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Selective immunoproteasome inhibitors with non-peptide scaffolds identified from structure-based virtual screening

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ABSTRACT

As a major component of the crucial nonlysosomal protein degradation pathway in the cells, the proteasome has been implicated in many diseases such as Alzheimer's disease, Huntington's disease, inflammatory bowel diseases, autoimmune diseases, multiple myeloma (MM) and other cancers. There are two main proteasome subtypes: the constitutive proteasome which is expressed in all eukaryotic cells and the immunoproteasome which is expressed in immune cells and can be induced in other cell types. Majority of currently available proteasome inhibitors are peptide backbone-based, having short half-lives in the body. It is highly desirable to identify novel, immunoproteasome-selective inhibitors with non-peptide scaffolds for development of novel therapeutics. Through combined virtual screening and experimental studies targeting the immunoproteasome, we have identified a set of novel immunoproteasome inhibitors with diverse non-peptide scaffolds. Some of the identified inhibitors have significant selectivity for the immunoproteasome over the constitutive proteasome. Unlike most of the currently available proteasome inhibitors, these new inhibitors lacking electrophilic pharmacophores are not expected to form a covalent bond with proteasome after the binding. These non-peptide scaffolds may provide a new platform for future rational drug design and discovery targeting the immunoproteasome.

Proteasome plays a central role in maintaining cellular homeostasis, controlling the cell cycle, removing misfolded proteins that can be toxic, and regulating the immune system.[1] This crucial protein degradation machinery has been implicated in multiple diseases such as Alzheimer's disease, Huntington's disease (HD), inflammatory bowel diseases (IBD), autoimmune diseases, multiple myeloma (MM) and other cancers.[2] In particular, proteasome has been recognized as a promising cancer target, and the Food and Drug Administration (FDA) has approved proteasome inhibitors bortezomib (Velcade®) in 2003 and carfilzomib (Kyprolis®) in 2012 for the MM treatment. The FDA approvals of these proteasome inhibitors as chemotherapeutic agents have dramatically improved the therapeutic landscape for patients with MM.[3] Despite the remarkable successes of these proteasome inhibitors in the clinic, intrinsic and acquired drug resistance remains a major clinical challenge. In addition, these drugs have failed to provide clinical benefit to patients with solid cancers,[4–6] further highlighting the need for next-generation of proteasome inhibitors.

There are two main proteasome subtypes: the constitutive proteasome (CP) which is expressed in all eukaryotic cells and the immunoproteasome (IP) which is expressed in immune cells and can be induced in other cell types. Constitutive proteasome contains three catalytic subunits denoted as β1, β2, and β5, whereas the three corresponding catalytic subunits of immunoproteasome are denoted as β1i, β2i, and β5i. The catalytic subunits responsible for the chymotrypsin-like (CT-L) activity (β5 and β5i) are thought to be most physiologically important and have been recognized as the key targets of bortezomib and carfilzomib.[7,8] However, these drugs have failed to achieve efficacy in patients with solid cancers despite strong indications of activity in preclinical animal models. The failure of these drugs has been attributed to their poor metabolic stability.[9] Further, recent reports revealed the importance of targeting the immunoproteasome catalytic subunit β5i in killing cancer cells.[8] Therefore, it is highly desirable to identify novel compounds with non-peptide scaffolds that can selectively inhibit the immunoproteasome β5i as the next-generation of therapeutic agents.

Here, we report the identification of a set of novel, selective inhibitors of the immunoproteasome with diverse non-peptide scaffolds through combined virtual screening and experimental

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studies targeting the catalytic subunit β5i (which is also known as LMP7).

Our virtual screening was based on our previously modeled three-dimensional (3D) structure of the immunoproteasome,[10–12] and performed on a compound library at Genomics Research Institute (GRI), the University of Cincinnati (UC). The UC/GRI compound library containing structural information for about 300,000 compounds was provided by Procter & Gamble (P&G) and belonged to a consortium including the University of Kentucky as a member. The virtual screening procedure utilized to screen the chemical compound library is essentially similar to that we used to identify small-molecule inhibitors of other proteins.[13,14] First of all, the ~300,000 compounds were first screened by performing rigid docking using FRED (OpenEye Scientific Software),[15] leading to identification of top-25,000 compounds. The subsequent energy-minimization and Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) binding energy calculations using Amber9 software[16] led to identification of the top-1000 compounds. Further structural analysis on possible interactions with key amino-acid residues (including Thr1, Ser21, Ser27, Gly47, Ala49, and Asp324) led to selection of the top-90 compounds that could inhibit the immunoproteasome.

The computationally selected 90 compounds were tested for their inhibitory activity against the CT-L activity of the immunoproteasome. The identified active compounds were also tested for their inhibitory activity against the CT-L activity of the constitutive proteasome. For the initial activity screening, the selected compounds were dissolved in DMSO and used at a concentration of 5 μM. Epoxomicin (1 μM), which reached the 100% inhibition, was used as a positive control. 20S human immunoproteasome and constitutive proteasome (Boston Biochem) were 2-fold diluted in an assay buffer (20 mM Tris/Cl, pH 8.0, 0.5 mM EDTA, 0.035% SDS).[17] Specifically, selected compounds were preincubated with 50 ng/well of the immunoproteasome or constitutive proteasome in a 96-well plate at room temperature for 90 min. 100 μM of Suc-LLVY-AMC, a fluorogenic peptide substrate for the CT-L activity, was then added to the wells. Fluorogenic signals of the free AMC (Ex: 360, Em: 460)[18] were recorded for 90 min. The initial reaction velocities (RFU/min) of each compound were calculated as a percentage of the positive control. All enzyme activity assays were carried out in triplicate.

According to the activity assays, nine of the tested compounds (**1**–**9**) showing the significant inhibition against the immunoproteasome are depicted in Figure 1 for their molecular structures and listed in Table 1 for their activity data. So, the hit rate of the virtual screening was 10%. Based on the activity data in Table 1, compounds **1**–**3** at 5 μM inhibited the CT-L activity of the immunoproteasome by about 36–85%, whereas these compounds at 5 μM inhibited the constitutive proteasome by only 2–20%. This indicates that compounds **1**–**3** are highly selective inhibitors for the immunoproteasome. In particular, compounds **1** and **2** at 5 μM inhibited the immunoproteasome by 85–62%, respectively. These most potent two compounds, along with compound **3**, were tested further for the dose-dependent inhibition (Fig. 2) in order to determine their IC_{50} values (Table 1) against the immunoproteasome. As shown in Table 1, the IC_{50} values for compounds **1** to **3** are 1.7, 4.9, and 22 μM, respectively. These compounds, particularly compounds **1** and **2**, are promising immunoproteasome inhibitors with non-peptide scaffolds.

Depicted in Figure 3 are the energy-minimized structures of the immunoproteasome binding with compounds **1** and **2**. As shown in Figure 3A, compound **1** has favorable hydrophilic interactions with amino-acid residues Thr1, Ser21, Ser27, and Gly47, including strong hydrogen bonds with the NH group of Ser21 backbone, hydroxyl group of Ser27 side chain, and carbonyl oxygen of Gly47 backbone. As shown in Figure 3B, compound **2** has favorable

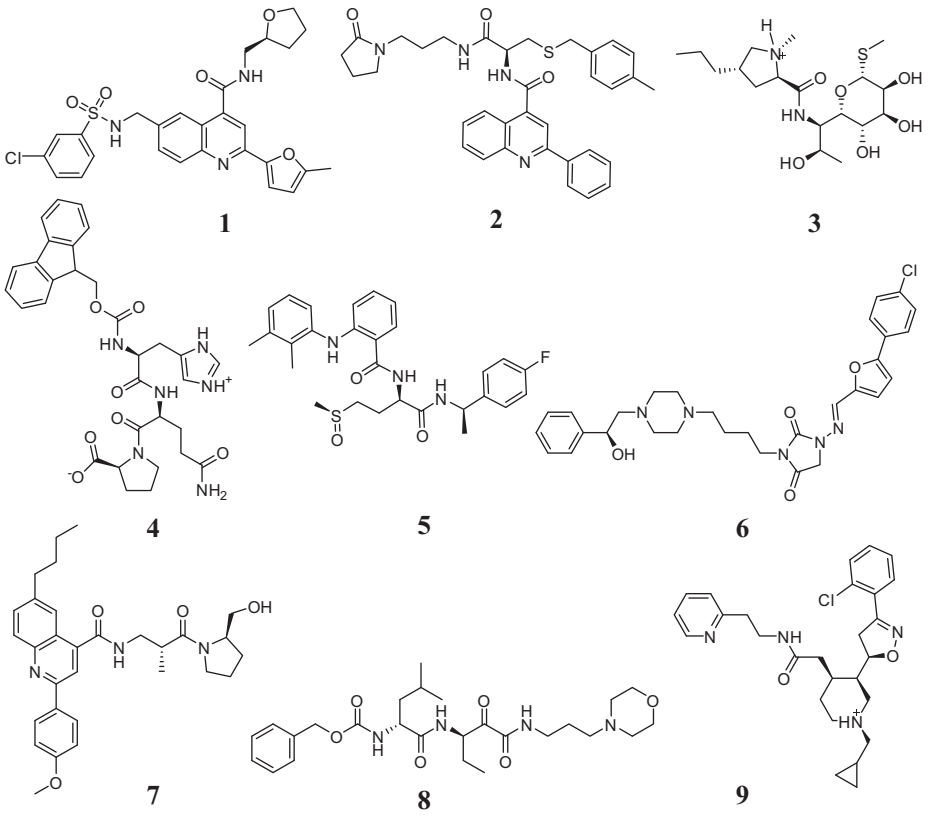

Figure 1. Molecular structures of the identified new inhibitors of the immunoproteasome.