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Discovery of Potent, Selective Human Granzyme B Inhibitors that Inhibit CTL Mediated Apoptosis

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Abstract—A novel class of small molecule human granzyme B inhibitors is reported. Compound **20** has a K_i of 7 nM against human granzyme B and blocks CTL mediated apoptosis with an IC_{50} of 3 micromolar.

Granzyme B is a serine protease localized in the granules of cytotoxic T lymphocytes (CTLs). Upon contact with a target cell the granules and perforin are released from the CTL and granzyme B penetrates into the target cell. The enzyme is responsible for activation of the apoptosis cascade, either by direct cleavage of apoptotic substrates or by activation of the caspases by processing of procaspase-3.[1] Granzyme B has also been implicated in the pathogenesis of autoimmune diseases.[2] In an effort to further elucidate the role of granzyme B in these processes we set out to design a small molecule granzyme B inhibitor.

Although granzyme B is a serine protease it is related to the caspase family of enzymes by its requirement for an aspartic acid in P1.[3] Screening of a positional scanning combinatorial library designed for the caspase enzymes revealed IEPD as the preferred tetrapeptide substrate sequence for granzyme B.[3a] We used this information as a starting point for inhibitor design and thus prepared the corresponding tetrapeptide aldehyde **1** (Fig. 1). Compound **1** was found to be a potent granzyme B inhibitor[4] having a K_i of 80 nM. We have previously described the X-ray crystallographic characterization of an enzyme/inhibitor complex of compound **1** and human granzyme B.[5] The structure clearly shows a covalent interaction between the active site serine and the P1 aldehyde carbon of **1** and this interaction is likely a major contributor to the binding energy.

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Based this consideration, and on examination of the library results, we chose to first explore P2–P3 region of the molecule while keeping the P1 Asp-aldehyde intact. The chemistry for preparation of the peptide aldehydes with the aspartic acid side chain has been previously reported.[6,11a] Table 1 shows the data from our initial SAR of these analogues. Removal of the P3 acid side chain resulted in a ~3-fold decrease in activity (**2**). The pyridone[7] derivative **3** has a K_i of 133 nM, about 2-fold more potent than **2**. Compound **4**, which incorporates a rigid bicyclic peptide mimic[8] in the P2–P3 position, has a K_i 122 nM, again about 2 fold better that **2**. Fusion of a phenyl ring onto the pyrrolidine as in compound **5** gave a significant increase in the activity, **5** having a K_i of 13 nM The most potent compound, **6**, which incorporates the features of both **4** and **5** into a rigid tricyclic scaffold,[9] is 10-fold more potent than the tetrapeptide **1**, having a K_i of 8 nM.

Compound **6** was then tested for its ability to inhibit CTL mediated apoptosis.[10] The assay employed human NK-92 natural killer cells acting on target K-562 cells whose DNA had previously been labeled with [^{125}I]iododeoxyuridine. After 1 h preincubation of both cell lines with compound, the cells were combined to give a ratio of 5:1 killer to target cells and a target cell concentration of 100,000 cells/mL in a total volume of 200 μL. After allowing 2 h for killing to develop, the cells were lysed with detergent and induction of apoptosis in the target cells was measured as the formation of soluble ^{125}I-DNA fragments, which in the absence of inhibition typically amounted to 25% of the total ^{125}I-DNA.

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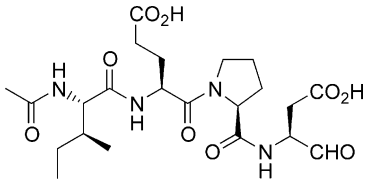
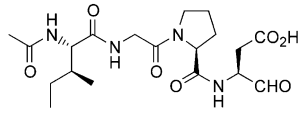
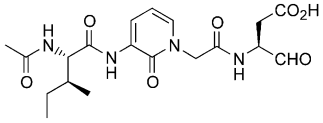
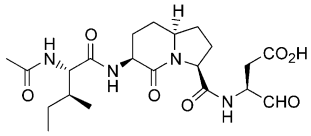
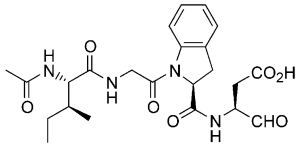
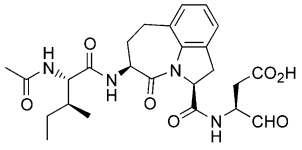


1 hGrB K_i = 80 nM

Figure 1. Tetrapeptide aldehyde IEPD-CHO.

Table 1. P2–P3 modifications of granzyme B inhibitors

Compd	Structure	GzmB K_i (nM)[a]
2		270
3		133
4		122
5		13
6		8

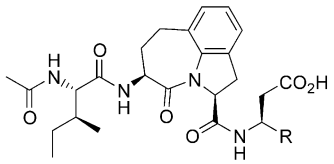
[a]Values are means of three experiments.

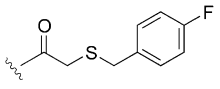
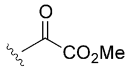
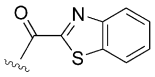
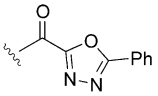
Compound **6** had an IC_{50} of 700 nM in this CTL mediated killing model. However, when tested against several of the caspases, in particular caspase 3 and caspase 8, compound **6** was also active (K_i = 71 and 350 nM, respectively). Importantly, caspase 3 inhibitors also inhibit apoptosis in this assay (data not shown). Thus the effects of compound **6** on apoptosis could not be conclusively attributed to granzyme B inhibition.

We then set out to improve the selectivity of these granzyme B inhibitors versus the caspase enzymes by modification of the P1 position. We began by examination of the electrophilic serine trap. These results are shown in Table 2.

Incorporation of a thiophenylpropyl ketone as has been described for cysteine protease inhibitors[11] resulted in a ~300-fold loss in activity. Compounds bearing other electrophilic serine traps such as keto-ester[12] **8**, keto-benzothiazole[13] **9**, or keto-oxadiazole[14] **10**, were potent granzyme B inhibitiors having K_i's of 16, 85 and 7 nM, respectively. However, these compounds were potent

Table 2. Modification of the serine trap



Compd	R	GzmB K_i (nM)[a]	Csp 3 K_i (nM)[b]
6	CHO	8	71
7		2300	n/t
8		16	202
9		85	143
10		7	26
11	H	580	n/a

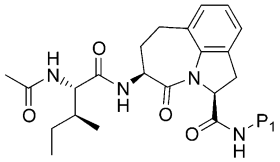
[a]Values are means of three experiments.
[b]n/t, not tested, n/a, <50% inhibition at 50 μM.

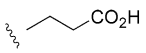
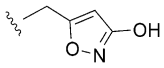
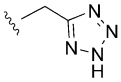
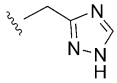
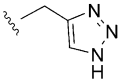
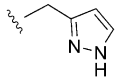
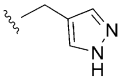
inhibitors of many of the capsase enzymes as exemplified by the data shown for caspase 3 (Table 2). Somewhat surprising was the activity of compound **11**. Although **11** did not have an electrophilic trap it was still a modestly potent granzyme B inhibitor. The K_i of 580 nM is 73-fold weaker than that of aldehyde **8** (K_i = 8 nM) however **11** showed no caspase inhibition at concentrations up to 50 micromolar.

In an effort to gain back some of the activity lost by removal of the electrophile the acid in the P1 position was replaced with heterocyclic acid bioisosteres[15] such as a hydroxyisoxazole (**12**) or tetrazole (**13**). This data is shown in Table 3. Compounds **12** and **13** had K_i's of 360 and 74 nM, respectively, while exhibiting no inhibition of any of the caspase enzymes. We now had in hand a compound that was equipotent to the tetrapeptide aldehyde **1** but had *no* electrophilic serine trap and *no* caspase activity.

We then examined a series of heterocyclic derivatives aimed at establishing which H-bond donor/acceptor features of the tetrazole were important for activity (see Table 3). As can be seen by comparing compounds **14**, **15**, **16**, and **17**, the 1,2,3 triazole substitution gives a 2-fold improvement over the tetrazole derivative **13**. None of the other derivatives showed any activity, highlighting the importance of the 1,2,3 arrangement of donor/acceptors. This is a rather remarkable finding given the strict requirement for an acidic residue in P1 because the 1,2,3 triazole (pK_a = 9.5) is neutral at physiologic pH. Gratifyingly, no caspase activity is observed with compound **15**.

Table 3. P1 modifications



Comp	P1	GzmB K_i (nM)[a]
11		580
12		360
13		74
14		n/a
15		38
16		n/a
17		n/a

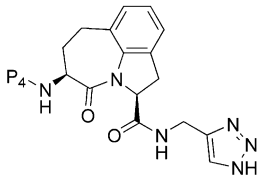
[a]Values are means of three experiments. n/a, <50% inhibition at 50 μM. All compounds were inactive against the caspase enzymes.

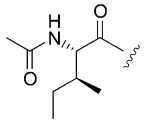
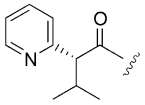
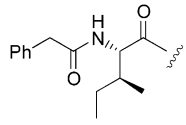
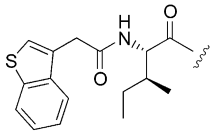
Analysis of compound **15** in the apoptosis inhibition assay (see Table 4) revealed that **15** would indeed block CTL mediated apoptosis. However, the IC_{50} was rather modest at 30 μM.

Finally we focused on optimization of the P4 position in order to improve the activity in cells. Some of these results are shown in Table 4. Replacement of the P4 acetyl isoleucine with a heterocyclic derivative gave compound **18**, which was 2-fold less active against the enzyme but equipotent in cells. By adding a hydrophobic group in the P5 position such as the phenyl group in compound **19** or the benzothiophene moiety in compound **20** we were able to improve the cellular activity 5- to 10-fold. Compound **19** was 13 nM against the enzyme and 6.3 μM in the apoptosis inhibition assay while compound **20** was 7 nM in the enzyme assay and inhibited CTL mediated apoptosis with an IC_{50} of 3.1 μM. Again compounds **18**, **19**, and **20** showed no caspase inhibition, underscoring the role of granzyme B in CTL mediated apoptosis.

In summary, we have discovered a novel class of selective small molecule granzyme B inhibitors that block CTL mediated apoptosis. A key feature of these compounds is the 1,2,3 triazole moiety, which proves crucial to the selectivity and cellular efficacy of these compounds. These results further support the role of granzyme B as a key mediator of apoptosis by cytotoxic T lymphocytes.

Table 4. Activity of P1 triazole derivatives



Compd	P4	GzmB K_i (nM)[a]	Apoptosis IC_{50} (μM)
15		38	30
18		75	30
19		13	6.3
20		7	3.1

[a]Values are means of three experiments.

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