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Dipeptidyl aspartyl fluoromethylketones as potent caspase-3 inhibitors: SAR of the P_2 amino acid

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Abstract—This article describes the synthesis and biological evaluation of a series of dipeptidyl aspartyl fluoromethylketones as caspase-3 inhibitors. Structure–activity relationship (SAR) studies showed that for caspase-3 inhibition, Val is the best P_2 amino acid. The SAR studies also showed that the Asp free carboxylic acid in P_1 is important for caspase inhibiting activities, as well as for selectivity over other proteases.

The caspases are a family of cysteine proteases that require aspartic acid residues at P_1 of substrates for cleavage. Twelve enzymes in this family have been identified,[1] and the structures of caspase-1, -3, -7 and -8 have been determined by X-ray crystallography.[2] Based on its biological function, caspases can be divided into two groups. One of the groups, represented by caspase-1, also known as interleukin-1β converting enzyme (ICE), plays an important role in cytokine maturation and inflammation.[3] The other group, represented by caspase-3, -8 and -9, are critical for death receptor signaling and execution of apoptosis. Among them, Caspase-3 has been identified as a key member of the executioner caspases.[4] Due to the important role of caspases in both inflammation and apoptosis, the discovery and development of caspase inhibitors has become a focus of research in recent years.[5]

Many potent caspase inhibitors have been prepared based on the tetrapeptide substrate structures of caspases. However, due to the peptidyl structure and the presence of carboxylic acid group(s), many of these inhibitors do not have good cell penetration and are not very active in cell assays.[6] One approach to address this problem is the preparation of conformationally constrained compounds, using heterocycles to replace the peptide backbone,[7] or the discovery of non-peptide inhibitors.[8] Another approach is to reduce the number of amino acids in the inhibitor, truncating the tetrapeptide to either a tripeptide, dipeptide,[9] or monopeptide, that is, substituted aspartic inhibitor.[10] We have reported that, starting with the DEVD tetrapeptide caspase-3 recognition site of PARP,[11] by reducing the number of amino acids to the VD dipeptide scaffold with a fluoromethylketone warhead, MX1013 (Z-Val-Asp-fmk) has been identified as a potent and broad-spectrum caspase inhibitor with IC_{50} values ranging from 5 to 20 nM for caspase-1, -3, -6, -7, -8 and -9.[12,13] MX1013 is more cell permeable than the corresponding tetrapeptide and tripeptide-fmk, as well as the widely used Z-VAD(OMe)-fmk, and shows good potency in protecting cells against apoptosis. MX1013 also is highly efficacious in several animal models of apoptosis, including rodent liver failure model, rat middle cerebral artery occlusion (MCAO) re-perfusion brain ischemia model and myocardial infarction model by IV administration,[13] and rodent endotoxin shock model.[14] Herein we report the synthesis and SAR studies of dipeptidyl aspartyl fluoromethylketones as caspase-3 inhibitors.

Scheme 1 shows the synthesis of the dipeptides Z-Val-Asp-fmk (**4**) and its methyl ester **5**. The intermediate **1** was prepared according to Revesz et al.[15] from *t*-Bu 3-nitropropionate and 2-fluoroethanol. Coupling of **1** with Z-Val gave amide **2**, which was oxidized by Dess–

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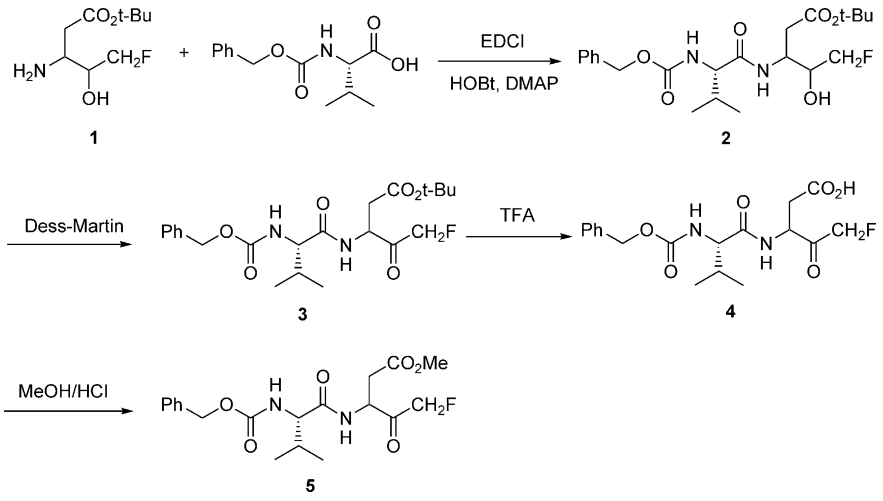


Scheme 1.

Martin reagent to produce the corresponding ketone **3**. Trifluoroacetic acid catalyzed cleavage of the *t*-Bu ester gave the free acid **4**. Acid **4** was then converted to methyl ester **5** by treatment with methanol in the presence of HCl. Other dipeptides were prepared similarly from the Z protected amino acid. The activity of the dipeptides to inhibit human recombinant caspase-3 was determined using a standard fluorometric assay.[13,16]

The caspase-3 inhibition activity (IC_{50}) of the dipeptides are summarized in Table 1. Compound **4** (Z-Val-Asp-fmk) showed an IC_{50} value of 0.03 μM in caspase-3. The P_2 position was explored by substituting the Val with other amino acids. When the side chain (isopropyl) of Val in **4** was replaced by a smaller group, such as a methyl (Ala, **11**) and a proton (Gly, **12**), the potencies decreased 20- and 63-fold, respectively. More bulky groups, such as an isobutyl (Leu, **9**) and a phenylmethyl (Phe, **10**), also resulted in compounds with reduced inhibition activities of 6–13 fold. Compound **6**, with a 2-butyl group (Ile), retains good activity and is about half as active as that of **4**, probably because the size of a 2-butyl (Ile) group is close to that of isopropyl (Val). The results showed that for caspase-3 inhibition, Val is the best amino acid at the P_2 position for these dipeptide inhibitors. This is consistent with the results of a combinatorial approach to define specific caspase tetrapeptide substrates, which showed that for caspase-3, Val is the best P_2 amino acid, and Ile is a good replacement for Val at the P_2 position.[17] The polar and large Lys (**13**) and Glu (**14**) as P_2 amino acid gave the least potent compound, indicating that polar and large size groups are not tolerated at P_2 position. This is also consistent with the results from the combinatorial approach, which showed that Lys and Glu are not preferred amino acid at the P_2 position for caspase-3.[17]

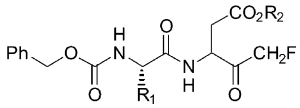
We also explored the replacement of P_2 Val by non-natural amino acids. When the isopropyl side group of Val was replaced by an ethyl (**15**), phenyl (**16**) and cyclohexyl (**17**), the compounds retain good activity and are about 1/3 as active as that of **4**. These compounds are all more active than **10**, which has a more bulky phenylmethyl side group. Compound **18**, with a 5-member thiophene ring, also is more active than **10**. These results are in agreement with the analysis of crystal structure of caspase-3 in complex with an Ac-DEVD tetrapeptide inhibitor, which suggested that a small and hydrophobic group in the P_2 position is best for caspase-3 inhibitors.[18]

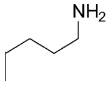
Introduction of an extra methyl group in the α-position of P_2 amino acid, as in compounds **19** and **20**, resulted in reduction of potency. Compound **20** is >70-fold less active than **4**. This large reduction of potency suggests that either the extra methyl could not be fit into the S_2 pocket, or the extra methyl group forces the isopropyl group into an unfavorable position, which could not interact with the S_2 pocket properly.

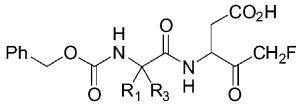
When the P_1 free carboxylic acid (**4**) was converted to a methyl ester (**5**), the potency in caspase-3 was reduced >35-fold, confirming that the carboxylic acid of Asp in the P_1 position is essential for the binding of the inhibitor to the caspase enzyme. Similarly, when **6** was converted to the methyl ester **7**, it also showed ~35-fold drop in potency.

Compounds **4** and **5** were then tested against other caspases and proteases for selectivity and the results are summarized in Table 2. Free acid **4** was found to have similar activity in caspase-1, -3, -6, -7, -8 and -9, but inactive in non-caspase proteases. Therefore, compound **4** is a broad-spectrum caspase inhibitor and is selective for caspases. Interestingly, the methyl ester **5** was found to be more active in non-caspase cysteine proteases calpain I and cathepsin B than in caspase-1 and -3, thus ester **5** is not selective for caspases. Both compounds **4** and **5** are not active (>100 μM) against serine proteases thrombin and factor Xa, indicating that the conversion of the acid **4** to ester **5** only affects their activity as cysteine protease inhibitors. These results indicate that for these dipeptides, the free carboxylic acid in the P_1 is not only important for the caspase inhibiting activity, but also important for their selectivity versus other cysteine proteases. Since ester prodrugs of caspase inhibitors have been used to increase cell permeability,[19] including Z-VAD(OMe)-fmk, Z-D(OMe)E(OMe)VD(OMe)-fmk, and Z-YVAD(OMe)-fmk, which have been reported to

Table 1. Caspase-3 activity of the dipeptide inhibitors



Entry	Name	R_1	R_2	Caspase-3 IC_{50} (μM)[a]
4	Z-Val-Asp-fmk		H	0.030
5	Z-Val-Asp(OMe)-fmk		Me	1.1
6	Z-Ile-Asp-fmk		H	0.070
7	Z-Ile-Asp(OMe)-fmk		Me	2.6
9	Z-Leu-Asp-fmk		H	0.20
10	Z-Phe-Asp-fmk		H	0.40
11	Z-Ala-Asp-fmk	Me	H	0.60
12	Z-Gly-Asp-fmk	H	H	1.9
13	Z-Lys-Asp-fmk		H	1.6
14	Z-Glu-Asp-fmk		H	14.
15	Z-Abu-Asp-fmk	Et	H	0.10
16	Z-Phg-Asp-fmk		H	0.10
17	Z-Chg-Asp-fmk		H	0.10
18	Z-2-Tha-Asp-fmk		H	0.15



Entry	Name	R_1	R_3	Caspase-3 IC_{50} (μM)[a]
19	Z-2-Me-Ala-Asp-fmk	Me	Me	1.4
20	Z-2-Me-Val-Asp-fmk		Me	2.3

[a] IC_{50} is determined as described in ref 13.

be non-selective cysteine protease inhibitors,[20] it is of critical importance to distinguish compounds with a P_1 free acid from the methyl ester prodrug when caspase inhibitors are used for in vitro and in vivo studies. If one is looking for specific inhibition of caspase and not inhibition of non-specific cysteine proteases, then it should be desirable to use an inhibitor with a free acid in the P_1 position rather than a methyl ester prodrug.

Table 2. Activity of compound **4** and **5** in different proteases

Compd	IC_{50} (μM)[a]	
	4	**5**
Caspase-1	0.020	0.9
Caspase-3	0.030	1.1
Caspase-6	0.018	ND
Caspase-7	0.007	ND
Caspase-8	0.007	ND
Caspase-9	0.005	ND
Cathepsin B	>10	0.3
Calpain-1	>10	0.12
Thrombin	>100	>100
Factor Xa	>100	>100

[a] IC_{50} is determined as described in ref 13.

In conclusion, we have synthesized a group of dipeptidyl aspartyl fluoromethylketones and evaluated their activity as caspase-3 inhibitors. We found that for caspase-3 inhibition, Val is the best in the P_2 position among the natural and non-natural amino acids explored for these dipeptidyl inhibitors. The introduction of an extra methyl group in the α-position of P_2 amino acid was found to result in large reduction in potency. We also found that the free carboxylic acid in the P_1 Asp is important not only for their activity as caspases inhibitors, but also for their selectivity over non-caspase proteases. It is therefore critical to distinguish the free acid from the ester prodrug when using caspase inhibitors for biological studies.

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