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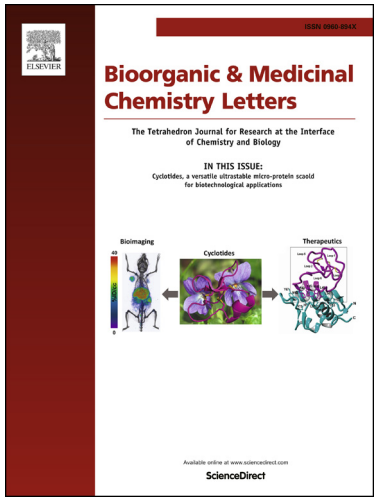

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Identification of 2-thioxoimidazolidin-4-one derivatives as novel noncovalent proteasome and immunoproteasome inhibitors

Rosanna Maccari[a], Roberta Ettari[a,*], Ilenia Adornato[a], Alexandra Naß[b], Gerhard Wolber[b], Alessandra Bitto[c], Federica Mannino[c], Federica Aliquò[c], Giuseppe Bruno[d], Francesco Nicolò[d], Santo Previti[a], Silvana Grasso[a], Maria Zappalà[a], Rosaria Ottanà[a]

[a]*Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Polo Annunziata, Viale SS. Annunziata, 98168 Messina (Italy).*
[b]*Department of Pharmaceutical and Medicinal Chemistry, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, (Germany).*
[c]*Department of Clinical and Experimental Medicine, University of Messina, Via C. Valeria, 98125 Messina, (Italy).*
[d]*Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale Ferdinando Stagno d'Alcontres, 31, 98166 Messina (Italy).*

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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 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.

* Corresponding author. Tel.: +39-090-6766554; fax: +39-090-6766402; e-mail: rettari@unime.it

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.

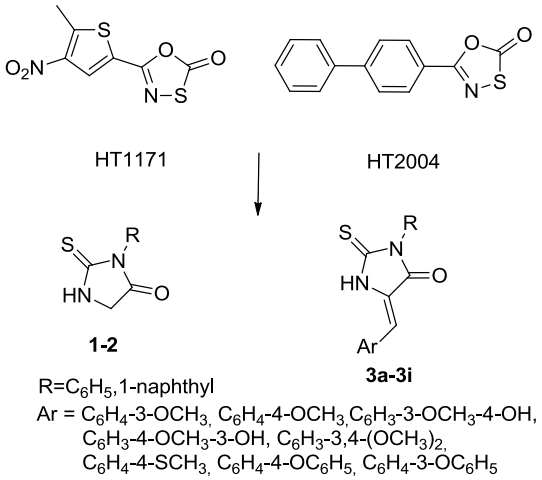
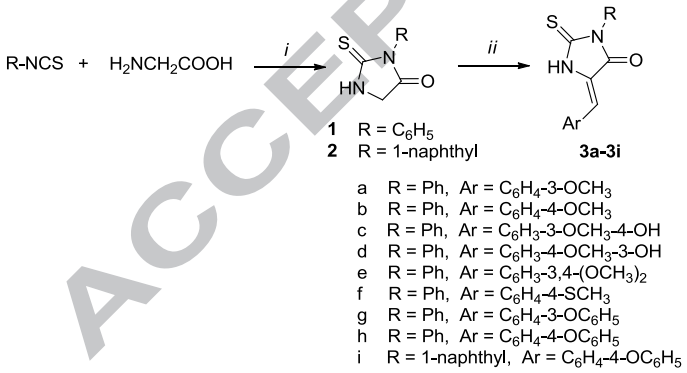


Fig. 1. Structures of model and target compounds **1, 2** and **3a-3i.**

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).



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

X-ray crystallographic studies of 5-(4-phenoxybenzylidene)-3-phenyl-2-thioximidazolidin-4-one **3h** (CCDC # 1811484), selected as representative, unambiguously attributed the *Z* configuration at the chiral axis of derivatives **3** (Figure 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-thiazolidindiones[17] and 2-phenylimino-4-thiazolidinones.[18]

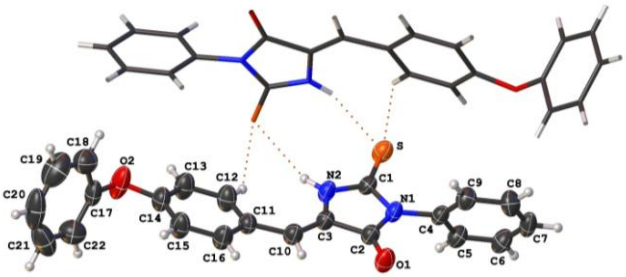


Fig. 2. ORTEP view of 5-(4-phenoxybenzylidene)-3-phenyl-2-thioximidazolidin-4-one **3h** along the crystallographic *b*-axis evidencing the H-interactions and the numbering scheme. Displacement ellipsoids are drawn at the 50% probability level while hydrogen size is arbitrary.

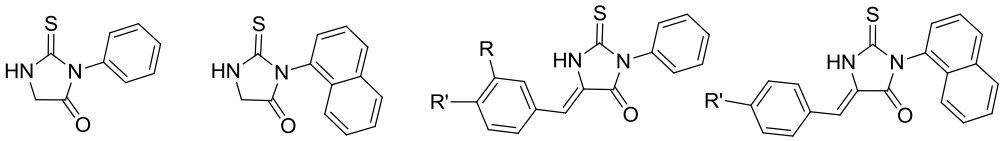
Compounds **1, 2** and **3a-3i** were tested for their inhibitory properties on 20S immunoproteasome and on 20S proteasome isolated from human spleen and from human erythrocytes, respectively, using an appropriate fluorogenic substrate for each proteolytic activity (Suc-Leu-Leu-Val-Tyr-AMC for β5i and 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 β5 subunits of the constitutive and immunoproteasomes (K_i =17.5 μM and 17.9 μM for β5i and β5c, respectively).

Conversely, the presence of a methoxy group at the position 4 of the arylidene portion allowed us to obtain a moderately selective β5i 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 β5i subunit while the inhibition of subunits β2i and β2c was favoured (Table 1).

Table 1.
Activity on proteasome and immunoproteasome core-particles of 2-thioxoimidazolidin-4-ones **1, 2** and **3a-3i.**



	% of inhibition at 50 μM or K_i (μM)							
Comp.	R	R'	β_{1c}	β_{2c}	β_{5c}	β_{1i}	β_{2i}	β_{5i}
1	-	-	32%	n.i.	47%	n.i.	n.i.	53%
2	-	-	47%	38%	n.i.	6%	n.i.	n.i.
3a	OCH_3	H	52%	36%	17.9±0.3	34%	18%	17.5±3.2
3b	H	OCH_3	56%	35%	45%	57%	29%	10.9±0.9
3c	OCH_3	OH	54%	32%	23.6±1.6	5%	6.71±0.58	11.5±0.2
3d	OH	OCH_3	28%	37%	45%	6.53±0.91	45%	2.35±1.16
3e	OCH_3	OCH_3	58%	29%	13.6±0.9	2.64±0.63	50%	2.01±0.17
3f	H	SCH_3	59%	20.0±1.1	28%	n.i.	4.38±0.37	50%
3g	OPh	H	n.i.	n.i.	39%	17%	32%	56%
3h	H	OPh	n.i.	5%	13%	n.i.	53%	58%
3i	-	OPh	9.64±0.98	57%	20%	n.i.	5.57±1.23	56%
MG-132	--	-	22.1±3.3	5.52±0.23	0.004±0.001	0.13±0.01	0.001±0.0002	0.07±0.01

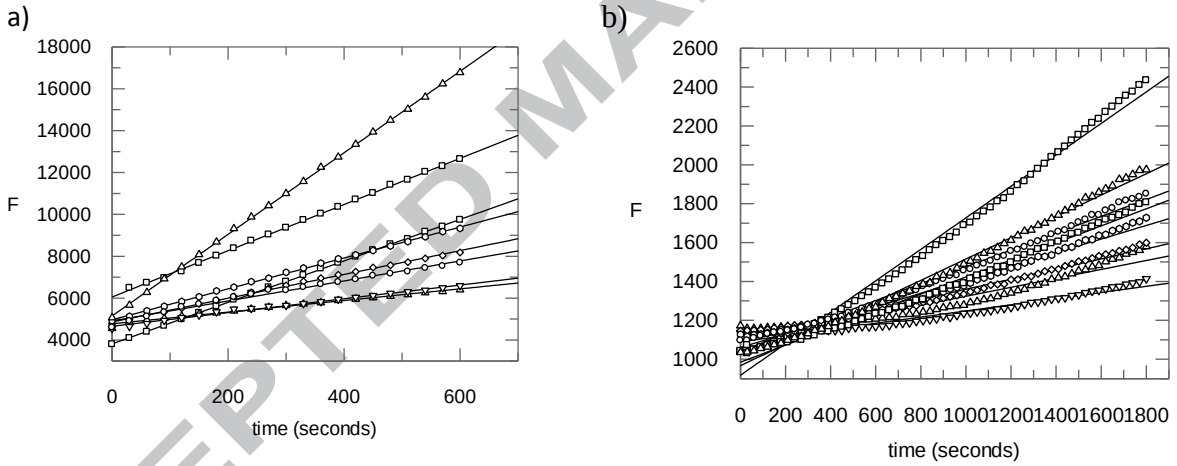


Fig. 3. Progress curves of substrate hydrolysis in the presence of the inhibitor **3e** tested against β5c subunit after an incubation time of 10 min (Fig. 3a) and 30 min (Fig. 3b). F = fluorescence units. Inhibitor concentrations (from top to bottom): 0, 0.1, 0.5, 1, 5, 10, 25, 50 μM.

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 analogue **3e** targeted β5i, β1i and β5c, with a ratio K_i β5c/ K_i β5i=~7 (Table 1).

Compounds **3d** and **3e** were the most active inhibitors, endowed with K_i values in the low micromolar range. 3,4-Dimethoxybenzylidene-substituted derivative **3e** was shown to be the most active compound of the series with K_i values of 2.01 μM and 2.64 μM towards β5i and β1i, respectively.

The replacement of small polar substituents such as methoxy or hydroxyl groups with a phenoxy moiety (compounds **3g-3i)** resulted in a general marked decrease of inhibitory potency. 3-Phenoxy- and 4-phenoxy-substituted analogues **3g** and **3h** showed poor or moderate inhibitory capability, whereas 3-(1-naphthyl)-substituted derivative **3i** showed appreciable inhibitory effects against subunits β1c and β2i with K_i values in the low micromolar range (Table 1). Overall, these inhibition results suggest that the presence of small polar groups could strongly contribute to stabilize the potential interactions with the S3 pocket of immuno subunits. However, docking studies reported below indicated a different binding mode of the molecule in the catalytic site.

The most interesting proteasome/immunoproteasome inhibitors **3a-3e** were selected to evaluate their growth inhibitory activity on human multiple myeloma cells (MM.1R). These cells are dexamethasone-resistant and show a poor response to chemotherapy being representative of patients in the later stages of the disease. Compounds **3a-3e** and MG132 were tested in triplicate at different doses (1-10-20-40-80-100 μM) for 24 h and the test was repeated 4 times. The cytotoxic effect of the compounds was determined by MTT assay; untreated cells were used as reference and assumed as the 100% of vital cells. MG132 was used as a positive control and showed an IC_{50} value of 14.4±1.0 μM. The IC_{50} values of compounds **3a-3e** (Table 2)

ranged from 19.4±1.1 μM (compound **3b**) to 40.7±1.2 μM (compound **3d**). The lack of a perfect correlation between enzyme inhibition and cellular activity suggests that these inhibitors could have additional targets at cellular level. However, a partial and non-homogeneous cellular penetration could not be ruled out.

Table 2.
IC_{50}values of tested compounds against MM.1R cells.

Comp.	IC_{50} (μM)
3a	25.9 ± 1.2
3b	19.4± 1.1
3c	20.2 ± 1.1
3d	40.7 ± 1.2
3e	36.7 ± 1.1
MG-132	14.4± 1.0

Docking experiments in the active site cavity of β5c (Figure 4) and β5i (Figure 5) subunits were carried out for compounds **3a**, **3d** and **3e**. Overall, the investigated compounds showed binding poses with the arylidene moiety and its substituents faced towards the catalytic Thr1 and the 3-aryl-2-thioxoimidazolidin-4-one moiety stretched out along the line of the backbone of known peptide inhibitors (see Figure 6 for orientation). The *N*-substituent is located in the extension of the S3 pocket.

Figure 4A shows interactions of the selected binding pose of compound **3a** with the catalytic cavity of subunit β5c. The pose is characterized by very good steric complementarity of the ligand to the catalytic cavity as well as several hydrogen bonding and hydrophobic interactions: the carbonyl group interacts with the side chain of Ser129 of the β6 chain, while the imidazolidinone NH shows an interaction with the backbone of Thr21. Additionally, the 3-methoxy group on the arylidene moiety shows a hydrogen bond to the side chain of the catalytic Thr1. Ala20, Ala27 and Ala49 of the β5c chain are in proximity of hydrophobic parts of the ligand and positively contribute to the binding. The slightly higher activity of 3,4-dimethoxy-substituted derivative **3e** can be explained by the favorable electrostatic complementarity of the additional 4-methoxy group with the side chain of Thr21, thus expanding the good sterical complementarity of **3a**.

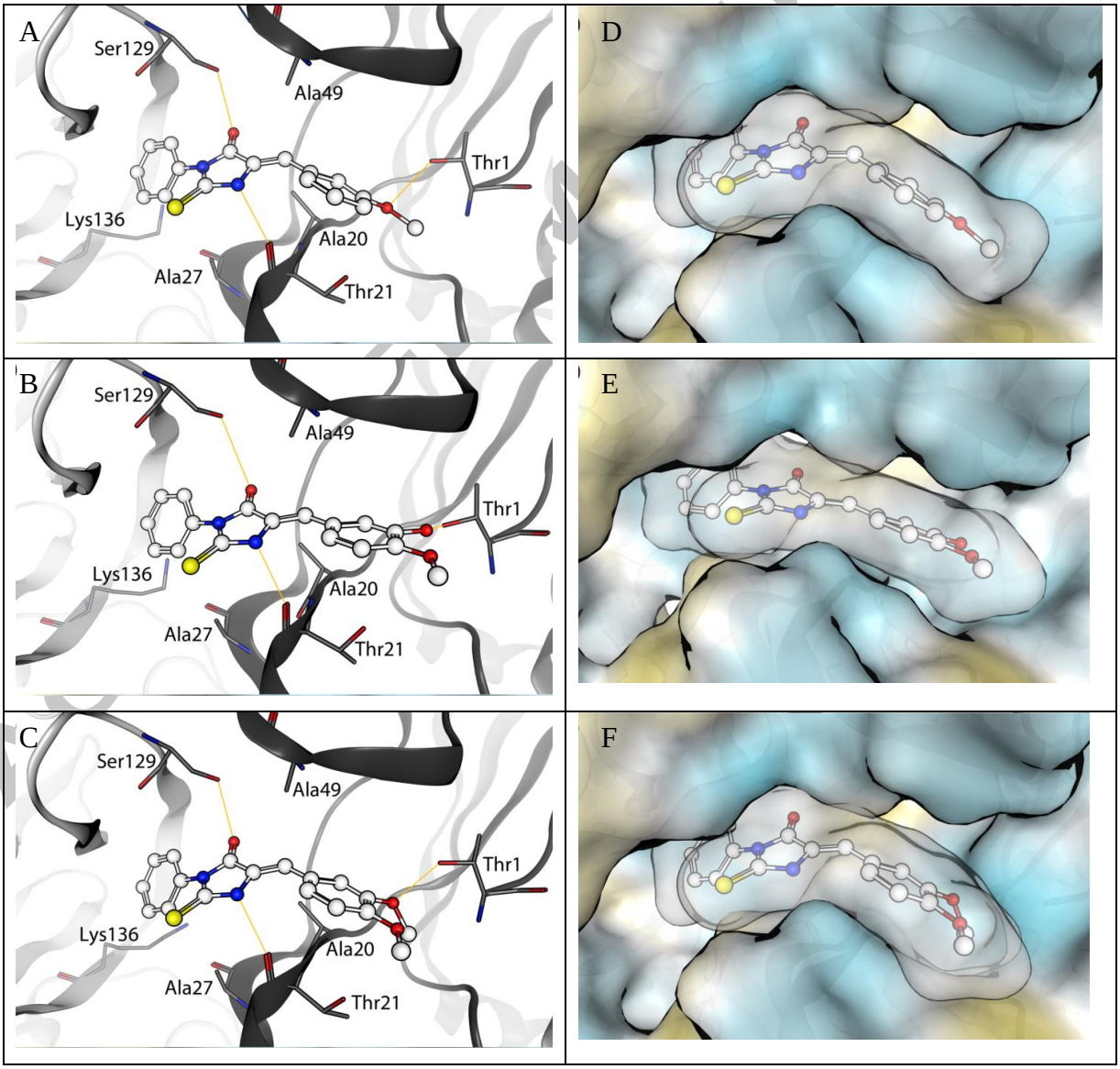


Fig. 4. Docking poses of compounds **3a**, **3d** and **3e** in the β5 subunit of the human constitutive proteasome. Electrostatic interactions: hydrogen bonds depicted as yellow lines (A, compound **3a**; B, compound **3d**; C, compound **3e**). Shape complementarity: surface coloring by hydrophilicity (cyan)/ lipophilicity (sand) (D, compound **3a**; E, compound **3d**; F, compound **3e**).

A hydroxyl group at 3-position of the arylidene moiety, however, leads to reduced steric complementarity of compound **3d** as depicted in Figure 4B/E, as a consequence of the hydrogen bonding interaction with Thr1. This also leads to the loss of the favourable electrostatic complementarity of the 4-methoxy group of the arylidene moiety. The combination of both effects could explain the reduced activity of this compound compared to the closely related compounds **3a** and **3e**.

Figure 5A shows interactions of compound **3a** with the immunoproteasome active site formed by the subunits β5i and β6. The position in the binding site is similar to that found in the corresponding constitutive proteasome pocket; however, in the immunoproteasome, the exchange of Thr21 to Ser21 leads to higher flexibility of the hydroxyl group at the protein side, favouring a position closer to Thr1. This leads to a weakening of the hydrogen bond formed in the constitutive proteasome with Ser129 (Ser130 in the immunoproteasome) of chain β6 in favour of the newly formed one between the hydroxyl group of Ser21 and the 3-methoxy group of the arylidene moiety of compound **3a**. The similar steric fit and position in the binding site explain the comparable activity of compound **3a** against the β5 subunits of both the constitutive and immunoproteasome.

Compound **3e** also shows similar binding pose in both binding pockets explored by our docking studies. Again, changes in the amino acid sequence from β5c to β5i, namely Thr21Ser, and the different position of the interacting Ala28 in the S3 pocket favour the binding affinity of compound **3e** towards immunoproteasome. This is also due to the additional hydrogen bonding interaction between the 4-carbonyl group and Ser130, as depicted in Figure 5C.

In the docking experiment, compound **3d** again showed an only slightly different binding pose characterized by several interactions depicted in Figure 5B. Again, hydrogen bonding interactions are formed with the backbone of Ser21 and the sidechain of Thr1; moreover, the 4-methoxy and 3-hydroxyl groups of the arylidene moiety establish additional hydrogen bond interactions with the sidechain of Ser21 and the backbone of Tyr169, respectively. The preferential binding of this compound to the immunoproteasome is therefore mainly introduced by the good fit of the arylidene substituents in close proximity to the catalytic Thr1, which is not possible in the constitutive proteasome due to reduced flexibility of Thr21 compared to Ser21.

Interestingly, the reported docking poses are able to explain the obtained SAR, also disclosing novel interactions with respect to those commonly reported in literature.[3]

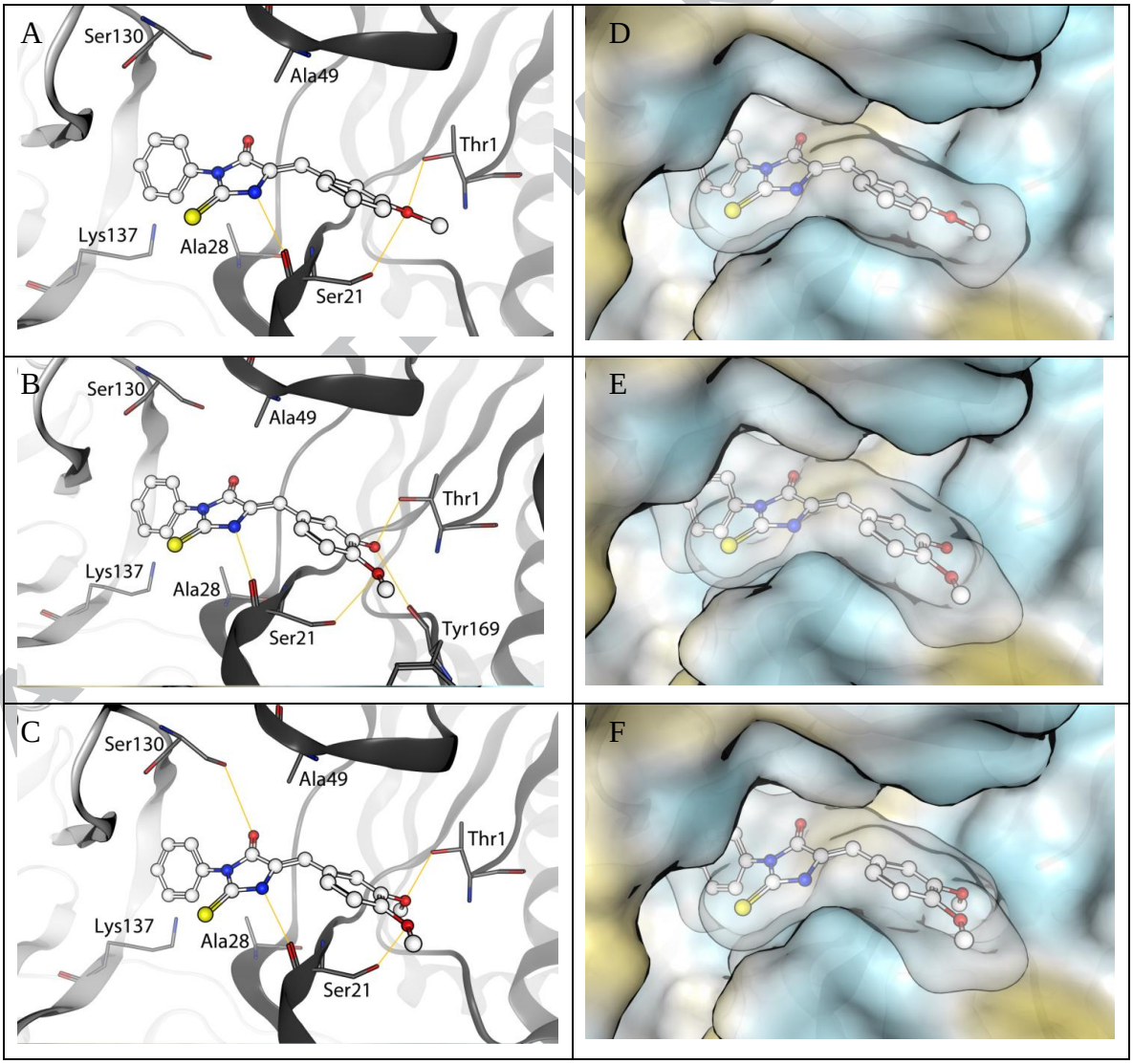


Fig. 5. Docking poses of compounds **3a**, **3d** and **3e** in the β5 subunit of the human immunoproteasome. Electrostatic interactions: hydrogen bonds depicted as yellow lines (A, compound **3a**; B, compound **3d**; C, compound **3e**). Shape complementarity: surface coloring by hydrophilicity (cyan)/ lipophilicity (sand) (D, compound **3a**; E, compound **3d**; F, compound **3e**).

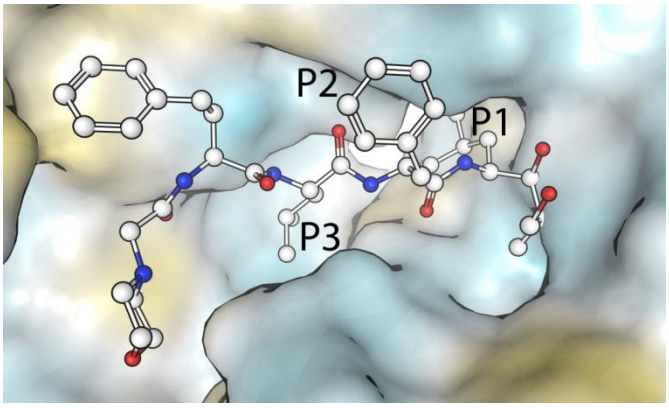


Figure 6. Crystal structure of the β5 subunit of the immunoproteasome in complex with a humanized version of subunit β6 and the covalent inhibitor carfilzomib; surface coloring by hydrophobicity (cyan)/ lipophilicity (sand).

In conclusion, in this work we tested a series of 3-aryl-5-arylidene-2-thioxoimidazolidin-4-one derivatives targeting human proteasome and immunoproteasome, as potential targets for the treatment of hematological malignancies. We identified several active inhibitors with K_i values in the low micromolar range towards one or two chymotrypsin-like activities of proteasome and immunoproteasome.

With respect to conventional proteasome inhibitors, which normally give rise to an irreversible inhibition of the target enzyme, (5*Z*)-3-aryl-5-arylidene-2-thioxoimidazolidin-4-ones **3** behaved as noncovalent inhibitors and might be devoid of all drawbacks and side-effects related to the irreversible inhibition. Thus, also considering their drug-like profile (see Supplementary data), they could be considered as a starting point for further optimization in order to identify more effective proteasome and immunoproteasome inhibitors for the treatment of hematological malignancies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/XXX/j.bmcl.XXXX

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Graphical Abstract

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

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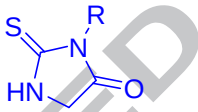
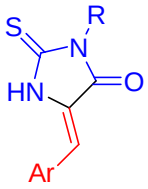
Rosanna Maccari[a] , Roberta Ettari[a,*], Ilenia Adornato[a], Alexandra Naß[b], Gerhard Wolber[b], Alessandra Bitto[c], Federica Mannino[c], Federica Aliquò[c], Giuseppe Bruno[d], Francesco Nicolò[d], Santo Previti[a], Silvana Grasso[a], Maria Zappalà[a], Rosaria Ottanà[a]

[a]*Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Polo Annunziata, Viale SS. Annunziata,98168 Messina (Italy).*
[b]*Department of Pharmaceutical and Medicinal Chemistry, Freie Universität Berlin, Königin-Luise-Str. 2+4, 14195 Berlin, (Germany).*
[c]*Department of Clinical and Experimental Medicine, University of Messina, Via C. Valeria, 98125 Messina, (Italy).*
[d]*Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale Ferdinando Stagno d'Alcontres, 31, 98166 Messina (Italy).*



1, R = C_6H_5,
2, R = 1-naphthyl



3a-h
R=C_6H_5 or 1-naphthyl

20.