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Discovery of novel aspartyl ketone dipeptides as potent and selective caspase-3 inhibitors

Yongxin Han,[a,*] André Giroux,[a] Erich L. Grimm,[a] Renée Aspiotis,[a] Sébastien Francoeur,[a] Christopher I. Bayly,[a] Daniel J. Mckay,[a] Sophie Roy,[b] Steve Xanthoudakis,[b] John P. Vaillancourt,[b] Dita M. Rasper,[b] John Tam,[b] Paul Tawa,[b] Nancy A. Thornberry,[c] Erin P. Paterson,[c] Margarita Garcia-Calvo,[c] Joseph W. Becker,[d] Jennifer Rotonda,[d] Donald W. Nicholson[b] and Robert J. Zamboni[a]

[a]*Department of Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research, Merck Frosst Canada & Co., PO Box 1005, Pointe-Claire-Dorval, Québec, Canada H9R 4P8*
[b]*Department of Biochemistry and Molecular Biology, Merck Frosst Centre for Therapeutic Research, Merck Frosst Canada & Co., PO Box 1005, Pointe-Claire-Dorval, Québec, Canada H9R 4P8*
[c]*Department of Enzymology, Merck Research Laboratories, Merck & Co., Inc., 126 E. Lincoln Ave., Rahway, NJ 07065, USA*
[d]*Department of Structure Biology, Merck Research Laboratories, Merck & Co., Inc., 126 E. Lincoln Ave., Rahway, NJ 07065, USA*

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Abstract—The discovery of a series of potent, selective and reversible dipeptidyl caspase-3 inhibitors are reported. The iterative discovery process of using combinatorial chemistry, parallel synthesis, moleculare modelling and structural biology will be discussed.

Recent studies towards understanding the molecular mechanisms of apoptosis have revealed the importance of a group of cysteinyl aspartate specific proteases, the caspases, in the programmed cell death process.[1] So far, 13 human members of caspases have been identified and these enzymes exist as dormant proenzymes which are processed to the catalytically active mature forms under certain conditions.[2] Caspases can be grouped into three subfamilies based on their substrate specificity[3] and cellular functions. Group I (1, 4, and 5) caspases are believed to be involved primarily in inflammation. Group II (2, 3, 6, and 7) and group III (2, 6, 8, 9, and 10) caspases are essential for apoptotic cell death. Caspase-3 (casp-3 for short), in particular, has been characterized as the dominant effector caspase involved in the proteolytic cleavage of a variety of protein substrates including cytoskeletal proteins, kinases and DNA repair enzymes during apoptosis.[2] Casp-3 knockout murine phenotype suggested the necessity of this enzyme during brain development[4] and recent studies have revealed its activation in many models of apoptosis.[5] The development of potent and selective casp-3 inhibitors has thus emerged as an attractive therapeutic target.[6]

Recent reports indicated that caspase inhibitors such as z-VAD-fmk, Boc-D-fmk and Ac-DEVD-fmk were effective in animal models of ischemia injury, burns, endotoximia, sepsis and neonatal hypoxia.[7,8] These inhibitors, however, are either irreversible pan-caspase inhibitors, inhibit other cysteine proteases,[9] or have poor whole cell activity (e.g., Ac-DEVD-CHO,[10] see Table 1) and in vivo stability.[11] In order to assess the importance of casp-3 activation in apoptosis, potent, selective and reversible inhibitors are highly desirable. In this communication, we will describe the iterative process that led to the discovery of such inhibitors.

Reversible $P_{1'}$ aldehyde and ketone casp-3 inhibitors incorporating DxVD, the preferred tetrapeptide motif recognized by casp-3, have been studied previously.[12] These compounds generally lack cell potency due to limited cell permeability. For example, Bz-DAVD-phenylpropyl ketone (**17**) was a potent inhbitor at rh-casp-3

* Corresponding author. Tel.: +1-514 428 3301; fax: +1-514 428 4900; e-mail: yongxin_han@merck.com

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Table 1. Inhibitor potency against casp-1, -3, -7 and -8 and in NT2 cells

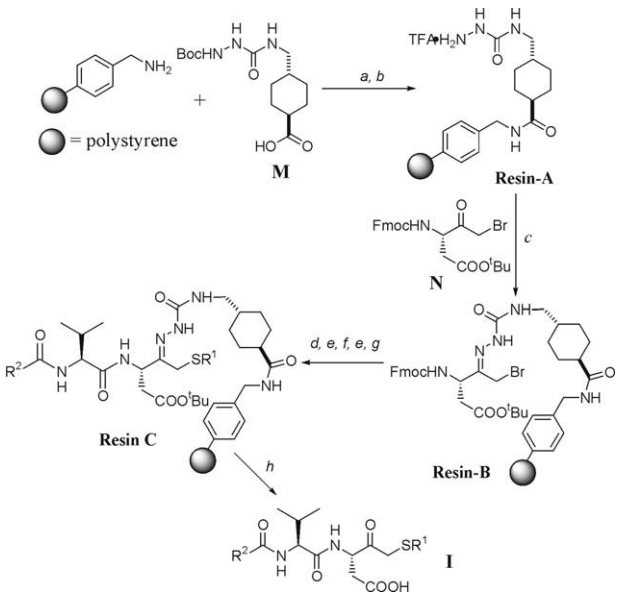
Compd	IC_{50}, μM[a]				
	Casp-1	Casp-3	Casp-7	Casp-8	NT2
1	6.0	0.048	3.2	6.6	10
2	0.86	0.14	1.5	8.7	63.0
3	2.6	0.083	1.4	3.3	55.0
4	2.6	0.13	1.8	7.8	32.0
5	5.0	0.073	2.5	2.4	9.0
6	1.8	0.063	>10	2	19.0
7	28	0.31	2.7	15.5	NT[b]
8	14	0.21	3.1	14	60.0
9	22	0.75	8.4	22.5	NT[b]
10	3.1	0.06	3.1	2.3	9.0
11	6.5	0.053	1.7	9.3	0.7
12	4.2	0.094	2.3	6.3	0.3
13	3.8	0.005	0.36	0.81	5.0
14	1.8	0.008	0.024	3.85	0.6
15	5.9	0.01	0.13	2.9	2.0
16	1.6	0.005	0.12	0.49	1.0
17	1.65	0.0018	0.0084	1.50	14.0
Ac-DEVD-CHO[c]	0.19	0.027	0.087	0.13	>100

[a] Average of 2–8 titrations.
[b] Not tested.

(IC_{50}, 1.8 nM) yet its potency was shifted dramatically in the whole cell assay (Table 1). Efforts to reduce the peptidic nature of these inhibitors by capping aspartic acid-phenylpropyl ketone with simple acids did not afford inhibitors with appreciable activity against recombinant human casp-3 (rh-casp-3). Preservation of the P_2 valine, however, was beneficial and resulted in inhibitors with high nano-molar potency, and thus represented a good starting point for inhibitor design.[13] In this communication, we will describe the development of two facile two-dimensional solid-phase combinatorial strategies that enabled the concomitant modification of both the $P_{1'}$ with heteroatom substitutions[14] and $P_2/P_3/P_4$ positions.

Central to this strategy was the direct attachment of bromomethyl ketone **N**[15] to semicarbazide resin-**A** in THF in presence of acetic acid as outlined in Scheme 1.[16] The semicarbazide linker **M**[17] serves the dual purpose of protecting the ketone functionality and anchoring the reactive bromide to solid support. This strategy simplifies significantly the synthetic sequence described in the original procedure by Webb and co-workers.[16] Nucleophilic displacement of the bromide with thiols in the presence of Hünig's base followed by standard Fmoc deprotection/coupling protocol for peptide synthesis afforded **Resin C**. The release of the final products from solid support was achieved by treatment with trifluoroacetic acid (TFA) in water (9/1, v/v) (instead of concentrated HCl).

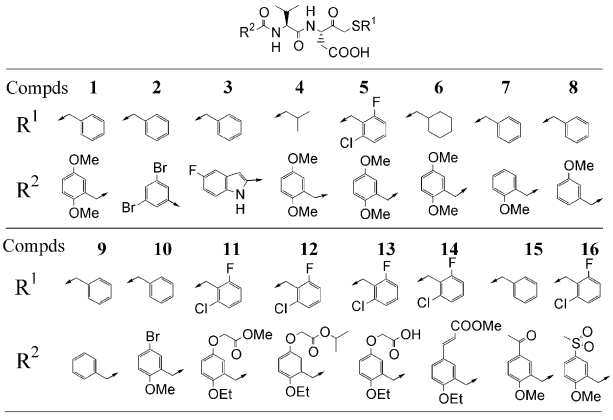
The initial library was constructed according to the split and pool protocol using the IRORI MacroKan® technology.[18] 10 Carboxylic acids (a–j) and 10 thiols (A–J) were chosen as building blocks (Fig. 1). The library rapidly identified the benzylthio (thiol D) and the isobutylthio (thiol J) moieties as the preferred groups in the $P_{1'}$ position while 2,5-dimethoxyphenyl-acetate (**1**), 3,5-dibromo-benzoate (**2**) and 5-F-indole-2-carboxylate (**3**) were the best capping acids at P_3–P_4. Four of the most



Scheme 1. General strategy for preparing dipeptide of structure **I**. Reagents and conditions: (a) EDCI, HOBt, DMF; (b) TFA, DCM; (c) AcOH, THF; (d) R^1SH, iPr_2NEt, DMF; (e) 20% piperidine in DMF; (f) Fmoc-V-OH, HATU, iPr_2NEt, DMF; (g) R^2COOH, HATU, iPr_2NEt, DMF; (h) TFA, H_2O (9:1 v/v).

potent compounds were re-synthesized and their inhibition against casp-1, -3, -7, and -8 determined (Table 1). All of the inhibitors were shown to be selective, fully reversible and competitive against rh-casp-3. Compound **1**, however, was the most potent among the four in the NT2 whole cell assay[19] with an IC_{50} of 10 μM while **2**, **3**, and **4** were less potent.

The enhanced cell potency of **1** can be attributed to its improved cell permeability (vs tetrapeptide **17**, Table 1). Using tritiated analogue ^{3}H-**1** and ^{3}H-**17** (prepared from the corresponding Br analogues), we were able to show that the intracellular concentration of **1** in NT2 cells was 6–20-fold higher than **17** (Fig. 2) (similar results were obtained in caco-2 monolayers).



Following the discovery of **1**, extensive SAR studies at the $P_{1'}$ position with more than 100 thiols were carried out using the ACT-496™ automated synthesizer and the best results were obtained with lipophilic thiols such as benzylthio, substituted benzylthio and cyclohexyl-methylthio

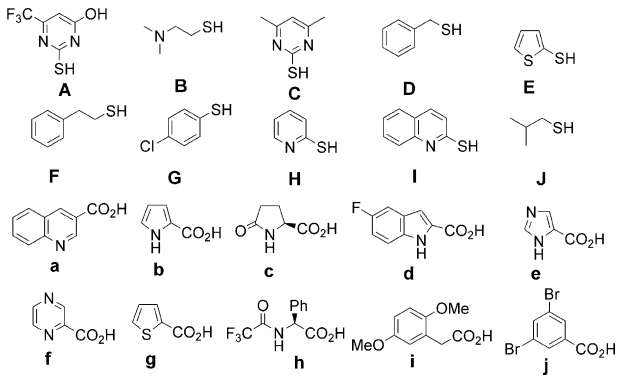


Figure 1. Building blocks for library 1 (**L-1**).

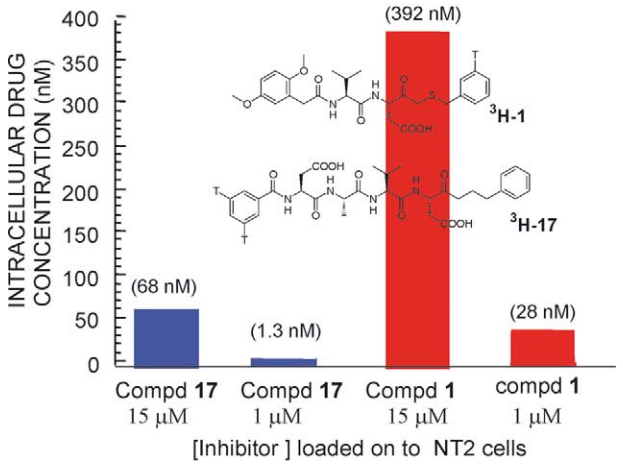
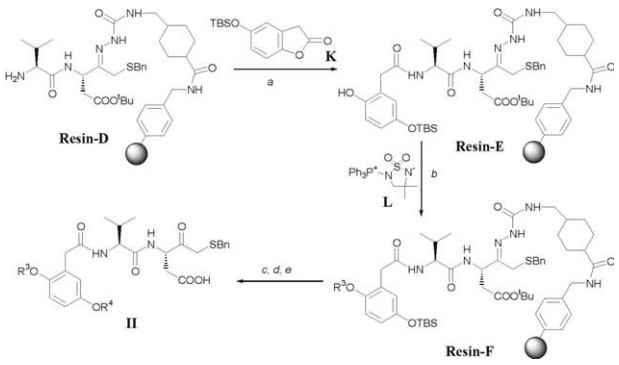


Figure 2. Cell permeability studies with tritiated compounds.

groups. In an another attempt, the 2,5-dimethoxyphenylacetate moiety was replaced with a variety of acids (substituted phenylacetate, benzoate, phenylpropionate, naphthoate, cinnamate, hetero-aromatic, aliphatic, etc.) and no significant improvement was obtained. We instead focused on the modification of both methoxy groups. We observed that both groups were required for optimal potency. Removal of either of the methoxy group resulted in 5-fold loss of activity (**7** and **8**) and removal of both groups gave compound **9** which was 15-fold less active. To further address the SAR at the two OMe positions, a two-dimensional library was constructed as outlined in Scheme 2.

This library was constructed using 10 alcohols at OR^3 and 50 alcohols at OR^4. Reaction of **resin-D** with lactone **K** in the presence of DMAP in DMF at 50 °C overnight gave phenolic intermediate **resin-E**. The phenol moiety thus generated was reacted with an alcohol R^3OH using reagent **L** as described by Castro et al.,[20] affording **resin-F**. After deprotection of the TBS group with TBAF and AcOH, the second phenolic OH was reacted with a second alcohol R^4OH under identical conditions and the final product **II** was released from solid support upon treatment with TFA in water.

A three-point titration (0.1, 1, and 10 μM) of this library in the NT2 whole cell assay quickly revealed that while a variety of groups were tolerated at OR^3, the most potent



Scheme 2. Modification of 2,5-dimethoxyphenylacetate moiety. Reagents and conditions: (a) reagent K, DMAP, DMF, 50 °C; (b) R^3OH, reagent L, toluene/DCM; (c) TBAF, AcOH, THF; (d) R^4OH, **L**, toluene/DCM; (f) TFA, H_2O.

compounds in the NT2 assay contained glycolate moieties at OR^4 (**11** and **12**). Furthermore, these compounds were not significantly shifted (intrinsic to whole cell) in comparison to other compounds tested. We speculated that the ester was acting as the pro-drug and was hydrolyzing to the corresponding acid **13** intracellularly. This was confirmed by incubating ^{3}H-**11** (prepared similarly as described for ^{3}H-**1**) in NT2 cells at 37 °C for 0.5 h. HPLC analysis revealed that the extracellular culture medium contained both ester **11** and acid **13**, while the intracellular content was exclusively acid **13**. Compound **13** was tested against rh-casp-3 and was found to be potent (IC_{50}: 5 nM) yet highly shifted in the NT2 assay (5 μM). Therefore, the enhanced whole cell activity of **11** and **12** was due to their improved cell permeability.

Modelling of acid **13** into the active site of rh-casp-3 suggested that the carboxylate residue of the glycolate would be interacting with the two neutral NH bonds from residues Phe381b and Asn342 in the S_4 pocket, similarly to the P_4 Asp of substrate Ac-DEVD-CHO. This was consistent with the X-ray structure of compound **10**,[21] the bromo analogue (Fig. 3). This structure

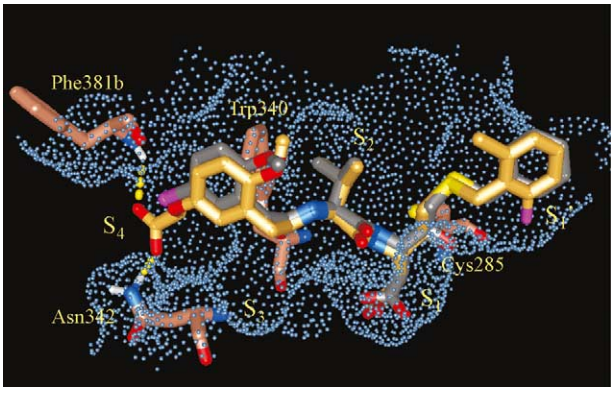


Figure 3. Compound **13** (gold) modelled into the active site of the X-ray structure of the complex between compound **10** (grey) and rh-casp-3 (pink); the two inhibitors virtually superimpose for their common structure from P_1 to P_3. In S_4, yellow dotted lines show the hydrogen bonding between the glycolate of compound **10** and the protein (Asn342 side chain and Phe381b main chain), the same hydrogen bonding present in the substrate Ac-DEVD-CHO. Above S_3, behind the plane of the figure, the edge of the Trp340 indole ring can be seen making an edge-to-face interaction with the phenyl ring for both compounds **10** and **13**.

confirmed that the $P_{1'}$, P_1, and P_2 residues of **10** bound similarly to tetrapeptide inhibitors.[11] The edge-to-face interaction of the phenyl group with Trp340 probably contributes to the enhanced potency of the phenylacetate moiety. The bromine atom was pointing straight to the S_4 pocket where we propose that the glycolate will be localized.

Extensive efforts were then directed towards finding a suitable neutral glycolate replacement at OR^4. A methyl ketone was found to be an effective replacement, giving compound **15** with good intrinsic activity against rh-casp-3 (IC_{50}: 0.01 μM) and reasonable whole cell potency (IC_{50}: 2 μM). Modelling suggested that a methyl sulfone moiety would make excellent interactions with the two neutral amide NH's at the S_4 pocket of casp-3. Indeed, compound **16** was found to have excellent intrinsic and whole cell potency with IC_{50}'s of 0.005 and 1 μM, respectively. As illustrated in the Table, compound **16** was also selective against other caspases tested.

In summary, a series of potent and selective casp-3 inhibitors were discovered employing iterative combinatorial strategies. The selectivity and potency profile of compounds such as **16** makes it a useful tool for studying the importance of casp-3 activation in cell based systems.

References and notes

1. Hengartner, M. O. *Nature* **2000**, *407*, 770.
2. Nicholson, D. W. *Cell Death Differ*. **1999**, *6*, 1028.
3. Thornberry, N. A.; Rano, T. A.; Peterson, E. P.; Rasper, D. M.; Timkey, T.; Garcia-Calvo, M.; Houtzager, V. M.; Nordstrom, P. A.; Rpy, S.; Champman, K. T.; Nicholson, D. W. *J. Biol. Chem*. **1997**, *272*, 11907.
4. Kuida, K.; Zheng, T. S.; Na, S.; Kuan, C.; Yang, D.; Karasuyama, H.; Rakic, P.; Flavell, R. H. *Nature* **1996**, *384*, 368.
5. Porter, A. G.; Janicke, R. U. *Cell Death Differ*. **1999**, *6*, 99.
6. Talanian, R. V.; Brady, K. D.; Cryns, V. L. *J. Med. Chem*. **2000**, *43*, 3351.
7. Hotchkiss, R. S.; Chang, K. C.; Swanson, P. E.; Tinsley, K. W.; Hui, J. J.; Klender, P.; Xantoudakis, S.; Roy, S.; Black, C.; Grimm, E.; Han, Y.; Nicholson, D. W.; Karl, I. E. *Nat. Immun*. **2000**, *1*, 496, and references cited therein.
8. Cheng, Y.; Deshmukh, M.; D'Costa, A.; Demaro, J. A.; Gidday, J. M.; Shah, A.; Sun, Y.; Jacquin, M. F.; Johnson, E. M.; Holtzman, D. M. *J. Clin. Invest*. **1998**, *101*, 1992.
9. Schotte, P.; Declercq, W.; van Huffel, S.; Vandenabeele, P.; Beyaert, R. *FEBS Lett*. **1999**, *442*, 117.
10. Nicholson, D. W.; Ali, A.; Thornberry, N. A.; Vaillancourt, J. P.; Ding, C. K.; Gallant, M.; Gareau, Y.; Griffin, P. R.; Labelle, M.; Lazebnik, Y. A.; Munday, N. A.; Raju, M. S.; Smulson, M. E.; Yamin, T.-T.; Yu, V. L.; Miller, D. K. *Nature* **1995**, *376*, 37.
11. Isatin analogues were reported to be selective caspase3/7 inhibitors (see: Lee, D.; Long, S. A.; Adams, J. L.; Chan, G.; Vaidya, K. S.; Francis, T. A.; Kirly, K.; Winkler, J. D.; Sung, C.-M.; Debouck, C.; Richardson, S.; Levy, M. A.; DeWolf, W. E., Jr.; Keller, P. M.; Tomaszek, T.; Head, M. S.; Ryan, M. D.; Haltiwanger, R. C.; Liang, P.-H.; Jan, C. A.; McDevitt, P. J.; Johanson, K.; Concha, N. O.; Chan, W.; Abdel-Meguid, S. S.; Badger, A. M.; Lark, M. W.; Nadeau, D. P.; Suva, L. J.; Gowen, M.; Nutall, M. E. *J. Biol. Chem*. **2000**, *275*, 16007). These compounds have potential drawbacks due to the high reactivity of the ketone carbonyl group.
12. Grimm, E.; Renaud, J.; Aspiotis, R.; Zamboni, R. WO 00/3262, June 8, 2000; *Chem. Abstr*. 2002:618121.
13. Aspiotis, R.; Bayly, C. I.; Becker, J.; Ethier, D.; Francoeur, S.; Margarita, G.-C.; Giroux, A.; Grimm, E.; Han, Y.; McKay, D. J.; Nicholson, D. W.; Paterson, E.; Rasper, D.; Renaud, J.; Retonda, J.; Roy, S.; Tam, J.; Tawa, P.; Thornberry, N.; Vaillancourt, J.; Xanthoudakis, S.; Zamboni, R. *Abstracts of Papers*, 224th ACS National Meeting, Boston, MA, USA, Aug. 18–22, 2002.
14. (a) It was reported previously that heteroatom substitutions at the $P_{1'}$ position greatly affected the activity, selectivity and reversibility of casp-1 inhibitors; see: Brady, K. D.; Giegel, D. A.; Grinnell, C.; Lunney, E.; Talanian, R. V.; Wong, W.; Walker, N. *Bioorg. Med. Chem*. **1999**, *7*, 621. (b) Graczyk, P. P. *Restorative Neurol. Neurosci*. **1999**, *14*, 1, and references cited therein.
15. Ketone **N** was prepared according to the literature procedure, see: Dolle, R. E.; Hoyer, D.; Prasad, C. V. C.; Schmidt, S. J.; Helaszek, C. T.; Miller, R. E.; Ator, M. A. *J. Med. Chem*. **1994**, *37*, 563.
16. A similar strategy using a carbazate linker has been reported, see: Lee, A.; Huang, L.; Ellman, J. A. *J. Am. Chem. Soc*. **1999**, *121*, 9907.
17. For the preparation of the Boc protected Webb linker 1, see: Webb, T. R. *J. Am. Chem. Soc*. **1992**, *114*, 3156.
18. Nicolaou, K. C.; Xiao, X.-Y.; Parandoosh, Z.; Senyei, A.; Nova, M. P. *Angew. Chem., Int. Ed. Engl*. **1995**, *34*, 2289.
19. The potency of compounds to inhibit camptothecin induced apoptotic cell death in the neuronal precursor (NT2) cells was determined as described by the testing kit from Roche Molecular Biochemicals, Cat. No. 1,920,685.
20. Castrol, J. L.; Matassa, V. G.; Ball, R. G. *J. Org. Chem*. **1994**, *59*, 2289.
21. Becker, J. W.; Rotonda, J.; Margarita, G.-C.; Bayly, C. I.; McKay, D. J.; Paterson, E.; Thornberry, N.; Giroux, A.; Grimm, E.; Han, Y.; Francoeur, S.; Renaud, J. *J. Med. Chem*., submitted for publication.