Final report

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Identification of non-peptidic inhibitors of the immunoproteasome using fragment based drug discovery methods

We followed a fragment-based approach to identify non-covalent and covalent inhibitors of the chymotrypsin-like β5i subunit of the immunoproteasome (iPS). Targeting the β5i subunit specific to iPS was motivated by the objective of finding inhibitors selective against the constitutive proteasome (cPS) thus reducing the side-effects associated with the inhibition of cPS. While the majority of the immunoproteasome inhibitors have peptide-like structure, we focused on non-peptidic inhibitors with the potential of improved pharmacokinetic properties.

Searching chemical starting points

Fragment-sized chemical starting points were searched with various methods detailed below.

1) Experimental non-covalent screening

The search for non-covalent inhibitors was started by screening our in-house fragment library in an inhibition assay. The library contained 900 fragment-sized compounds (average MW: 180±66 Da, average heavy atom count: 13±5) compiled in our group. Our Slovenian partner performed the screening, and the evaluation was done jointly. IC_{50} values were determined for the best 15 compounds. Based on the measured activities and chemical tractability 6 compounds belonging to different chemical series were selected for further investigations. These compounds served as a basis for purchasing and synthesizing 90 close analogues, primarily chloro- and methoxy derivatives for inhibitory activity measurements and for structure-activity relationship (SAR) studies. Based on these results a single series with condensed [6+5] type scaffold was selected for detailed investigations.

2) Experimental covalent screening

a) We developed and screened our in-house cysteine reactive fragment library consisting of ~600 electrophilic compounds. Out of this set, compounds with 10 different reactive warheads (isothiocyanates, cyanamides, maleimides, vinyl sulfones, haloacetyl compounds etc.) were found to inhibit iPS. Based on inhibitory activity and novelty a single series with benzisothiazolinone scaffold was selected for further investigations.

b) We developed and tested our in-house heterocyclic electrophilic fragment library of 84 fragments against iPS and 14 hits were identified. Our Slovenian partners developed libraries around the vinylpyridine and chlorobenzothiazole hits with 68 and 71 compounds, respectively. These libraries were tested and several hits were identified. Heterocyclic fragments were selected for further investigations based on inhibitory profile and synthetic feasibility.

3) Structure-based covalent virtual screening:

a) Potential covalent binder boronic acids were taken from commercial compound libraries of the ZINC and eMolecules databases and their screening was performed in Budapest. A hierarchical screening cascade was developed that allowed us to screen over hundred thousand of compounds and to select 32 virtual hits for experimental testing. The inhibitory activity of five compounds was experimentally confirmed [1].

b) Commercially available cyclic boronic acids with 5- and 6-membered rings were also subjected to virtual screening against the β5i subunit of iPS. Best scored 12 compounds were assayed and 3 exhibited lower than 50% remaining activity at 100 μM concentration. However, the lack of dependent inhibition did not validate their follow-up [2].

4) Structure-based non-covalent virtual screening

The ZINC database was used as a source of potential non-covalent ligands and the virtual screening campaign was performed in Ljubljana. 40 compounds were selected, purchased, and tested in in vitro inhibitory assay but none of them exhibited inhibition.

Hit optimization

Hits identified in the screening campaigns described above were further investigated to select compounds suitable for optimization.

BenzXazole series

Screening campaign 1) resulted in compounds containing benzoxazole-2-thione, benzimidazole-2-thione and benzothiazole-2-thione scaffolds (collectively called benzXazoles). Chloro scans around the benzene-ring were performed for all three series to further explore SAR. Altogether, 18 compounds were either purchased or synthesized. 6- and 7-Cl-substitution showed beneficial effect on the activity. A second catalogue similarity search was performed and identified 29 compounds out of which 8 exhibited significant activity at 10 micromolar concentration. *N*-methylation of benzimidazole-2-thiones increased affinity. Single digit micromolar inhibitory activity was observed for several compounds including 6-substituted analogues.

The tautomer distribution between thiol and thione forms was investigated by ^{13}C, ^{15}N NMR, IR and UV spectroscopy and it was found that the dominant form in each series is the thione tautomer.

The inhibition assay performed by our Slovenian partner used full iPS and β5i specific substrate. In order to confirm the binding of the benzXazole series to the β5i subunit microscale thermophoresis (MST) experiments were performed. Single-point measurements were carried out for 5 compounds and dissociation constants were determined for additional 4 compounds including benzo[*d*]imidazoles, -oxazoles and thiazoles. These studies confirmed the binding of all three subseries to the β5i subunit.

A synthetic plan to further explore structure-activity relationships was developed. The synthesis of *N*-methylated compounds was performed in Ljubljana, while the 6- and 7-substituted analogues were synthesized in Budapest. 42 benzXazoles substituted either in the 6 or 7 positions were synthesized and assayed. It was found that although small substituents in the 6- and 7-positions are beneficial for iPS inhibition, larger substituents diminish activity. Highest activity compounds inhibited the β5i subunit of the human iPS in single digit micromolar range.

Since 1-Me substitution was found to increase the inhibitory potency of benzo[*d*]imidazole-2(3*H*)-thiones, 10 compounds with other substituents in position 1 were synthesized and assayed. Single point measurements at 100 μM concentration were performed and IC_{50} values were determined for the most active compounds. No activity improvement with respect to the methylated compounds was found.

Since selective inhibition of the iPS without significantly affecting the constitutive proteasome (cPS) is a desired feature to reduce unwanted side effects, inhibitory activity against the β5 subunit of the cPS was investigated for selected compounds. Interestingly, these compounds showed significant selectivity toward the β5i subunit of the iPS. A potential source of this selectivity is the interaction with Cys48 specific to iPS and this prompted us to further investigate the inhibitory mechanism of these compounds.

Studies on the inhibitory mechanism

The reactivity of the series was investigated to explore potential covalent mechanism of inhibition. No reactivity toward gluthatione in pH range 7-9, toward *N*-benzoylthreonine and toward a nonapeptide was observed. Moreover, inhibition was found slightly or not time dependent. The mechanism of action of the benzXazole series was further investigated by our Slovenian partner. Assays for possible redox activity of compounds were performed. Three different assays, each with a distinct mechanism, namely H_2O_2 generation, formation of ROS, and free radicals were used. The majority of the compounds showed no significant redox activity.

The disulfide forming ability of the compounds was investigated with 2-nitro-5-thiobenzoic acid (TNB^{2-}). It was found that benzXazoles facilitate the disulfide formation between TNB^{2-} units but only a very small amount of the mixed disulfide between benzXazoles and TNB^{2-} was observed.

Inhibition of iPS by benzXazoles was investigated in reducing environment using dithiothreitol (DTT). The most active compounds showed no inhibition under such conditions.

The above experiments together with the observed iPS selectivity suggest that the benzXazoles may be able to form disulfide bond with Cys48 of the β5i subunit of iPS. However, our attempt to detect the β5i

labelling by MS was unsuccessful. We hypothesized that the interaction between benzXazoles and Cys48 contribute to the inhibition, but the disulfide formation is transient.

Warhead change

The mechanism of action was further investigated by testing new compounds where the thione (thiol) moiety is replaced with either methyl sulfone, vinyl sulfone, chloroacetamide or nitrile warheads. These compounds inhibit β5i and the inhibition is preserved in reducing environment. Moreover, 1,2-benzoxazole-2-carbonitrile exhibits time dependent inhibition of iPS and selectivity against the β1 and β2 subunits of both iPS and cPS. Ellman's assay and MS experiments suggest covalent binding to Cys48. These viable chemical starting points are suitable for further investigation and the development of β5i-selective probes for non-cancer indications related to the iPS [3].

We synthesized 16 benzoxazole-2-carbonitrile derivatives to explore the SAR of this series. Systematic substituent scans around the fragment core resulted in compounds with single digit micromolar inhibition. Experimental reactivity studies against N-acetylcysteine and glutathione just as quantum chemical reaction barrier calculations (B3LYP/6-311++G**) showed that the substituents do not affect the covalent reactivity of the nitrile warhead. This finding suggests that substituents affect inhibitory activity primarily by influencing the non-covalent recognition. Considering the small size of the inhibitors this finding underlines the importance of the non-covalent step in the covalent mechanism of action [4].

Attempt to generate bidentate inhibitors

Compounds were designed that contain both boronic acid and benzoxazole-2-carbonitrile reactive groups, the latter was identified in our studies as Cys48 binding covalent iPS inhibitors. The boronic acid warhead and the connected moiety was taken from inhibitors whose structural data in complex with iPS are available. Molecular modeling was used to design molecules containing boronic acid warhead and a benzoxazole-2-carbonitrile group, the former binding to Thr1 and the latter occupying a position near to the iPS specific Cys48. Compounds with vinyl-thiazole group identified as iPS inhibitor in the screening of heterocyclic electrophilic fragments (Screening 2b) were also combined with boronic acid warhead. 14 compounds were synthesized, and the most active ones showed inhibitory activity below micromolar IC_{50}. Ellman's assay proved cysteine binding, however, these compounds showed no significant benefit in terms of activity compared to inhibitors with a single warhead [4].

Benzisothiazolinones

Chloro-scan on benzisothiazolinones from screening 2a) was initiated to further explore SAR; two compounds were ordered, and two others were synthesized. Although activity was promising, flat structure-activity relationship (SAR) was observed and initial synthetic efforts to expand SAR were unsuccessful. Moreover, these compounds were found to be promiscuous covalent binders both in the literature and by us. Taking also into account that covalent mechanism was assumed for the compounds identified in screening 1) the work with benzisothiazolinone scaffold compounds was not continued.

Boronic acids

Screening 3a) resulted in 5 confirmed boronic acid hits. Two of them with the lowest residual activity in β5i inhibitory assay were subjected to IC_{50} measurements. Concentration dependent inhibition with double digit micromolar IC_{50}s were observed. The compounds exhibited time-dependent inhibitory activity in line with the covalent mechanism of action. Microscale thermophoresis experiments confirmed binding to the β5i subunit. The binding mode of the inhibitors were investigated by computational docking and was found to be similar to the non-peptidic non-covalent Roche inhibitor (WO 2014086701 A1). Slight selectivity (2-3 fold) against the constitutive proteasome was measured for the two compounds. One of these lead-like boronic acid derivatives is a suitable starting point for chemical optimization [1].

Methodological developments

Experimental characterization of cysteine reactivity

The reactivity of compounds toward protein nucleophiles is a key factor in the design and development of targeted covalent inhibitors. We investigated computational and experimental technics to characterize compound reactivities. This included a comparative kinetic analysis of a variety of cysteine surrogates against a designed set of electrophilic fragments equipped with a range of warheads was performed. Our study contained seven different thiol models and 13 warheads resulting in a reactivity matrix analyzed thoroughly. We found that the reactivity profile might be significantly different for various thiol models. Comparing the different warheads, we concluded that glutathione (GSH) provided the best estimate of reactivity with highest number of true positives identified [5]. We also showed that cysteine specificity can be characterized by an oligopeptide assay and the combined use of the GSH and oligopeptide assays is useful in the investigation of the reactivity profile of covalent warheads [6].

Experimental assessment of tractable cysteines

The assessment of tractable cysteines is another key factor in designing cysteine targeting covalent inhibitors. We developed a toolbox of fragments containing a 3,5-bis(trifluoromethyl)phenyl core that was equipped with chemically diverse electrophilic warheads showing a range of reactivities. We characterized the library members for their reactivity, aqueous stability and specificity for nucleophilic amino acids. By screening this library against a set of enzymes amenable for covalent inhibition, we showed that this approach experimentally characterized the accessibility and reactivity of targeted cysteines [7].

Covalent virtual screening of large compound collections.

Virtual screening of covalent inhibitors is a time and resource intensive process primarily owing to the chemical bond formation between the ligand and the protein. We developed a hierarchical screening cascade that combines non-covalent and covalent docking steps and allows the handling of large

collections as we exemplified by the screening ~100000 compounds against the β5i domain of iPS. The successive application of screening steps with increasing complexity led to the identification of 5 experimentally confirmed covalent hits out of the 32 compounds tested and this represents a 16% hit rate [1], [8], [9].

Computational affinity prediction of covalent inhibitors

We developed a protocol for the computational prediction of the activity of covalent inhibitors. Covalent inhibition can be characterized by the binding free energy of the non-covalent step and the reaction barrier of the covalent step. Our protocol includes thermodynamic integration to calculate ligand binding free energy differences for the non-covalent step, and mixed quantum mechanical/molecular mechanical potential of mean force calculations to calculate reaction barriers. Calculated quantities can be compared to experimental affinity data to validate the computational model and can be used to predict affinities for designed compounds. This complete free energy characterization of the inhibition was applied to several systems including iPS [10], [11].

Publications acknowledging grant SNN125496

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