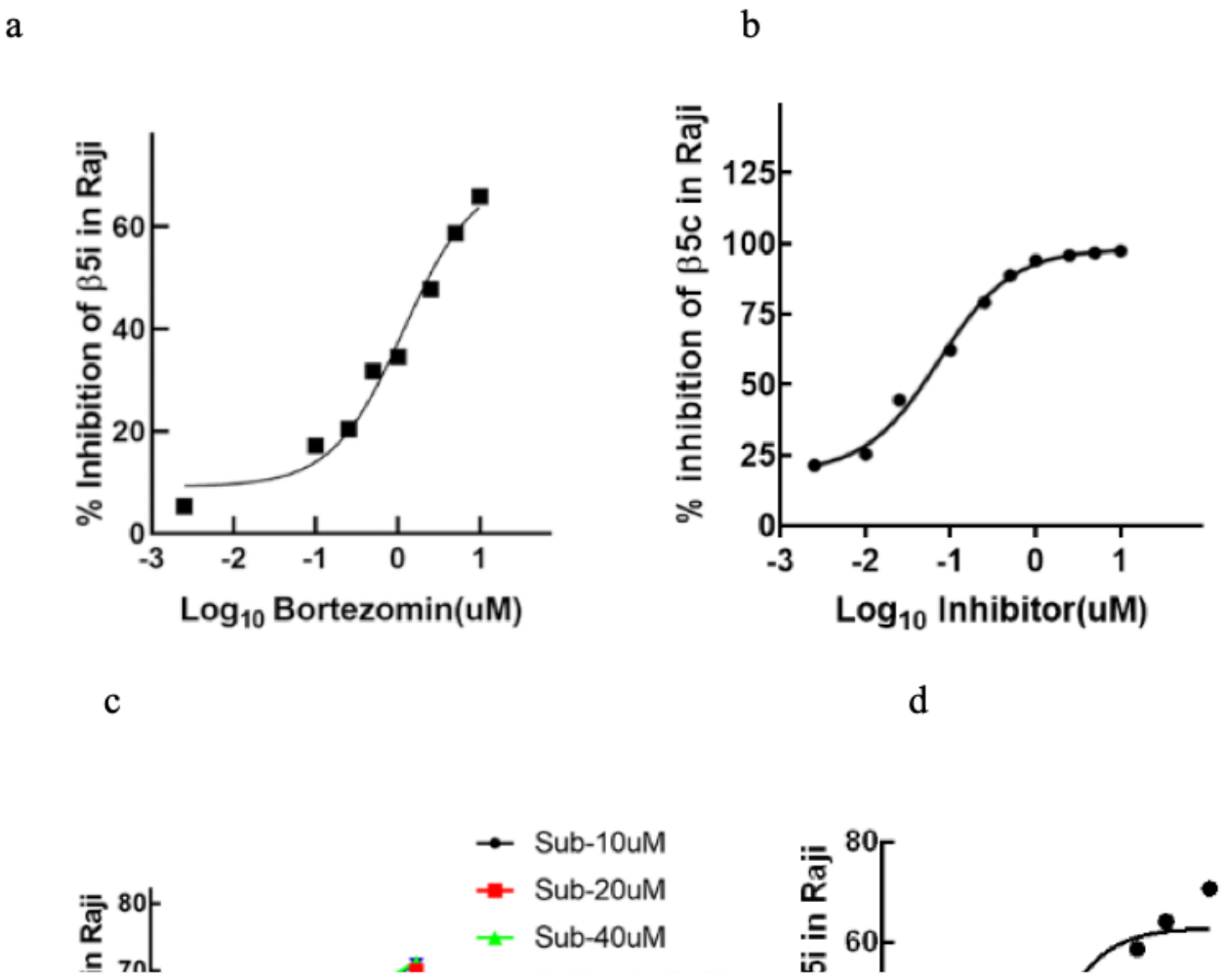

Figure 2

Optimization of detergents in cell lysis buffer.

Figure 2-a, The IC50 of bortezomib is shown in digitonin combined with NP-40 buffer.

Figure 2-b, The IC50 of bortezomib was reduced to 72 μM in optimized lysis buffer.

Figure 2-c, Comparison of the concentration of substrates in the system and the proportion of cell lysate responding to the kinetic rate.

Figure 2-d, Making the fresh substrate working solution at room temperature improves the efficacy of the reaction in vitro.

Figure 3

Figure out the key step and the best way of β5i assay.

Figure 3-a, the higher concentration of DMSO in the reaction dramatically reduces the efficacy of the reaction.

Figure 3-b, 3-c, Comparable IC50 is acceptable due to different calculations with the endpoint of measurement and kinetic slope during the reaction.

Figure 3-d, the ideal IC50 comes from different cell lines with bortezomib and ONX-0914. Figure 3-e, the cell lysate of this protocol is compatible with the assay of β5c.