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Oxamyl dipeptide caspase inhibitors developed for the treatment of stroke

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Abstract—Structural modifications were made to a previously described acyl dipeptide caspase inhibitor, leading to the oxamyl dipeptide series. Subsequent SAR studies directed toward the warhead, P2, and P4 regions of this novel peptidomimetic are described herein.

The caspases[1] are a family of cysteine proteases with aspartic acid specificity at P1 involved in both cytokine maturation and apoptosis.[2] Caspase 1 (interleukin-1β converting enzyme, ICE) is involved in the induction of inflammation by catalyzing the cleavage of the pro-form of IL-1β. Other caspases may play a role in the regulation of apoptosis, either as apoptosis signaling molecules or as downstream effectors. Inhibition of caspases, either broad spectrum or caspase specific, would be of therapeutic value[3] in the treatment of inflammatory and degenerative diseases, such as rheumatoid arthritis, ALS, Alzheimer's disease, Parkinson's disease, and stroke. Up to now there have been few reports in the literature on caspase inhibitors, other than a broad body of work associated with caspase 1.[3]

Our aim was to develop a series of novel, peptidomimetic, broad spectrum caspase inhibitors with low nanomolar activity. These compounds could then be optimized and developed as therapeutics for CNS disorders, such as stroke, traumatic brain injury, and others.

A series of acyl dipeptide aldehydes was previously prepared[4] as peptidomimetic caspase inhibitors. The naphthyloxyacetyl dipeptide **1** showed broad spectrum caspase inhibition[5] (Table 1). Results from our modeling studies suggest that the aryl ether oxygen of compound **1** may occupy the space occupied by the P3 amide-NH of tetrapeptide inhibitors.[6] Replacement of the ether oxygen in compound **1** by NH (**2**) led to a dramatic enhancement in potency against the caspases tested[7] (Table 1).

Conversion of the methylene in the amino-amide moiety of compound **2** to a carbonyl generated the oxamide **3**.[8] Of the three analogues, the oxamide **3** showed the best enhancement in potency against caspases 1, 3, and 9. Compound **3** was also significantly more potent than the parent ether analogue (**1**) in the monocyte[8,9] assay (Table 1). Due to this improved cellular activity, along with the observed relative chemical instability of analogues such as amine **2**, an SAR study was initiated on the oxamyl dipeptide series of caspase inhibitors.

Our first approach was to prepare various irreversible warhead analogues using both valine and leucine at P2,[10] based on our previous work with the acyl dipeptide series[5,7,11] (Scheme 1). The term 'warhead' is used here to describe the electrophilic moiety that covalently interacts

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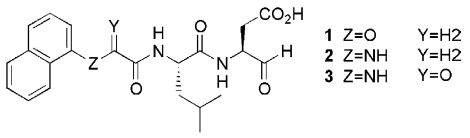
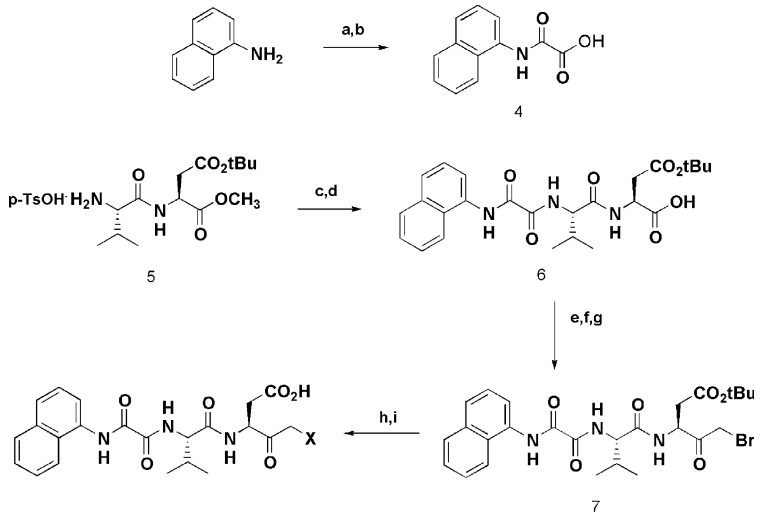
Table 1. Transition from acyl dipeptide to oxamyl dipeptide



Compd	Enzyme assays[a] IC_{50} (μM)					Monocyte assay
	mCsp-1[b]	Csp-3	Csp-6	Csp-8	Csp-9	% inhib @ 10 μM
1	0.570	0.135	0.940	0.770	2.72	17%
2	0.033	0.013	0.037	0.007	0.160	18%
3	0.027	0.010	1.50	0.179	0.230	79%[c]

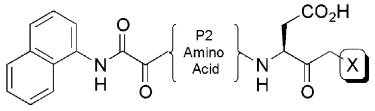
[a]Assay conditions are described in Refs. 8 and 14.
[b]Murine caspase-1 enzyme. Other enzyme and cellular data are from human.
[c]$IC_{50} = 3.08$ μM.



Scheme 1. Reagents and conditions: (a) Methyl chlorooxoacetate (1.1 equiv), triethylamine (TEA) (1.1 equiv), 0 °C to room temperature (rt), 1 h; (b) 1 N LiOH (1.2 equiv), 1,4-dioxane, rt, 1 h, 78% overall; (c) **4** (1 equiv), HATU (1 equiv), DIEA (3 equiv), *N*-methylpyrolidinone (NMP)/CH_2Cl_2 (1:1), rt, 2 h, 73%; (d) 1 N LiOH (1.02 equiv), 1,4-dioxane, rt, 1 h; (e) isobutyl chloroformate (1.1 equiv), N–Me morpholine (NMM) (1 equiv), THF, −10 °C, 30 min; (f) 1.5 equiv CH_2N_2/Et_2O, 0 °C, 1 h, 62%; (g) HBr (aq 48%) THF, 0 °C, 15 min; (h) Nucleophile (X), KF (3 equiv), DMF, rt 16 h; (i) TFA/CH_2Cl_2/anisole, (1:1:0.5), rt, 1 h.

with the active site of the enzyme and its prime side substitution. These analogues were prepared[8] by acylation of 1-aminonaphthalene with methyl chlorooxoacetate, then basic hydrolysis to yield the oxamic acid, **4**. Coupling to the *p*-tosylate salt of amine **5**[8] using HATU followed by basic hydrolysis gave the oxamyl dipeptide **6**. Three-step conversion to the bromomethyl ketone employing diazomethane[12] afforded the key intermediate **7**. This compound could then be reacted with the nucleophile of choice employing modified Finklestein conditions,[13] followed by acidic hydrolysis of the *tert*-butyl ester to yield the inhibitors (Tables 2 and 3).

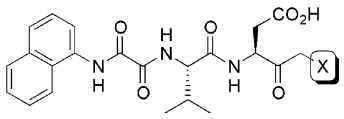
Table 2. Warhead SAR, valine versus leucine



Compd	X	P2	Csp-3 k_3/K_i (M^{-1} s^{-1})	Csp-8 k_3/K_i (M^{-1} s^{-1})	Cellular assays[a] IC_{50} (μM)	
					Con A	Jurkat
8	OCO(2,6-Cl_2-Ph)	Leu	282,834	112,166	0.50	1.3
9	OCO(2,6-Cl_2-Ph)	Val	740,466	119,197	0.35	0.0043
10	O(2,3,5,6-F_4-Ph)	Leu	180,453	177,219	2.58	2.5
11	O(2,3,5,6-F_4-Ph)	Val	288,748	447,761	0.55	0.0061
12	O(2,4,6-F_3-Ph)	Leu	85,300	145,011	4.61	0.535
13	O(2,4,6-F_3-Ph)	Val	340,398	189,358	0.30	0.0052

[a]Assay conditions are described in Refs. 8 and 14.

Table 3. Warhead SAR, P2=valine



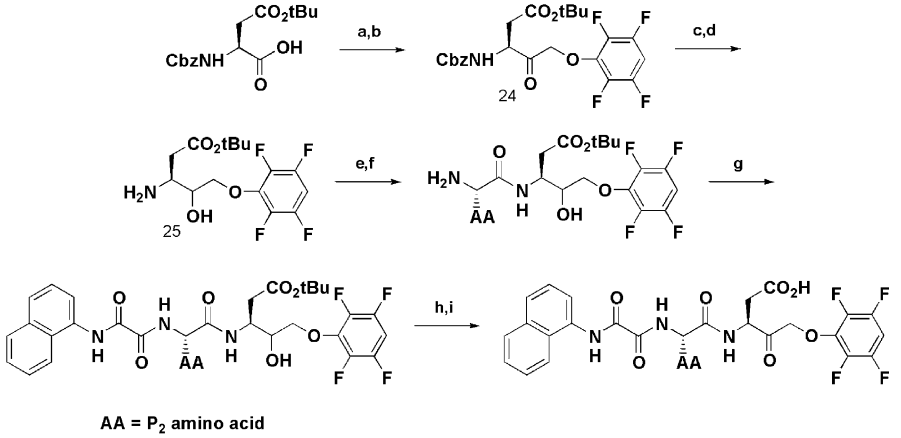
Compd	X	Enzyme assays[a] k_3/K_i (M^{-1} s^{-1})				Cellular assays[a] IC_{50} (μM)	
		mCsp-1[b]	Csp-3	Csp-6	Csp-8	THP-1	Con A
9	OCO(2,6-Cl_2-Ph)	1,052,775	740,466	234,318	119,197	0.52	0.35
11	O(2,3,5,6-F_4-Ph)	892,596	288,748	142,903	447,761	0.770	0.55
13	O(2,4,6-F_3-Ph)	879,183	340,398	37,438	189,358	0.40	0.30
14	$OPOPh_2$	2,950,000	876,478	222,420	2,447,090	0.65	0.65
15	$OPO(CH_3)Ph$	1,404,853	529,376	165,406	847,197	0.09	2.00
16	O(2,6-F_2-Ph)	1,295,045	492,401	49,970	311,465	0.48	0.45
17	O(2-CF_3-4-pyrimidyl)	1,069,270	293,818	13,349	60,347	0.25	2.00
18	O(5-CO_2CH_3-3-isoxazyl)	532,101	264,194	12,805	88,785	0.28	9.00
19	OSOPh	2969	342	459	1	8.30	>50
20	O(2-naphthyl)	155,894	9455	31	20	1.93	7.30
21	O(1-naphthyl)	378,200	11,653	268	12	3.06	2.70
22	O-Ph(4-Ph)	58,032	1659	0	0	1.12	>50

[a] Assay conditions are described in Refs. 8 and 14.
[b] Murine caspase-1 enzyme. Other enzyme data are from recombinant human.

Given our experience with irreversible inhibitors and their ability to block cell death in cellular models of apoptosis,[11,14] we focused primarily on warheads that were potentially irreversible in nature.[11,12] The results of this SAR (Table 2) indicated that valine at P2 was better than leucine, especially with respect to cellular activity. Some of these derivatives exhibited extremely potent activities for inhibition of cell death in the Jurkat cellular assay.[9] Additional warhead analogues were then prepared, using only valine at P2 (Table 3).

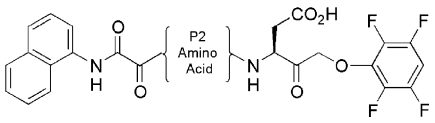
The dichlorobenzoyloxy (**9**),[12b] polyfluorophenoxy (**11**, **13**), and diphenylphosphinyl (**14**)[15] warhead analogues in particular exhibited excellent broad spectrum enzymatic and cellular activities (see Table 3). From these analogues, the 2,3,5,6-tetrafluorophenoxy warhead (**11**) was selected for further study.

Additional P2 analogues of the tetrafluorophenoxy warhead were prepared as shown in Scheme 2.[8] Commercially available *Z*-Asp(O-*t*Bu)-OH was converted to the bromomethyl ketone.[12] Displacement with the potassium salt of 2,3,5,6-tetrafluorophenol gave compound **24**. Reduction of the ketone and hydrogenolysis afforded the key intermediate **25**, which could then be coupled to the protected amino acid of choice. Deprotection of the amine and coupling to the oxamic acid **4** yields an oxamyl dipeptide alcohol. Dess–Martin oxidation and subsequent hydrolysis of the *tert*-butyl ester gave the series of analogues in Table 4. The SAR of this set of analogues was evaluated as before by comparing broad spectrum caspase inhibition and activity in cellular models. The valine derivative was optimal when considering cellular activity. From the perspective of broad spectrum caspase inhibition and cellular activity,



Scheme 2. Reagents and conditions: (a) (i) isobutyl chloroformate (1.2 equiv), NMM (1.1 equiv), THF −10 °C, 20 min; (ii) 3 equiv CH_2N_1/Et_2O, 0 °C, 15 min; (iii) HBr (aq 48%) THF, 0 °C, rt, 30 min, used crude; (b) 2,3,5,6-tetrafluorophenol, (1.14 equiv), KF (3.2 equiv), DMF, rt, 16 h, 95%; (c) $NaBH_4$ (0.74 equiv) EtOH, 0 °C, 1 h, 94%; (d) H_2 (1 atm), 10% Pd/C, MeOH, rt, 4 h, 93%; (e) Fmoc or Cbz amino acid, HATU (1.2 equiv), DIEA (2 equiv), CH_2Cl_2, rt, 16 h; (f) 10% piperidine/DMF, rt, 1 h or H_2 (1 atm), 10% Pd/C, MeOH, rt, 4 h; (g) **4** (1.5 equiv), HATU (1.2 equiv), DIEA (2 equiv), CH_2Cl_2, rt, 16 h; (h) Dess–Martin periodinane (2.8 equiv), CH_2Cl_2, rt, 30 min; (i) TFA/CH_2Cl_2/anisole (1:1:0.2), rt, 30 min.

Table 4. P2 SAR



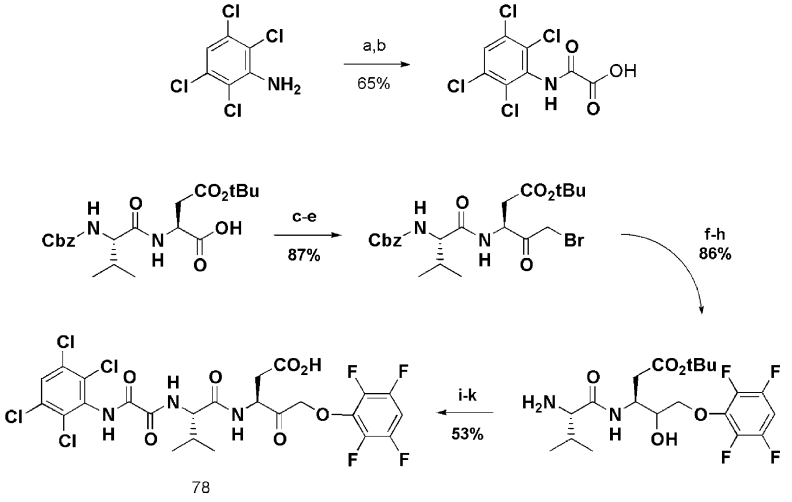
Compd	Amino acid	Enzyme assays[a] k_3/K_i (M^{-1} s^{-1})				Cellular assays[a] IC_{50} (μM)	
		mCsp-1[b]	Csp-3	Csp-6	Csp-8	THP-1	Con A
11	Valine	892,596	288,748	142,903	447,761	0.77	0.55
10	Leucine	253,514	180,453	83,651	177,219	2.13	2.58
26	Homoproline	35,955	9849	11,557	19,481		16
27	Cyclohexyl glycine	1,220,081	153,367	170,466	388,557	1.44	1.5
28	Phenylalanine	1,285,268	42,326	136,846	1,025,631	1.26	2.7
29	*tert*-Butyl glycine	2,323,255	98,551	160,076	456,491	0.27	2.9
30	(4,5-Benzo)proline	912	511	5583	1136	22.14	50
31	Phenyl glycine	1,075,916	275,734	229,873	1305,449	2.77	0.5
32	norLeucine	2,571,212	148,881	174,831	1,834,206	1.49	1
33	homoPhenylalanine	2,680,884	62,940	1,082,958	1,826,721	7.53	3
34	*t*Bu-alanine	1,537,859	96,254	160,779	234,028	0.84	2
35	Aminocyclopentane carboxylic acid	767,316	70,631	8227	51,317	0.93	2.5

[a] Assay conditions are described in Refs. 8 and 14.
[b] Murine caspase-1 enzyme. Other enzyme data are from human.

the P2 *tert*-butyl and phenyl glycine analogues (**29** and **31**) were quite potent. Other bulky P2 amino acids, such as phenylalanine and homophenylalanine (**28** and **33**) showed potent caspase inhibition but limited cellular activity (Table 4).

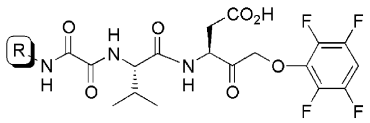
The synthesis of compound **78** is outlined in Scheme 3 as an example of how the analogues in Table 5 were prepared.[8] This involves preparation of the oxamic acid, using methyl chlorooxoacetate and the amine of choice, followed by ester hydrolysis. The valinyl-aspartyl tetrafluorophenoxy methyl ketone portion of the inhibitor is prepared by converting *Z*-Val-Asp(O*t*Bu)-OH to its bromomethyl ketone derivative, followed by substitution with the potassium salt of 2,3,5,6-tetrafluorophenol. After reduction of the ketone and deprotection of the amine, the requisite oxamic acid is then coupled. Dess–Martin oxidation of the alcohol and subsequent *tert*-butyl ester hydrolysis yields the oxamyl dipeptide.

This SAR study involved an extensive investigation of the oxamyl terminal group effect on in vitro activity. The results of this P4 SAR are summarized in Table 5. As can be seen from the table, several analogues exhibited potent in vitro activities and were considered candidates for in vivo analysis. Alkyl oxamides showed modest activity; the *t*-octyl and adamantyl analogues (**38** and **40**) indicated a preference for bulk, although the methyl



Scheme 3. Reagents and conditions: (a) methyl chlorooxoacetate (1.1 equiv), (TEA) (1.1 equiv), 0 °C, rt, 1 h; (b) 1 N LiOH (1.2 equiv), 1,4-dioxane, rt, 1 h, (65%, two steps); (c) isobutyl chloroformate (1.1 equiv), (NMM) (1 equiv), THF, −10 °C, 30 min; (d) 1.5 equiv CH_2N_2/Et_2O, 0 °C, 1 h; (e) HBr (aq 48%) THF, 0 °C, rt, 15 min (87% three steps); (f) KO(2,3,5,6-Fr)Ph, NaI, acetone, rt; (g) $NaBH_4$; MeOH/THF, 0 °C; (h) H_2 (1 atm), 10% Pd/C, MeOH, rt, 4 h; (i) (2,3,5,6-Cl_4-Ph)NHCOCOOH (1.5 equiv), HATU (1.2 equiv), DIEA (2 equiv), CH_2Cl_2, rt, 2 h; (j) Dess–Martin (1.25 equiv), CH_2Cl_2, rt, 15 min; (k) TFA/CH_2Cl_2/anisole, (1:1:0.5), rt, 1 h.

Table 5. P4 SAR



Compd	R	Enzyme assays[a] K_3/K_i (M^{-1} s^{-1})				Cellular assays IC_{50} (μM)			
		mCsp-1[b]	Csp-3	Csp-6	Csp-8	Jurkat	THP-1	Con A	SKW 6.4
36	Me	16,846	149,284	126,292	21,165	0.11	1.23	7	1.04
37	*n*-Heptyl	87,791	47,953	52,600	21,407	1.28	7.71	7	7.80
38	*t*-Octyl	241,885	243,190	444,855	643,095	0.013	1.51	2	0.41
39	Cyclohexyl	126,294	164,873	142,818	92,584	0.06	0.72	6.12	1.78
40	Adamantyl	171,800	68,859	128,669	154,580	1.76	1.79	3.5	0.72
41	Ph	158,722	327,155	274,616	281,943	0.43	0.07	1.5	0.94
42	$PhCH_2$	259,386	388,350	218,579	124,16	0.0771	0.57	1.5	0.91
43	3,4,5-Trimethoxybenzyl	314,557	156,637	119,655	71,761	1.55	3.5	9	18.33
44	1-Naphthyl-CH_2	166,660	55,558	35,050	37,831	0.52	0.37	0.8	1.59
45	$PhCH_2CH_2$	345,243	243,319	317,122	206,906	4.7	0.08	3.5	0.61
46	Ph_2CH	441,760	67,743	54,920	119,087	>200	0.17	7.3	
11	1-Naphthyl	892,596	288,748	142,903	447,761	0.0061	0.77	0.5	1.61
47	2-Naphthyl	625,087	395,393	483,945	588,771	0.0007	0.7	3.28	0.38
48	1,2,3,4-H_4-1-naphthyl	46,041	20,525	21,079	18,719	0.37	2.91	2.4	0.86
49	5,6,7,8-H_4-1-naphthyl	904,928	138,864	132,427	1,150,059	0.175	1.10	2	1.07
50	(1-Naph)(Me)	236	2	78	0	>100		>50	>100
51	4-Cl-1-naphthyl	18,802	4282	2319	9825	0.03	0.61	0.75	2.45
52	1-Anthryl	17,606,236	260,083	263,666	1,917,858	0.01	0.22	0.85	1.96
53	2-Anthryl	43,267	141,709	191,060	39,064	0.15	0.66	0.55	0.75
54	(2-Ph)-Ph	2,593,143	158,011	335,251	1,269,323	0.2	0.16	2.5	0.48
55	2-Phenoxy-Ph	1,502,281	78,594	202,138	933,186	0.66	1.77	2.5	1.78
56	3-Phenoxy-Ph	190,759	244,710	185,911	82,742	1.15	1.85	1.1	2.00
57	2-Methoxy-Ph	369,375	100,239	98,510	441,478	0.31	0.4	2.5	1.20
58	2-Benzyl-Ph	1,156,360	187,179	213,969	935,882	0.0090	0.18	2	1.56
59	Ph_2-N	1,234,278	168,506	130,873	666,672	0.19	0.78	4.87	2.24
60	1-Ph,1-Bz-N	5,594,712	104,026	123,918	526,289	0.07	0.63	2.3	2.01
61	Ph-NH	59,480	113,104	143,499	70,373	2.35	6.58	20	9.90
62	$PhCH_2O$	36,278	78,333	66,233	46,344	30.8	>32	15	
63	5-Indanyl	289,114	189,287	146,052	305,616	0.041	1.28	1	0.27
64	2-*t*Bu-Ph	955,812	268,006	424,103	2,302,210	0.0022	1.85	5.68	0.34
65	2,5-di-*t*Bu-Ph	210,683	104,370	308,387	1,338,965	0.017	2.64	2	1.87
66	4-*n*-Heptyl-Ph	11,732	22,681	18,592	18,657	0.27	6.6	>50	13.00
67	2-CF_3-Ph	1,942,965	842,242	745,467	1,626,372	0.0017	0.82	0.65	0.23
68	2-F-Ph	194,786	221,052	191,812	247,890	0.097	0.68	0.97	0.50
69	4-F-Ph	106,344	*8,161,648*	247,449	179,386	0.019	1.21	2	0.92
70	2-Cl-Ph	2,019,148	1,173,738	654,890	2,137,136	0.016	0.16	0.3	0.11
71	2-Br-Ph	7,157,535	1,274,281	*504,107*	2,346,190	0.356	0.54	0.4	1.27
72	2-I-Ph	1,980,934	563,535	373,103	2,328,063	0.081	1.5	1	1.54
73	2,6-di-F-Ph	277,593	322,524	280,613	396,449	0.136	0.38	1	1.63
74	2-F;4-I-Ph	>10,000,000	206,962	132,506	83,952	0.01	0.41	0.7	0.15
75	2,3,4,5-F_4-Ph	266,379	113,021	62,630	58,875	0.04	0.57	3.5	1.19
76	2,3,4,6-F_4-Ph	415,922	207,576	23,953	125,846	0.11	0.27	2.1	0.58
77	2,3,4,5,6-F_5-Ph	236,329	141,958	104,258	66,174	0.39	0.47	2.9	14.02
78	2,3,5,6-Cl_4-Ph	3,908,091	279,263	98,718	375,045	0.22	1.05	0.66	0.41
79	4-Pyridyl (TFA salt)	13,844	54,288	42,761	23,128	15.8	5.67	30	30.10
80	2-Pyridyl (TFA salt)	22,030	117,608	64,055	42,243	0.14	1.43	9.4	2.20
81	2-Pyrazyl (TFA salt)	27,578	149,427	124,444	50,371	0.17	1.44	5.9	19.09

[a] For assay conditions, see Refs. 8 and 14.
[b] Murine caspase-1 enzyme. Other enzyme data are from human.

oxamide showed reasonable cellular activity. In another set of analogues ranging from phenyl, benzyl, phenethyl to tetrahydronaphthyl derivatives, the 1- and 2-naphthyl (**11** and **47**), 1- and 2-anthryl (**52** and **53**), and the 5,6,7,8-H_4-1-naphthyl (**49**) stood out as particularly active compounds. This is consistent with what was learned in the acyl dipeptide caspase inhibitor SAR.[7] In general, 2-substituted phenyl oxamides show the best overall activity, as well as other halogenated phenyl oxamides. Modeling of some these caspase inhibitors in caspase 1, as well as crystal structures of various inhibitors bound to caspase 3 (unpublished results), show that the key interactions in the P3/P4 region are primarily between Arg 207 and the oxamide functionality itself. More specifically, in a crystal structure of an inhibitor bound to caspase 3, a 2.8 Å interaction

between the carbonyl of Arg 207 and the distal oxamyl NH was noted, as well as a 2.7 Å interaction between the amide hydrogen of Arg 207 and the proximal oxamyl carbonyl. It was difficult to propose defined interactions between the phenyl ring of substituted phenyl oxamide analogs and residues in the P4 pocket, such as His 342 and Arg 383. This is not surprising given that the P4 pocket of caspase 1 is not a very well defined binding pocket, generally picking up hydrophobic interactions. This lack of defined phenyl interactions is borne out in the methyl oxamide (**36**). Although not as potent as the phenyl oxamide (**41**), or as the benzyl oxamide (**58**), the methyl oxamide is in the same range and possesses similar cellular activity. This is further reinforced by the loss of activity seen with the methylated 1-naphthyl oxamide (**50**). New analogues with more diverse substitution of the phenyl ring should prove interesting.

It was noted that the potency of caspase inhibition and cellular activity do not correlate well. Lack of potency in these cellular assays with analogues having good caspase activity could be attributable to a number of factors, ranging from protein binding, cell permeability to the compounds stability in a 24 h assay run at 37 °C. Other reasons for the poor correlation between overall caspase inhibition and the cell assays are that some caspases are more relevant to particular cell assays than others. For example, the monocyte and THP-1 assays are solely dependent on caspase 1. On the other hand, although caspase 8 is highly relevant to the Jurkat assay, it is not the only caspase involved. When evaluating in vitro data of this type, it is best to look at general trends.

Based on in vitro activities (namely cellular data) analogue **78** was one of several inhibitors selected for testing in vivo. This inhibitor showed an 18% reduction in infarct size when dosed iv at 30 mg kg^{-1} in a rat permanent MCAO (Middle Cerebral Artery Occlusion) model.[16]

A new, novel class of oxamyl dipeptide caspase inhibitors were prepared. The SAR reveals that the activity of this series can be optimized using either polyfluorophenoxy, dichlorobenzoyloxy, or diphenylphosphinyl as the warhead, valine, or *tert*-butyl valine at P2, and 2-substituted phenyl oxamides or halogenated phenyl oxamides at P4. These analogues are potent, broad spectrum caspase inhibitors (low nanomolar activity) that are active in cellular models of apoptosis and show promise in vivo. The nature of the SAR and the limited diversity of the analogues discussed here show that much work remains in exploring this series of inhibitors.

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