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PEPTIDYL β-HOMO-ASPARTALS: SPECIFIC INHIBITORS OF INTERLEUKIN-1β CONVERTING ENZYME AND ITS HOMOLOGUES (CASPASES)

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Abstract: Inhibition of interleukin-1β converting enzyme (ICE), apopain, papain, thrombin and trypsin with substrate like peptidyl L- and D-α-aldehydes and their L-β-homo-aldehyde analogues was investigated. The L-β-homo-aspartals appear to be specific inhibitors for ICE and its homologues; the other enzymes were not inhibited with such L-β-homo aldehydes. Papain shows tolerance for D-residues at P_1 depending on their chiral stability.

Interleukin-1β, an inflammatory mediator released from human monocytes, is synthesized as a precursor, which is cleaved by a cytoplasmic cysteine protease termed interleukin-1β converting enzyme (ICE), at an Asp-Ala bond to produce the active cytokine. ICE is also synthesized in monocytes as a precursor, proICE, which is activated autocatalytically. ICE appeared to be the first member of a new cysteine protease family - for its unusual specificity, atypical sensitivity to class-specific inhibitors and unique structure.[1] The first homolog of ICE was CED-3, a protein required for programmed cell death (apoptosis) in the nematode *Caenorhabditis elegans*.[2] The further homologues isolated from mammalian cells,[3] such as apopain/CPP32,[4] also initiated apoptosis. The human members of the ICE/CED-3 family have recently been named *caspases*,[5] indicating their cysteine protease character and P_1 aspartic acid specificity; e.g. ICE = caspase-1, apopain = caspase-3. A serine protease with similar specificity is granzyme B,[6] which also induces apoptosis.[7]

The potent ICE inhibitors, substrate related peptidyl aspartals[1] and aspartyl (acyloxy)methanes[8] follow the usual structural scheme for cysteine protease inhibitors as they comprise a substrate portion for recognition by the enzyme and a nucleophilic attackable/substitutable group which can react with the catalytic thiolate of the active site. Peptide aldehydes are not selective inhibitors *per se*; they inhibit both cysteine and serine proteases.[9]

According to a recent study on the above-mentioned inhibitors[10] the aspartyl (acyloxy)methane analogues having the β-homo-aspartyl residue, NH-CH(CH_2COOH)-CH_2-CO (hAsp), are inactive suggesting the key role of Asp α-CO in P_1 recognition, and ICE accepts both D- and L-Asp at the P_1 position of (acyloxy)methanes and aldehydes; this tolerance for D-stereochemistry at a P_1 is unprecedented for the cysteine protease superfamily.

The mechanism proposed for the inhibition of cysteine proteases by peptidyl (acyloxy)methanes assumes that the thiolate can react directly with the C=O (*a*) as well as the adjoining (acyloxy)methyl group (*b*).[9] In view

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of path *b*, one may expect that the thiolate can attack the β-CO of hAsp and also reach and contact the α-proton of the temporary bound P_1-D-Asp, which is directed like the α-CO of P_1-L-Asp. The first reaction brings about the reversible inhibition of ICE (lack of such interaction with the hAsp-OAc analog studied may be due to steric hindrance) and the second one results in proton abstraction, which can lead, *via* asymmetric induction, to the formation of the L-aspartyl-thiomethane product.

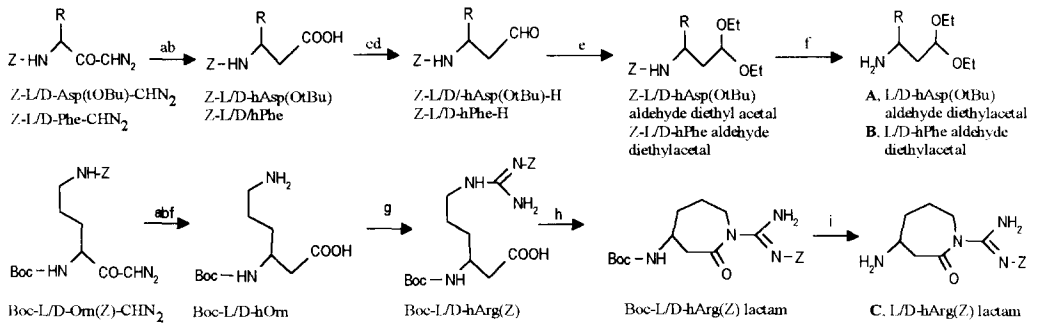
Starting from these findings and speculations we examined the inhibiting activity of some peptide β-homo-aldehydes (hXaa-H) and their parent α-aldehydes (Xaa-H) against ICE, apopain, papain, thrombin and trypsin. The compounds included in this study are presented in Table 1. The parent α-aldehydes, except for **4a**, are known from the literature: **1a**,[1] **2a**,[3] **3a**[11] and **5a**.[12] To the best of our knowledge, peptidyl β-homo-aldehydes have not been prepared yet.

Table 1. Peptide Aldehyde Inhibitors of ICE (**1**), Apopain (**2**) Papain (**3**,**4**) and Thrombin/Trypsin (**5**) Having α-Amino Aldehyde or β-Homo-Aldehyde[a] at P_1

	P_n-.....-P_2-	P_1		
		a	**b/d**[b]	**c**
1	Ac-Tyr-Val-Ala-	-Asp-H	-hAsp-H[b]	—
2	Ac-Asp-Glu-Val-	-Asp-H	-hAsp-H	—
3	Z-Arg-Ile -	Phe-H	-hPhe-H[b]	-D-Phe-H
4	iBoc-Phe -	-Arg-H	-hArg-H	-D-Arg-H
5	Boc-D-Phe-Pro-	-Arg-H	-hArg-H	-D-Arg-H

[a]hXaa-H, NH-CH*R*-CH_2-CHO; iBoc, 2-methyl-propyloxy-carbonyl. [b]D-hXaa isomer indicated by **d**, was also prepared.

The compounds listed in Table 1 were prepared in our laboratory by using conventional procedures starting from the C-terminal components *tert*-butyl-β-homo-aspartal diethylacetal (**A**), β-homo-phenylalaninal diethylacetal (**B**) and N^G-benzyloxycarbonyl-β-homo-arginine lactam (**C**) (Scheme 1). For the β-homo-aldehyde analogues **1b**, **1d**, **2b**, **3b** and **3d** the C-terminal amino acids were converted into Z-protected α-amino acyl diazomethanes, which were rearranged to β-homo-amino acids, whose 3,5-dimethylpyrazolide derivatives were reduced by $LiAlH_4$ to the aldehyde function that was protected as diethylacetals, and finally the Z was removed for further coupling. For the β-homo-arginals **4b** and **5b**, Boc-hArg(Z), prepared from Boc-hOrn(Z), was converted into the lactam, and the Boc was removed for coupling. The peptidyl N^G-Z-β-homo-arginine lactams thus obtained were reduced by $LiAlH_4$ to peptide aldehydes and the N^G-Z removed.[13]



Scheme 1. R = $CH_2COOtBu$ or C_6H_5-CH_2. Reagents: (a) Ag_2O/MeOH, reflux; (b) NaOH/MeOH, H^+; (c) coupling with 3,5-dimethylpyrazole *via* mixed anhydride with isobutyl chloroformate; (d) $LiAlH_4$/THF; (e) $HC(OEt)_3/H^+$; (f) H_2/Pd; (g) Z-NH=C(SMe)-NH_2; (h) mixed anhydride with isobutyl chloroformate; (i) HCl/EtOAc.

The ICE inhibiting activity was assessed in LPS-stimulated human whole blood as described earlier.[14] Inhibition of the other proteases was determined by the amidolytic method using known fluorogenic or chromogenic substrates.[15]

Protease inhibiting activities are presented in Table 2. For ICE-inhibition, the known tetrapeptide

Table 2. Protease Inhibiting Activity (IC_{50}, μM) of Peptidyl L- and D-α-Amino-Aldehydes Compared with their L-β-Homo-Aldehyde Analogues[a]

		P_1: α, NH-CH*R*-CHO; β, NH-CH*R*-CH_2CHO[b]			Potency ratios	
Protease	Peptide aldehyde	**a**: Lα	**b**: Lβ	**c**: Dα	Lα:Lβ	Lα:Dα
ICE	**1**, Ac-Tyr-Val-Ala-P_1	2.000 ± 0.60	2.90 ± 1.0[c]	ND[d]	1.45	~1[d]
Papain	**3**, Z-Arg-Ile-P_1	0.070 ± 0.01	65.00 ± 0.8[c]	8.50 ± 1.00	929	121
Papain	**4**, iBoc-Phe-P_1	0.130 ± 0.02	105.00 ± 5.0	12.50 ± 1.50	808	96
Thrombin	**5**, Boc-D-Phe-Pro-P_1	0.009 ± 0.001	0.19 ± 0.02	5.44 ± 0.22	21	604
Trypsin	**5**, Boc-D-Phe-Pro-P_1	0.003 ± 0.0001	1.76 ± 0.08	1.79 ± 0.09	587	597

[a]ICE inhibiting activity was assessed in LPS-stimulated human whole blood; the inhibition of other proteases was estimated by the amidolytic method using known fluorogenic or chromogenic substrates.

[b]*R* means -CH_2COOH, $C_6H_5CH_2$- and -$(CH_2)_3$-NH-C(NH)-NH_2 for **1**, **3** and **4-5**, respectively.

[c]The Dβ isomers showed no inhibition at 100 μM (**1d**) and 500 μM (**3d**).

[d]ND, not determined. Such diastereomeric inhibitor pairs were found equipotent against ICE (cf. ref. 10).

aldehyde (**1a**) and its β-homo-aldehyde analog (**1b**) are almost equiactive, the Lα:Lβ potency ratio is 1.45. For the others, however, the β-homo-aldehyde analogues are not inhibitory or only produce as much inhibition as their side-chains and backbone can generate. It is moderate in the case of papain and trypsin but significant for thrombin. It is probably due to the high thrombin-affinity of **5** even without C-terminal functionality as a serine trap, like D-Phe-Pro-Agm.[17] The influence of the D-aldehydes is different. The Lα:Dα potency ratios illustrate neither trypsin nor thrombin can tolerate D-arginal at P_1. On the other hand, papain, the cysteine protease, appears to have a tendency to accept D-aldehydes at P_1 depending on the residues' chiral stability, i.e. on the lability of their α-hydrogen, which is influenced by side chain and increases, in the present instance, in the order of Phe < Arg < Asp (cf. Ref. 18). The ICE-inhibiting diastereomeric peptide pairs have been found equipotent.[10]

Regarding the inhibitory mechanism of peptidyl (acyloxy)methanes or, in general, peptidyl X-methanes, these findings suggest that the catalytic thiolate of ICE can directly attack the carbonyl carbon as well as the adjoining X-methyl group seeing that it reacts with the carbonyl carbon of both normal α-aspartal and β-homo-aspartal. On the other hand, the thiolate of papain-like cysteine proteases attacks only the carbonyl carbon of peptidyl X-methanes having P_1-L residue and cannot react directly with the X-methyl group. However these enzymes can contact the α-hydrogen of a P_1-D residue and so they may show tolerance for D-stereochemistry in proportion to the lability of the α-C-H bond. Thus the observed tolerance of ICE for D-stereochemistry at P_1 may arise from its cysteine protease nature and the aspartyl residue's high tendency for racemization.

To obtain more information about the sensitivity of caspases to β-homo-aspartals we examined the inhibition of the amidolytic activity of THP.1 cell lysate on the known ICE and apopain substrates,[15] *C1*- and *C3*-aspartyl-AMC, respectively (Table 3). (i) By using these substrates ICE-like and apopain-like activities of the

Table 3. Inhibitory Potencies (IC_{50}, M) of Peptidyl Aspartals (Asp-H) and β-Homo-Aspartals (hAsp-H) on the Amidolytic Activity of THP.1 Cell Lysate on ICE (Caspase-1) and Apopain (Caspase-3) Substrates *C1*-Asp-AMC and *C3*-Asp-AMC, Respectively[a]

	C1 = Ac-Tyr-Val-Ala[b]		*C3* = Ac-Asp-Glu-Val[b]	
Substrate	*C1*-Asp-H (**1a**)	*C1*-hAsp-H (**1b**)	*C3*-Asp-H (**2a**)	*C3*-hAsp-H (**2b**)
C1-Asp-AMC	$6.8 \pm 1.4 \cdot 10^{-5}$	$14.2 \pm 6.3 \cdot 10^{-5}$	$7.0 \pm 2.8 \cdot 10^{-5}$	NI[c]
C3-Asp-AMC	$1.9 \pm 0.2 \cdot 10^{-4}$	NI[c]	$2.23 \pm 0.70 \cdot 10^{-8}$	$4.38 \pm 0.56 \cdot 10^{-7}$

[a] ICE- and apopain-like activities of the lysate of 10^8 cells are ~1.0 and ~5.5 pkat, respectively.

[b] hAsp-H:Asp-H IC_{50} ratios for identical inhibitor/substrate sequences: *C1*, 2.09; *C3*, 19.6.

[c] No inhibition at 10^{-4}M.

lysate of 10^8 cells were assessed to be about 1 and 5.5 picokatals, respectively. (ii) The homo-aspartal to aspartal IC_{50} ratios for identical sequences *C1* and *C3* are 2.9 and 19.6, respectively, which may indicate that β-homo-aspartal fits into the active center of ICE somewhat better than into that of apopain. Accordingly, the architecture of the active centers of these two caspases are somewhat different. (iii) Inhibition of the cleavage of the ICE substrate, *C1*-Asp-AMC, by ICE inhibitors, **1a** and **1b** is 3-2 orders of magnitude less efficient than that of the apopain substrate by the corresponding apopain inhibitors, **2a**, **2b**. (iv) The normal α-aldehydes, **1a** and **2a**, inhibit the amidolysis of both substrates but differently, each inhibitor is more efficient with the sequentially identical substrate. These substrate-dependent IC_{50} ratios are less than 3 for **1a** and more than 3000 for **2a**. Moreover the corresponding β-homo-aspartals, **1b** and **2b**, only inhibit the cleavage of sequentially identical substrates. In other words, a peptidyl-β-homo-aspartal can compete with a peptidyl α-aspartyl-amide on condition that their peptide structures are identical. However, such restrictions might relate only to peptide substrates because, as shown in Table 2, **1a** and **1b** reduced IL-1β release, which originate from the cleavage of the native protein substrate of ICE, with similar efficacy. Thus, it is very likely that **2b** will also inhibit the action of apopain on its native protein substrates. It has long been known that protease inhibition by peptide aldehydes is substrate-dependent and that in some instances it can be substantial; in the case of thrombin inhibition with D-Phe-Pro-Arg-H the K_i values measured with fibrinogen and D-Phe-Pro-Arg-pNA were 0.075 and 700 μM, respectively.[19]

In conclusion, this study has shown the following. (i) Substrate related peptidyl L-β-homo-aspartals are specific inhibitors for caspases with potencies similar to those of the L-α-aspartal-containing analogues. (ii) The L-β-homo analogues of peptide aldehyde inhibitors of cysteine and serine proteases such as Z-Arg-Ile-hPhe-H for papain or Boc-D-Phe-Pro-hArg-H for trypsin and thrombin are not inhibitory. (iii) In contrast to the serine proteases, papain appears to accept a D-aldehyde at P_1 in proportion to the residues' chiral stability. Thus ICE's tolerance for D-stereochemistry at P_1 may arise from its cysteine protease nature and the aspartyl residue's high tendency for racemization.

References and Notes

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13. The novel key components, Z-L- and D-hAsp(tOBu)-aldehyde diethylacetal [P-$\mathbf{A_L}$ & P-$\mathbf{A_D}$, (R)- and (S)-3-benzyloxycarbonylamino-4-(*tert*-butoxycarbonylamino)-butyraldehyde diethylacetal] for **1b**, **2b**, and **1d**, respectively; Z-L- and D-hPhe-aldehyde diethylacetal [P-$\mathbf{B_L}$ & P-$\mathbf{B_D}$, (S)- and (R)-3-benzyloxycarbonyl-amino-4-phenylbutyraldehyde diethylacetal] for **3b**, and **3d**, respectively, and *tert*-butoxycarbonyl-hArg(Z)-lactam, [P-**C**, (S)-1-benzyloxycarbonylamidino-2-oxo-4-*tert*-butoxycarbonylamino-hexamethyleneimine] for **4b** and **5b**, and the final products **1a-5c** were characterized by ^{1}H NMR and FAB MS. $[M+H]^+$ values of FAB MS (M_r) of the key compounds and final products were as follows: P-$\mathbf{A_L}$, 396 (395.5); P-$\mathbf{A_D}$, 396 (395.5); P-$\mathbf{B_L}$, 372 (371.5); P-$\mathbf{B_D}$, 372 (371.5); P-**C**, 405 (404.5); **1a**, 493 (492.5); **1b**, 507 (506.6); **1d**, 507 (506.6); **2a**, 503 (502.5); **2b**, 517 (516.5); **3a**, 553 (552.7); **3b**, 567 (566.7); **3c**, 553 (552.7); **3d**, 567 (566.7); **4a**, 406 (405.5); **4b**, 420 (419.5); **4c**, 406 (405.5); **5a**, 503 (502.6); **5b**, 517 (516.6); **5c**, 503 (502.6).
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15. The substrates used, i.e. Z-Phe-Arg-AMC[17] (papain), Tos-Gly-Pro-Arg-pNA, Chromozym-TH (thrombin & trypsin), Ac-Tyr-Val-Ala-Asp-AMC[1] (ICE) and Ac-Asp-Glu-Ala-Asp-AMC[4] (apopain), were prepared by conventional methods in our laboratory.
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