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Identification of 2-thioxoimidazolidin-4-one derivatives as novel noncovalent proteasome and immunoproteasome inhibitors

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ABSTRACT

This paper describes the design, synthesis, and biological evaluation of 2-thioxoimidazolidin-4-one derivatives as inhibitors of proteasome and immunoproteasome, potential targets for the treatment of hematological malignancies. In particular, we focused our efforts on the design of noncovalent inhibitors, which might be a promising therapeutic option potentially devoid of drawbacks and side-effects related to irreversible inhibition. Among all the synthesized compounds, we identified a panel of active inhibitors with K_i values towards one or two chymotrypsin-like activities of proteasome (β5c) and immunoproteasome (β5i and β1i subunits) in the low micromolar range. Docking studies suggested a unique binding mode of the molecules in the catalytic site of immunoproteasome proteolytic subunits.

The ubiquitin-proteasome system (UPS) is a key pathway involved in the intracellular protein turnover in eukaryotic cells. In normal cells, proteasome proteolytic activity is responsible for a regular cell cycle progression; thus, defects in UPS can lead to uncontrolled cell proliferation and tumor development. 20S proteasome core shows a barrel-like structure, composed of four stacked rings, each containing seven subunits, i.e. α1–α7 in the two outer rings, β1–β7 in the two inner rings.[1] The proteolytic activities are located into β1, β2, and β5 subunits, which are responsible for the caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (ChT-L) activities, respectively.[2]

In addition to the constitutive proteasome (c20S), vertebrates possess a specialized form of proteasome, named immunoproteasome (i20S), which is predominantly expressed in lymphocytes and monocytes, and is responsible for the regulation of major histocompatibility complex (MHC) class I antigen presentation.[3] Under the stimuli of IFN-γ and TNF-α, the constitutive subunits β1c, β2c and β5c are replaced by the newly formed immuno counterparts β1i, β2i and β5i.[4] β2i and β5i subunits maintain the same substrate specificity of β2c and β5c ones; on the contrary, β1i mainly performs a ChT-L activity whereas its caspase-like activity is reduced to background levels.[5]

It has been demonstrated that i20S is highly expressed in cells of hematopoietic origin, including multiple myeloma (MM) cells; thus, the inhibition of i20S could be a promising strategy to treat MM.[6] In this regard, Parlati et al. clearly assessed that selective inhibition of single β5i or β5c subunit is insufficient to produce an antitumor response, whereas inhibition of both β5i and β5c is required to induce an antitumor effect in MM, non-Hodgkin lymphoma, and leukemia cells, without causing cytotoxicity in non-transformed cells.[7] At present, both non-selective and selective immunoproteasome inhibition has been validated as potential strategy for the treatment of MM.[8]

In the last years, our research group has been widely involved in the development of novel peptidomimetics as inhibitors of the ChT-L activity of constitutive proteasome.[9–15] In particular, we focused our efforts on the design of noncovalent inhibitors, which might be a promising therapeutic option because potentially devoid of drawbacks and side-effects related to irreversible inhibition.

Starting from these considerations, and assuming as lead compounds oxathiazolones HT1171 and HT2004 (Fig. 1), which were reported to irreversibly inhibit both β5i and β5c subunits with a

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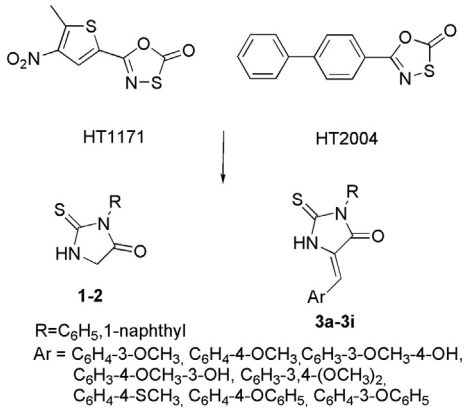


Fig. 1. Structures of model and target compounds **1**, **2** and **3a–3i**.

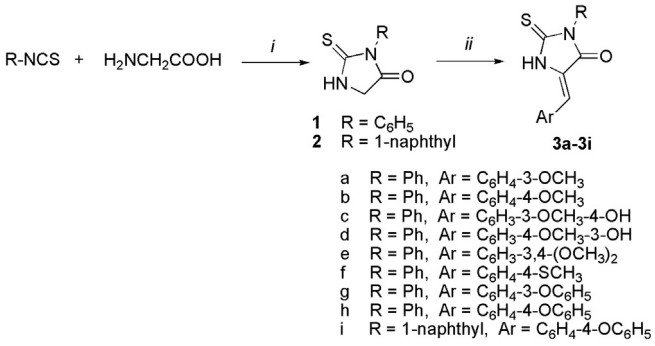
preference for β5i,[16] we planned to assess the inhibitory activity of a panel of 3-aryl-2-thioxoimidazolidin-4-ones **1**–**2** and (5*Z*)-5-arylidene-3-aryl-2-thioxoimidazolidin-4-ones **3a–3i** (Fig. 1) against constitutive proteasome and immunoproteasome.

The selected molecules are endowed with a nonpeptide structure that gives them a great stability in solution and, in addition, could be promising as potential immunoproteasome inhibitors because they possess structural features that make them suitable for the binding to i20S. In fact, the i20S subunits responsible for ChT-L activity show a strong preference for bulky hydrophobic groups able to fit into the S1 pocket, in particular aromatic for β5i and branched for β1i; conversely, both ChT-L activities have the same preference for small polar groups able to establish key interactions with their S3 site.

3-Aryl-2-thioxoimidazolidin-4-ones **1** and **2** were synthesized by reacting glycine with phenyl- or 1-naphthyl-isothiocyanate. The subsequent Knoevenagel condensation of compounds **1** and **2** with the appropriate arylaldehyde, in the presence of piperidine as a base, afforded (5*Z*)-3-aryl-5-arylidene-2-thioxoimidazolidin-4-ones **3a–3i** in high yields (Scheme 1).

The structures of compounds **1**–**3** were confirmed by analytical and spectroscopic data (^{1}H and ^{13}C NMR) and by X-ray diffraction study (see Supplementary data).

X-ray crystallographic studies of 5-(4-phenoxybenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one **3h** (CCDC # 1811484), selected as representative, unambiguously attributed the *Z* configuration at the chiral axis of derivatives **3** (Fig. 2). This is consistent with the presence of only one set of signals in ^{1}H NMR spectra, which indicated that, in the reported experimental conditions, compounds **3** were obtained as the most stable *Z* geometric isomers, similarly to previously reported 5-arylidene substituted 2,4-thiazolidinediones[17] and 2-phenylimino-4-thiazolidinones.[18]



Scheme 1. *Reagents and conditions: i,* EtOH/H_2O,Δ. *ii,* arylaldehyde, piperidine, EtOH, Δ.

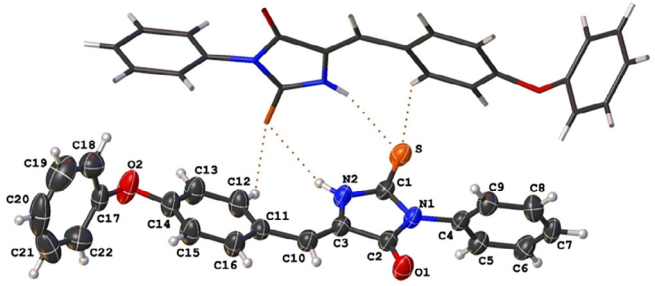


Fig. 2. ORTEP view of 5-(4-phenoxybenzylidene)-3-phenyl-2-thioxoimidazolidin-4-one **3h** along the crystallographic *b*-axis evidencing the H-interactions and the numbering scheme. Displacement ellipsoids are drawn at the 50% probability level while hydrogen size is arbitrary.

Compounds **1**, **2** and **3a–3i** were tested for their inhibitory properties on 20S immunoproteasome and on 20S proteasome isolated from human spleen and from human erythrocytes, respectively, using an appropriate fluorogenic substrate for each proteolytic activity (Suc-Leu-Leu-Val-Tyr-AMC for β5i and β5c; Boc-Leu-Arg-Arg-AMC for β2i and β2c; Ac-Pro-Ala-Leu-AMC for β1i and Z-Leu-Leu-Glu-AMC for β1c). First, compounds underwent a preliminary screening on each proteolytic subunit at 50 μM. An equivalent volume of DMSO was used as a negative control, and MG-132, Z-Leu-Leu-Leu-al, a reversible inhibitor of both proteasome and immunoproteasome, as positive control.

Compounds able to inhibit the enzymatic activity by more than 60% were characterized in detail: continuous assays were thus performed (progress curve method, at seven different concentrations, ranging from those that minimally inhibited to those that fully inhibited the immunoproteasome or the proteasome subunit) to determine the K_i values reported in Table 1. All compounds were shown to inhibit immunoproteasome and proteasome in a reversible manner, as demonstrated by the analysis of the progress curves at seven different concentrations (see e.g. **3e** tested against β5c subunit, Fig. 3). As a matter of fact, after an incubation time of 10 min (Fig. 3a) and 30 min (Fig. 3b) the obtained linear progress curves put in evidence a time-independent inhibition. On the contrary, in time-dependent inhibition the progress curves follow an exponential equation.[19] Reversibility of inhibition was also confirmed by measuring the recovery of enzymatic activity after dilution of the enzyme–inhibitor complex with assay buffer (see Supplementary data).

The 3-aryl-2-thioxoimidazolidin-4-ones **1** and **2** did not pass the initial screening, whereas the insertion of the 5-arylidene moiety generally resulted in consistent inhibitory effects. In the series of (5*Z*)-5-arylidene-3-aryl-2-thioxoimidazolidin-4-ones **3**, we observed that the presence of a methoxy group at the position 3 of the arylidene moiety (compound **3a**) led to similar binding affinity towards both *β*5 subunits of the constitutive and immunoproteasomes (K_i = 17.5 μM and 17.9 μM for *β*5i and *β*5c, respectively).

Conversely, the presence of a methoxy group at the position 4 of the arylidene portion allowed us to obtain a moderately selective *β*5i inhibitor (i.e. **3b**, K_i = 10.9 μM); the presence of a methylthio group in the same position (i.e. compound **3f**) led to a loss of potency towards the *β*5i subunit while the inhibition of subunits *β*2i and *β*2c was favoured (Table 1).

Overall, compounds bearing two polar groups on the arylidene moiety were active against two or three chymotrypsin-like activities of immunoproteasome and proteasome, but with a different trend of selectivity. 4-Hydroxy-3-methoxybenzylidene substituted compound **3c**, was proven to be active on both β5i and β5c, with a 2-fold higher activity against β5i. Its 3-hydroxy-4-methoxybenzylidene isomer **3d** showed a preferential binding (~20-fold) towards subunit β5i over β5c, whereas 3,4-dimethoxybenzylidene