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Dipeptidyl aspartyl fluoromethylketones as potent caspase inhibitors: SAR of the *N*-protecting group

Sui Xiong Cai,* Lufeng Guan, Shaojuan Jia, Yan Wang, Wu Yang, Ben Tseng and John Drewe

Maxim Pharmaceuticals, 6650 Nancy Ridge Drive, San Diego, CA 92121, USA

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Abstract—This article describes the synthesis and biological evaluation of a group of *N*-protected Val-Asp-fmk as caspase inhibitors. The protecting group was found to contribute to caspase-3 inhibiting activity, and compounds with a large group such as Cbz are more active than compounds with a small group such as Ac. Compounds with more hydrophobic protecting groups were found to be more active in cell apoptosis protection assays, probably due to increased cell permeability. MX1122, 2,4-di-Cl-Cbz-Val-Asp-fmk, is identified as a potent broad-spectrum caspase inhibitor and is selective for caspases versus other proteases, with good activity in the cell apoptosis protection assays as well as good efficacy in the mouse liver apoptosis model.

Apoptosis is a highly regulated process of cell death and caspases play a pivotal role in apoptosis by mediating a series of proteolytic reactions that cleave many important proteins within the cells, and lead to cell death.[1] Caspases are cysteine proteases with strict substrate specificity requiring aspartic acid as P_1 amino acid.[2] It is well known that excessive apoptosis is responsible, at least in part, for a variety of diseases, including liver diseases,[3] brain ischemia,[4] myocardial infarction,[5] and neurodegenerative disorders such as Huntington's disease and Alzheimer's disease.[6] Therefore the discovery and development of caspase inhibitors could potentially lead to effective anti-apoptotic drugs for the treatment of various diseases.[7–9]

Many caspase inhibitors have been designed and synthesized based on substrate specificity of caspases. These include tetrapeptide,[10] tripeptide,[11] dipeptide,[12] or monopeptide based inhibitor,[13] as well as conformationally constrained compounds using heterocycles to replace the peptide backbone,[14] and the discovery of non-peptide inhibitors.[15] Some of these are specific to different caspases, including many of the tetrapeptide based inhibitors; while others are active against all the caspases, including many of the dipeptide based inhibitors. Since apoptosis plays an important function for the elimination of billions of unwanted and excessive cells daily,[16] it might be more preferable to use a specific caspase inhibitor for the treatment of chronic diseases, such as Huntington's disease and Alzheimer's disease. However, because multiple caspases are activated in the apoptosis pathway, a broad-spectrum caspase inhibitor should be more effective to inhibit all the caspases and to achieve maximum inhibition of apoptosis, especially for the treatment of acute diseases, such as stroke, sepsis and myocardial infarction.

We have reported the identification of MX1013 (Cbz-Val-Asp-fmk) as a potent and broad-spectrum caspase inhibitor, with potent in vivo activities (IV or IP administration) in several animal models of apoptosis, including rodent liver failure model, rat middle cerebral artery occlusion (MCAO) re-perfusion brain ischemia model, myocardial infarction model,[17,18] and a rodent endotoxin shock model.[19] These data suggest that the dipeptide structure of MX1013 provides a good combination of in vitro and in vivo activities for a caspase inhibitor, which might be useful for the design of novel caspase inhibitors.

It has been known that for inhibition of caspase enzyme, caspase inhibitors based on tetrapeptide are more potent than the corresponding tripeptides, and tripeptides are

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* Corresponding author. Tel.: +1 858 202 4006; fax: +1 858 202 4000; e-mail: scai@maxim.com

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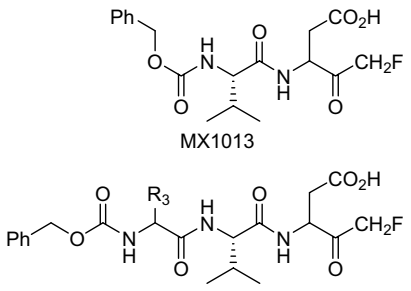


Chart 1.

more potent than the corresponding dipeptides.[20] To maintain the preferred Val-Asp dipeptide scaffold and to explore the P_3 subsite, we examined the replacement of the Cbz protecting group in MX1013 with other groups. As shown in Chart 1 comparing the structure of the dipeptide inhibitor MX1013 with a tripeptide inhibitor, the benzyl of the Cbz protecting group of MX1013 could potentially bind to the same subsite as the side chain (R_3) of P_3 amino acid of a tripeptide inhibitor. Herein we report the synthesis and biological evaluation of a group of *N*-protected Val-Asp-fmk as caspase-3 inhibitors.

The dipeptide Ac-Val-Asp-fmk (**5a**) was prepared similar to MX1013 as reported previously.[18] Ac-Val (**1a**) was coupled with amine **2**[21] to give amide **3a**, which was oxidized by Dess–Martin reagent to produce the corresponding ketone **4a**. Trifluoroacetic acid catalyzed cleavage of the *t*-Bu ester gave the free acid **5a** (Scheme 1). Other dipeptides **5b**–**t** were prepared similarly from *N*-protected Val **1b**–**t**.

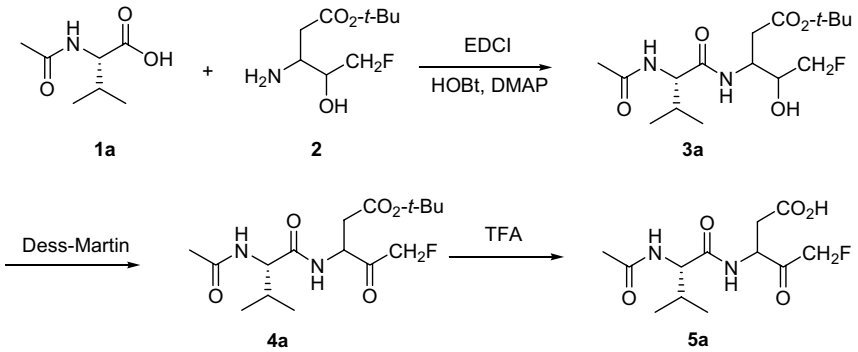
Propionyl-Val (**1b**)[22] was prepared by reaction of Val with propionyl chloride (**7b**) in NaOH aqueous solution (Scheme 2). *N*-protected Val **1c**–**f** were prepared similarly by reaction of Val with the corresponding commercial available acyl chloride.

Cyclopentylmethoxycarbonyl-Val (**1g**) was prepared in two steps (Scheme 3). Reaction of cyclopentylmethanol (**6g**) and phosgene in diethyl ether with *N*,*N*-diisopropylethyl amine produced cyclopentylmethyl chloroformate (**7g**),[23] which was reacted with Val in NaOH aqueous solution to produce **1g**. *N*-protected Val **1h**–**t** were prepared similarly in two steps starting from the corresponding commercial available alcohols.

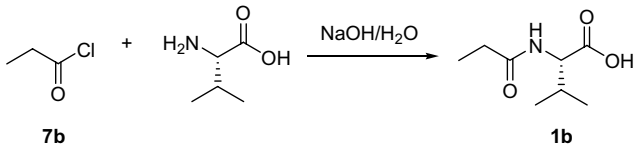
Benzyl-glutaryl-Val-Asp-fmk (**5u**) and glutaryl-Val-Asp-fmk (**5v**) was prepared as shown in Scheme 4. Glutaric acid monobenzyl ester (**8**)[24] was prepared from reaction of glutaric anhydride with benzyl alcohol. Acid **8** was coupled with Val-*O*-*t*-Bu in THF with EDC, HOBT and DMAP to produce benzyl-glutaryl-Val-*O*-*t*-Bu (**9**), and the *t*-Bu protecting group was removed by TFA to produce benzyl-glutaryl-Val (**10**). Benzyl-glutaryl-Val-Asp-fmk (**5u**) was prepared in three steps by coupling of acid **10** with amine **2** to give amide **11**, followed by oxidation to ketone **12** and then deprotection. Glutaryl-Val-Asp-fmk (**5v**) was prepared from benzyl-glutaryl-Val-Asp(*O*-*t*-Bu)-fmk (**12**) by deprotection of the benzyl and *t*-Bu groups in a AcOH solution of HBr.[25]

The activity of the dipeptides to inhibit human recombinant caspase-3 was determined using a standard fluorometric assay,[17,26] and the results are summarized in Table 1. Compound **5a** (Ac-Val-Asp-fmk) had an IC_{50} value of 250 nM in caspase-3, which was about eight-times less active than MX1013 (Cbz-Val-Asp-fmk), indicating that the Cbz protecting group contributes to the activity of MX1013. Compound **5b** and **5c** were found to be about 3- and 4-fold more potent than **5a**, suggesting that a larger group is preferred as the protecting group. Interestingly, compound **5d** is about 2-fold more potent than **5b**, and MX1013 is 3-fold more potent than compound **5e**, suggesting that an oxygen might be preferred over a CH_2 group as the linker. Compound **5f** and **5g** both have similar activity as that of MX1013, indicating that the Ph group can be replaced by a non-aromatic ring and a non-ring structure. Interestingly, compound **5h**, with a phenethyloxy group, was >3-fold less active than MX1013; while compound **5i**, with a phenylpropyloxy group, was found to be only slightly less potent than MX1013.

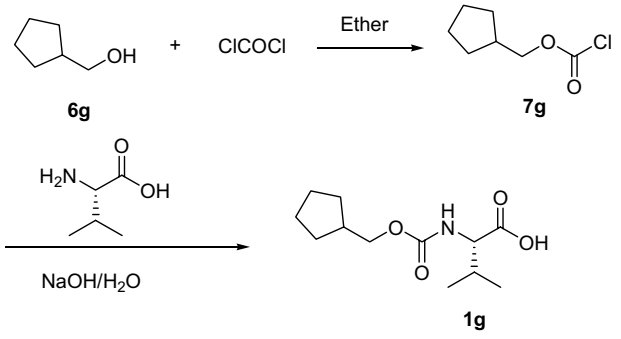
Since the preferred P_3 amino acid for caspase-3 is glutamic acid,[27] we also explored the incorporation of the side chain of glutamic acid into the protecting group. Compound **5v**, with the protecting group matching the side chain of glutamic acid, was found to be 5-fold more potent than **5a**, but slightly less active than MX1013,



Scheme 1.



Scheme 2.

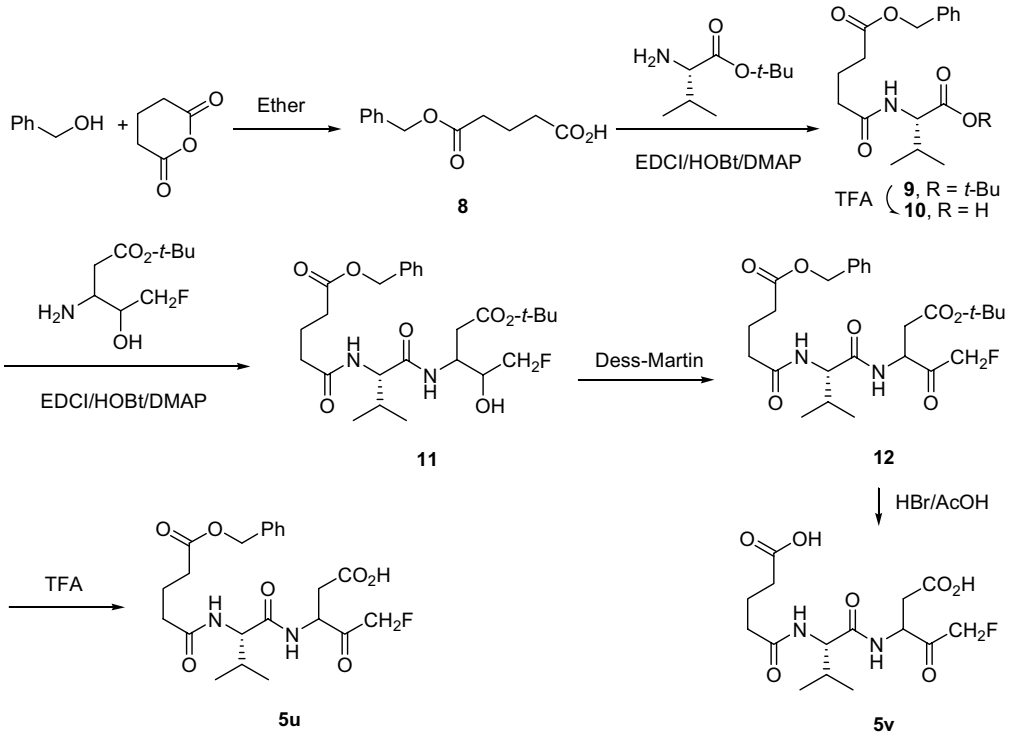


Scheme 3.

suggesting that the glutamic acid side chain may not be able to bind effectively to the P_3 subsite without part of the tripeptide backbone, probably due to the important hydrogen bond formed between the P_3 amide NH and the backbone carbonyl of Arg[207].[28] Interestingly, the benzyl protected compound **5u** was found to be about as potent as MX1013, indicating that a large group can be tolerated in that position.

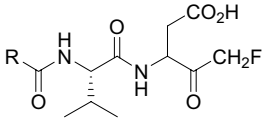
We then explored MX1013 analogs with chloro and fluoro substituted on the Ph of the Cbz group, with the goal of increasing the hydrophobicity of the compounds, which might make them more cell permeable. As shown in Table 1, the mono and dichloro, and mono and difluoro substituted analogs **5j**–**t** all have similar potency as MX1013 in the caspase-3 assay, with IC_{50} values ranged between 15 and 38 nM, indicating that the chloro and fluoro groups have little effects on caspase-3 activity.

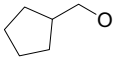
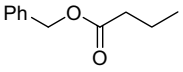
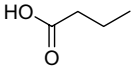
Several of these compounds were tested in the HeLa cell apoptosis protection assays.[17] The assays measure the protecting effects of caspase inhibitors against apoptosis induced by TNF-α in HeLa cells. The viability of the cells was quantified by uptake of calcein AM, and reported as the concentration of inhibitor that provides 50% of cell protection (Table 2). Compound **5d**, which is about as potent as MX1013 in the caspase-3 asaay, was found to be about half as potent as MX1013 in the cell protection assay, suggesting that the additional hydrophobicity of the Ph group in MX1013 might make the compound more cell permeable relative to compound **5d**. Compound **5g**, which has similar caspase-3 enzyme activity as that of **5d**, and has an additional cyclopentyl group comparing to **5d**, also was found to be more potent than **5d** in the cell protecting assay. Several compounds with fluoro or chloro substituted on the Ph of the Cbz group were found to be more active in the cell protecting assay than MX1013. Compound **5p** (MX1122), with a di-chloro substituted Ph group, was found to be >2-fold more potent than MX1013 in the cell protection assay, confirming that increasing hydrophobicity can increase cell permeability of these



Scheme 4.

Table 1. Caspase-3 inhibiting activity of the dipeptide inhibitors



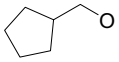
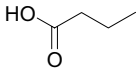
Entry	R	IC_{50}[a] (nM)
5a	CH_3	250
5b	CH_3CH_2	81
5c	$PhCH_2$	61
5d	CH_3O	37
5e	$PhCH_2CH_2$	98
5f		35
5g		30
5h	$PhCH_2CH_2O$	110
5i	$PhCH_2CH_2CH_2O$	46
5j	2-Cl–$PhCH_2O$	36
5k	3-Cl–$PhCH_2O$	36
5l	4-Cl–$PhCH_2O$	34
5m	2-F–$PhCH_2O$	38
5n	3-F–$PhCH_2O$	29
5o	4-F–$PhCH_2O$	28
5p	2,4-di-Cl–$PhCH_2O$	25
5q	3,4-di-Cl–$PhCH_2O$	21
5r	2,5-di-Cl–$PhCH_2O$	15
5s	2,4-di-F–$PhCH_2O$	35
5t	3,4-di-F–$PhCH_2O$	30
5u		33
5v		50
MX1013	$PhCH_2O$	30

[a] IC_{50} is determined as described in Ref. 17.

dipeptide inhibitors. Interestingly, compound **5v**, which has an extra carboxylic acid group and is slightly less active than MX1013 in the caspase-3 assay, was not active in the cell protection assays up to 2000 nM, at least 8-fold less active than MX1013, indicating that the extra acidic group results in major reduction of cell permeability.

Compounds **5p** was then tested against other caspases and proteases for selectivity and the results are summarized in Table 3. Compound **5p** was found to have similar activity in caspase-1, -3, -7, -8, and -9. Testing against other proteases, compound **5p** was found to be about 200-fold, 500-fold, and >5000-fold selective for caspase-3 versus Calpain-1, caspase-3 versus Cathepsin B, and caspase-3 versus Factor Xa, respectively. Therefore, similar to MX1013,[18] compound **5p** is a broad-spectrum caspase inhibitor and is selective for caspases versus other proteases. Since it is known that the P_4 amino acid in tetrapeptide caspase substrates is the key determinant of specificity between caspases,[27] dipeptide based caspase inhibitors, including MX1013 and compound **5p**, which do not have the P_4 and P_3 amino acids,

Table 2. Cell apoptosis protection activity and Caspase-3 inhibiting activity of the dipeptide inhibitors

Entry	R	Caspase-3 IC_{50} (nM)	50% Cell protection[a] (nM)
5d	CH_3O	37	500
5g		30	300
5o	4-F–$PhCH_2O$	28	150
5p	2,4-di-Cl–$PhCH_2O$	25	100
5q	3,4-di-Cl–$PhCH_2O$	21	150
5r	2,5-di-Cl–$PhCH_2O$	15	100
5v		50	>2000
MX1013	$PhCH_2O$	30	250

[a] Concentration of inhibitor that provided 50% of cell protection is determined as described in Ref. 17.

Table 3. Inhibiting activity of compound **5p** (MX1122) against different proteases

Enzyme	IC_{50}[a] (nM)
Caspase-1	26
Caspase-3	25
Caspase-7	10
Caspase-8	6
Caspase-9	9
Calpain-1	~5000
Cathepsin B	~10,000
Factor Xa	>100,000

[a] IC_{50} is determined as described in Ref. 17.

are expected to be broad-spectrum and inhibit all the caspases.

Compound **5p** also was tested in a mouse liver apoptosis model.[17,29] The liver apoptosis model, which is based on the fact that hepatocytes express high levels of cell death receptor Fas and can be activated by agonistic antibodies, is useful for the determination of the in vivo anti-apoptotic efficacy of caspase inhibitors. In this model, mice were injected IV with anti-Fas monoclonal antibody, followed 5 min later by IV administration of various doses of compound **5p** formulated in an aqueous vehicle containing 50 mM Tris–HCl. The number of surviving animals in each group was determined at 1, 3, 6, 24 h, and 5 days post-injection, and was expressed as a percentage of the total number of animals in each group. As shown in Table 4, mice injected with 8 μg of anti-Fas antibody and without treatment were all dead by the 1 h time point. In contrast, the lowest dose of compound **5p** tested (0.25 mg/kg) protected 100% of the mice from the lethal effects of anti-Fas antibody at the 1 and 3 h time point, and 83% (5/6), 33% (2/6), and 16% (1/6) at the 6, 24 h, and 5 days time point, respectively. At 0.5 mg/kg dose, the 24 h and 5 days time point protection was increased to 50% (3/6). At 1 and 10 mg/kg dose, **5p** protected 100% of the mice at all the time points, indicating that **5p** truly protected the mice from the initial injury caused by anti-Fas antibody and is highly active in this in vivo model of apoptosis. The activity of compound **5p**

Table 4. Survival of mice with compound **5p** (MX1122) treatment after induction of apoptosis by anti-Fas antibody

Dose of **5p** (mg/kg)	% Survival ($n = 6$)				
	1 h	3 h	6 h	24 h	5 days
0	100	0	0	0	0
0.25	100	100	83	33	16
0.5	100	100	83	50	50
1	100	100	100	100	100
10	100	100	100	100	100

Mice were injected intravenously (IV) with 8 μg of anti-Fas antibody (clone JO-2; Pharmingen), followed 5 min later with IV injection of compound **5p** at the indicated doses or by vehicle. Six mice were treated in each group and the number of surviving mice was monitored for up to 5 days.

in this model is similar to that of MX1013, which also provides 100% protection at all time points at dose of 1 mg/kg.

In conclusion, we have synthesized a group of *N*-protected Val-Asp-fmk and evaluated their activity as caspase-3 inhibitors. We found that the *N*-protecting group contributes to the potency of these caspase-3 inhibitors and many different groups are tolerated in that position. The introduction of chloro and fluoro groups in the phenyl ring of Cbz protecting group did not affect their potency as caspase-3 inhibitors. In the cell apoptosis protecting assays, compounds with more hydrophobic protecting group were found to be more active, suggesting that the increased hydrophobicity makes them more cell permeable. Compound **5p** (MX1122), 2,4-dichloro-Cbz-Val-Asp-fmk, was found to be a potent and broad-spectrum caspase inhibitor. It is selective for caspases among the proteases tested and is highly active in the cell apoptosis protection assays. MX1122 also was found to be highly efficacious in the mouse liver apoptosis model. A broad-spectrum caspase inhibitor such as MX1122 is expected to be useful for the treatment of acute diseases, such as brain ischemia and myocardial infarction, where it is desirable to have fast and efficient inhibition of all caspases.

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