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A multistep virtual screening approach to identify proteasome inhibitors

Proteasome plays a fundamental role in the protein turnover by degrading misfolded, abnormal or damaged proteins, previously labeled through addition of a polyubiquitin chain. An aberration of this proteolytic system could result in different types of hematological malignancies. An overexpression of protesome has been detected in multiple myeloma (MM) cells, and, in this context, selectively targeting the chymotrypsin-like (ChT-L) activity of 20S proteasome has emerged as a promising approach for anticancer therapy, in particular for the treatment of MM. To date, three proteasome inhibitors have been approved by the FDA for the treatment of MM: the peptide boronates bortezomib and ixazomib, and the peptide epoxyketone carfilzomib. Despite the wide use of these proteasome inhibitors in the clinic, their toxicity and off-targets interactions remain a great limitation for their use. On the contrary, noncovalent inhibitors, devoid of a reactive warhead, may lack of several drawbacks that are related to covalent inhibition.

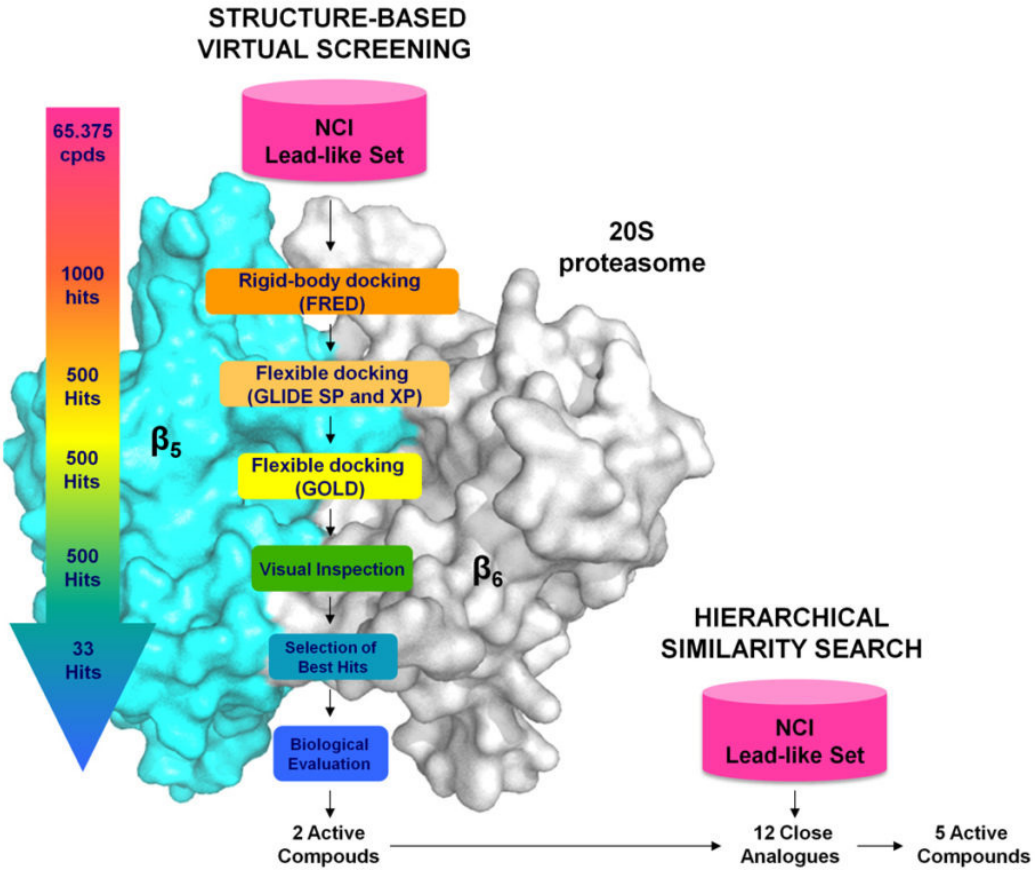

Fig. 1. Flow chart of the multistep hierarchical SBVS strategy implemented in this work.

In an effort to identify novel noncovalent inhibitors of the mammalian 20S proteasome, we screened the NCI (National Cancer Institute) lead-like library (~65,375 compounds) using a multistep hierarchical

structure-based virtual screening (SBVS) approach (Fig. 1). Using this strategy, we discovered two compounds (**1** and **2**, Fig. 2) that noncovalently inhibit the ChT-L activity of the mammalian 20S proteasome with K_i values of 2.18 μM and 2.12 μM respectively. Since a co-inhibition of another proteasome proteolytic activity is strongly required to enhance an antitumor response, we identified compound **1** as promising lead compound, because of its ability to co-inhibit the caspase-like (C-L) activity with K_i=8.59 μM, differently to compound **2** which inhibited the sole ChT-L activity.

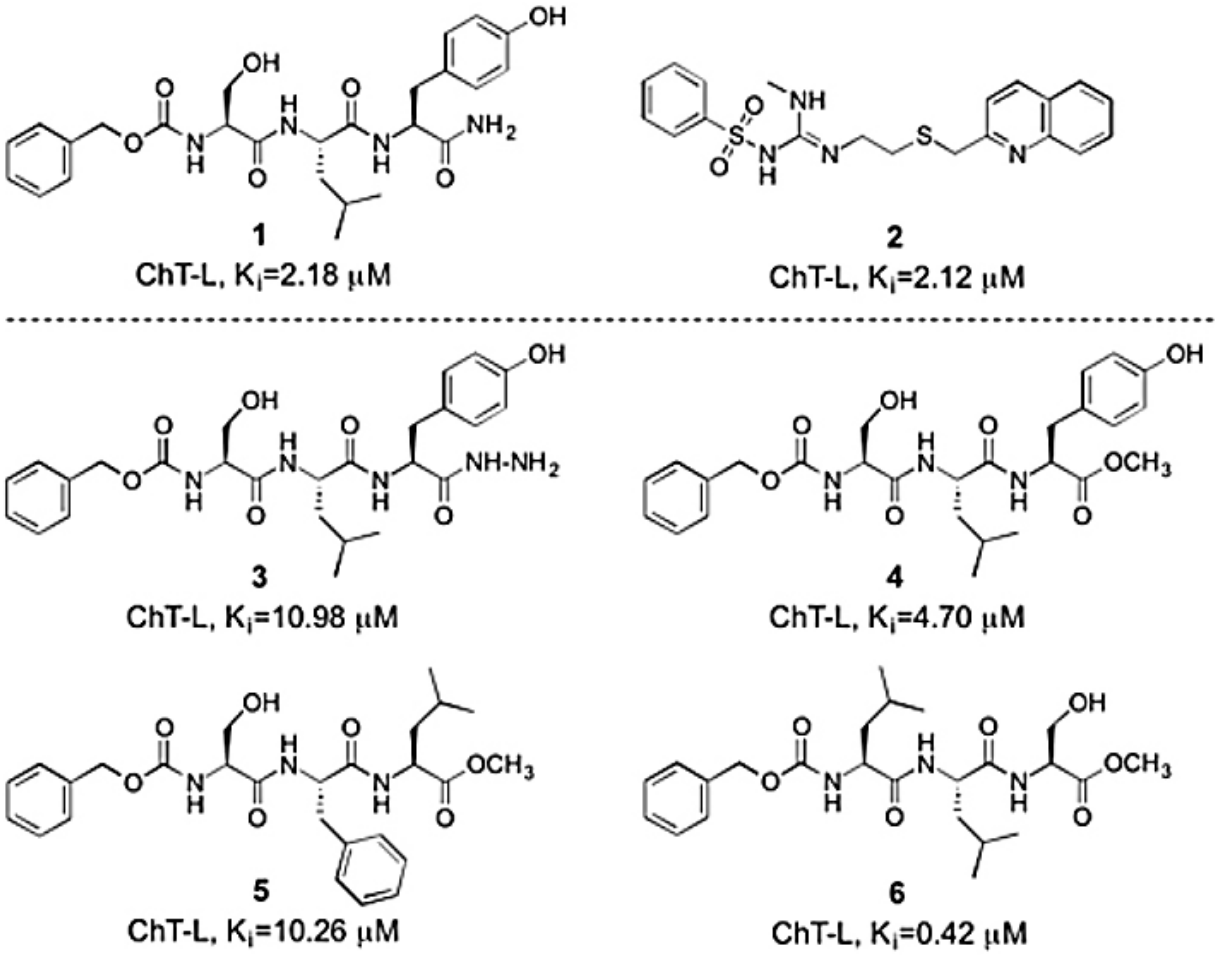

Fig. 2. Molecular structures of the new identified 20S proteasome inhibitors1-6.

A subsequent hierarchical similarity search over the full NCI database with the most active tripeptide-based inhibitor **1** led to the identification of an additional set of 12 ligands, four of which (**3-6,** Fig. 2) exhibited K_i values in the low micromolar range for the ChT-L activity. Compound **6** was the most active inhibitor, with a binding affinity towards the ChT-L activity in the submicromolar range (K_i=0.42 μM). We solved the structure in solution of the most active inhibitor **6** by NMR spectroscopy and elucidated its binding mode into the ChT-L, C-L and T-L active sites of the yeast 20S proteasome by applying docking experiments. Substrate selectivity of compound **6** for the ChT-L subunit seems to be dictated by the interaction of the inhibitor's P1 side chain with the active site's S1 specificity pocket. On the contrary, the

S1 pocket in C-L site is positively charged by the guanidinium group of R45 and therefore does not favor the hydrophobic methyl ester group of **6**. In T-L subunit, G45 results in a more spacious unstructured S1 pocket in comparison with C-L and ChT-L subunits, allowing for motion and flexibility of the P1 side chains of the bound ligand. The cytotoxic effects of the active compounds were also evaluated on dexamethasone resistant (MM.1R) human multiple myeloma cells, which are less responsive to chemotherapy, and are representative of patients in the later stages of the disease. The obtained IC_{50} values ranged from 16.2 µM to 29.8 µM, with the most active compound being compound **6** (IC_{50}=16.2 µM). A western blot analysis of ubiquitin detected changes in total ubiquitin levels due to an increase of ubiquitinated protein levels that accounts for proteasome inhibition. Also in this case, compound **6** was proven to be the most effective at 20 µM, thus being worthy to be considered a promising lead compound for the development of new effective drugs for the treatment of MM.

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