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SELECTIVE INHIBITION OF THE CHYMOTRYPSIN-LIKE ACTIVITY OF THE 20S PROTEASOME BY 5-METHOXY-1-INDANONE DIPEPTIDE BENZAMIDES

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Abstract: Potent inhibitors of the 20S proteasome that contain a novel indanone head group coupled to di and tripeptides are described. These compounds are the first proteasome inhibitors have demonstrated high selectivity for the chymotrypsin-like activity of the 20S proteasome.

The 26S proteasome is recognized as one of the central enzymes of nonlysosomal protein degradation. Most significantly, the proteasomal protein degradation of key regulatory proteins, such as the cyclins and Iκ-B, has been identified as a mandatory step in controlling the respective signaling pathways for cell cycling and activation of NFκ-B.[1] Inhibiting proteasomal degradation of these important signal proteins may prove useful in controlling diseases, such as cancer and inflammation, that are driven by these signaling cascades.

The proteasome is a highly conserved cellular structure that is responsible for the ATP-dependent proteolysis of most cellular proteins.[2] The archaebacteria proteasome exhibits only chymotrypsin-like proteolytic activity,[3] however the eukaryotic proteasome contains at least five identifiable proteolytic activities. Three of these activities are similar in specificity to chymotrypsin, trypsin, and peptidylglutamyl peptidase. The other two proteolytic activities demonstrate a preference for cleavage of peptide bonds on the carboxyl side of branched chain amino acids and toward peptide bonds between short chain neutral amino acids.[4]

The proteasome is a threonine protease, and as such, can be inhibited by an electrophilic 'head group' connected to small peptide fragment that binds to the substrate recognition site. Common classes of serine/threonine protease inhibitors include peptide aldehydes, trifluoromethyl ketones, boronates, chloromethyl ketones, α,β-epoxy ketones, and α-keto amides.[5] There have been recent reports of potent inhibitors of proteasome using peptide boronates, aldehydes and α,β-epoxyketones.[6] The inherent drawbacks of these traditional electrophilic peptide inhibitors are that they may suffer from being too reactive, unstable, nontarget specific, or membrane impermeable. These properties tend to make the prototypical serine protease inhibitors poor drug candidates.

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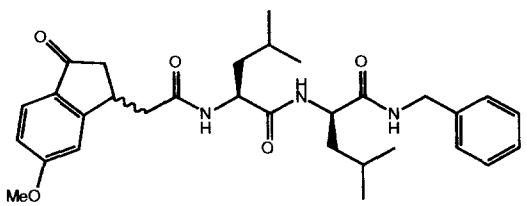
In an attempt to avoid the problems associated with traditional serine protease inhibitors, a search for inhibitors that possess a less reactive, but potentially hydratable, head group coupled to a high affinity peptide fragment was initiated. Among motifs examined was 5-methoxy-1-indanone-3-acetic acid. Herein we report a series of 5-methoxy-1-indanone-3-acetic acid based proteasome inhibitors that are highly selective for the chymotrypsin-like activity of the 20S proteasome.

ALLN (Ac-Leu-Leu-Nle-H) is a known non-selective, potent inhibitor of the 20S proteasome. ALLN analogs, with 5-methoxy-1-indanone-3-acetic acid as the intended head piece replacement for the electrophilic norleucinal moiety, were prepared and screened for inhibition proteasomal chymotrypsin-like proteolytic activity. This screening effort resulted in the identification of a new class of substrate-competitive, proteasome inhibitors. The structure of representative example of these novel proteasome inhibitors is shown in Figure 1.

Figure 1



Indanone-Leu-d-Leu-benzamide(**17**)

Library Generation:

An initial peptide screening library containing approximately 400 compounds was prepared using standard FMOC solid-phase synthesis on Rink amide resin. The library contained both dipeptides and tripeptide analogs that were N-capped with 5-methoxy-1-indanone-3-acetic acid. No amino acid mixtures were employed, since a diastereomeric mixture of peptides naturally results from the use of racemic indanone. Efforts to separate and purify each isomer of the diastereomeric mixtures have proved futile. Synthetic efforts to prepare each isomer will be reported in due course. C- terminal modifications were done using solid-phase FMOC chemistry using Wang Resin. After cleavage from the Wang resin, the free carboxylic acid was coupled to the desired amine using standard solution-phase techniques.[7]

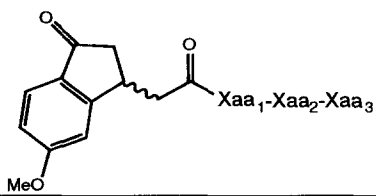
Discussion:

All compounds were initially screened for inhibition for proteasomal chymotrypsin-like activity. Inhibitory data for a subset of compounds are shown in Table 1. Compound **1** showed competitive inhibition, which suggests that the indanone is binding in the active site. Unlike ALLN and other known inhibitory peptide aldehydes, all active compounds generated in this study proved to be highly selective for the chymotrypsin-like activity of the 20S proteasome.[8]

None of these compounds inhibited the other four proteasomal proteolytic activities (IC_{50} >20 μM), nor did these compounds display Calpain I inhibitory activity (ALLN vs Calpain I has an IC_{50} of 7 μM).

The data suggest that inhibition of proteolyic activity by these peptidal compounds is lost with just slightest the modification in structure. The active di- and tripeptide analogs displayed a preference for D or L leucine in the Xaa_1 subsite and sole preference for D leucine in the Xaa_2 subsite. The optimum side chain stereochemistry of these inhibitors is L-leu-D-leu. The most potent inhibitor identified in the initial library was compound 11 with an IC_{50} of 0.2 μM. Unfortunately, 11 proved inactive in a cell proliferation assay.

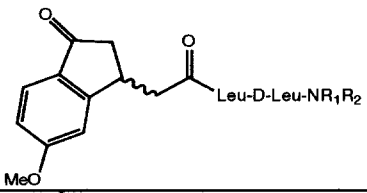
Table 1



Compound	Xaa_1	Xaa_2	Xaa_3	IC_{50} (μM)
ALLN				0.15
1	D-Leu	D-Leu-NH_2		2.2
2	D-Leu	Leu-NH_2		>20
3	Leu	D-Leu-NH_2		>20
4	Leu	Leu-NH_2		>20
5	D-Phe	D-Leu-NH_2		>20
6	D-Val	D-Leu-NH_2		>20
7	D-Leu	D-Val-NH_2		>20
8	norVal	D-Leu	Gly-NH_2	>20
9	D-Leu	D-Leu	Gly-NH_2	>20
10	D-Leu	Leu	Gly-NH_2	10-20
11	Leu	D-Leu	Gly-NH_2	0.2
12	Leu	Leu	Gly-NH_2	>20

Encouraged by the results seen with tripeptide 11, a library of derivatized dipeptides was prepared. Simple C-terminal derivatives were prepared to examine effects of this substitution on potency and antiproliferative activity. Inhibitory data on selected examples from the second library are shown in Table 2. The results show that only a C-terminal benzyl amide derivative was a suitable replacement for the terminal amino acid. Even though the enzyme inhibitory potency of compound 17 was less than that seen for compound 11, compound 17 displayed antiproliferative activity (IC_{50} = 7 μM) against a murine macrophage (RAW) cell line. Like the glycine derivatives, the benzylamide variants also displayed demanding SAR requirements for the L-Leu-D-Leu motif (data not shown).

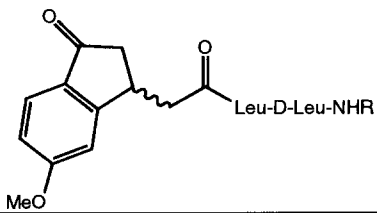
Table 2



	R_1	R_2	IC_{50} (μM)
13	-CH_2-CH_2-O-CH_2-CH_2-		>20
14	-CH_2-CH_2-CH_2-CH_2-CH_2-		>20
15	-CH_2-CH_2-CH_2-CH_2-		>20
16	Isopropyl	H	>20
17	Benzyl	H	0.93
18	Hydroxyethyl	H	>20
19	Benzyl	Me	>18
20	Adamantyl	H	>18
21	Phenyl	H	>19
22	Phenethyl	H	>18
23	Cyclohexylmethyl	H	>18

The results described above clearly suggested that a library of methylene aryl and heterocycle analogs of **17** should be prepared. Table 3 lists data on some of these benzyl derivatives. Substituted aryl groups were well tolerated, with electron withdrawing groups (**26**) having deleterious effect on activity relative to electron donating substituents (**25, 27**). Indane analog **24**, with its rigidly constrained substituted methylene position, displayed good

Table 3



	R	IC_{50} (μM)
24	1-Indanyl	0.89
25	4-Methoxybenzyl	0.71
26	4-Nitrobenzyl	>17
27	Piperonyl	0.34
28	2,6-Difluorobenzyl	0.14
29	Pentafluorobenzyl	>16
30	Furfuryl	1.9
31	Diphenylmethyl	>16
32	4-Phenylbenzyl	0.49
33	2-Phenylbenzyl	>16
34	4-Benzyloxybenzyl	16
35	Benzofurfuryl	8.7

inhibitory activity despite being a four component mixture. Compound **32** showed good inhibition suggesting that there is space to accommodate some bulk at the four position of the aryl group, although data on analogs **31** and **34** indicates that this area is not without some subtle spatial constraints. Bulky substitution at the 2-position caused a loss of activity (compound **33**). Electron rich heterocyclic analogs **30** and **35** show a lesser degree of activity than the electron rich aryl analogs **25** and **27**. The most potent compound identified from this library was 2,6-diflurobenzyl analog **28**. Surprisingly, the pentafluoro analog **29** was inactive. Like all of the other inhibitors reported here, **28** is a highly selective, competitive inhibitor of the chymotrypsin-like activity of the 20S proteasome. In addition analog **28** is a potent antiproliferative compound (IC_{50} = 8 μM) against RAW cells.

In summary, we have identified a series of novel 5-methoxy-1-indanone-3-acetic acid peptide derivatives that are potent, competitive inhibitors of the chymotrypsin-like activity of the 20S proteasome. The extremely tight SAR indicates a strong preference for lipophilic side chains L-Leu and D-Leu in the Xaa_1 and Xaa_2 positions, respectively, of the peptide portion of these inhibitors. In addition, the C-terminal benzylamide and substituted benzylamide substitution yields the most potent inhibitors which also display antiproliferative activity against RAW cells. All these novel indanone based inhibitors displayed selectively for the chymotrypsin-like activity of the 20S proteasome and none inhibited Calpain I. With this series of selective inhibitors in hand, we are continuing to explore the general biological consequences of inhibiting the proteasome, and are working toward elucidating any special biology attributable to chymotrypsin-like activity of the 20S proteasome.

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7. For example: 10 mg of FMOC-d-Leu-Wang resin (Nova Biochem) was loaded into fritted syringe. The resin was deprotected with 20% piperidine in DMF for 30 min. The resin was washed 5 times with DMF, 5 times with methanol, and 5 times with DMF. A solution of FMOC-Leu, EDC•HCl, HOBT in DMF was drawn up and the resin was agitated for 6 h. The solution was removed and the

resin washed 5 times with DMF, 5 times with methanol and 5 times with DMF. The resin was deprotected with 20% piperidene in DMF as previously described. Coupling of 5-methoxy-indanone-1-acetic acid, was accomplished using EDC•HCl, HOBT in DMF. The reaction mixture was agitated 6 h, before washing with DMF, methanol and DMF. Cleavage of the dipeptide carboxylic acid was achieved using 95% water/5% TFA for 2 h. The cleavage mixture was concentrated to drynesss. The residue was taken up in DCM and a solution of benzylamine, EDC•HCl and HOBT in DCM was added. The mixture was allowed to stir for 4 h, followed by a normal aqueous workup. The organic layers were concentrated to an off white solid.

8. For complete experimental details see: Lum, R. T.; Meyer, S. M.; Nelson, M. G.; Schow, S. R.; Shiffman, D.; Wick, M. M.; Joly, A. In Press *Biochem. Pharm.*
General chymotrypsin like assay conditions are as follows: 1 μg purified 20S proteasome protein in 200 μl reaction buffer (50mM HEPES, 0.1% sodium dodecyl sulfate, pH 7.5). The proteolytic reaction was initiated by the addition of 50 μM fluorogenic peptide substrate (succinyl-leu-leu-val-tyr-7-amino-4-methyl coumarin) and allowed to progress for 15 min at 37 °C. The reaction was terminated by the addition of 100 μl of 100 mM acetate buffer, pH 4.0. The rate of proteolysis is directly proportional to the amount of liberated aminomethyl coumarin that was measured by fluorescent spectroscopy (EX 370 nm/ EM 430 nm).