Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 2, pp. 241-246, 1994

0960-894X/94 $6.00+0.00

0960-894X(93)E0066-A

BENEFICIAL REPLACEMENT OF THE P_1 PHENYLALANINE SIDE CHAIN IN HIV-1 PROTEASE INHIBITORS OF THE DIFLUOROSTATONE TYPE

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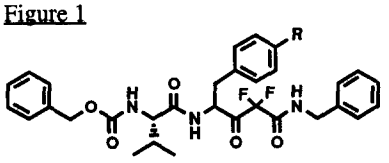
Abstract : An O-benzyltyrosyl P_1 side chain on HIV-1 protease inhibitors of the difluorostatone type confers increased potency and an improved cytotoxic index in infected cells. A number of carboxy or amino termini modifications are permitted for instance, carboxy termini tertiary amides.

Numerous peptidomimetic analogues of the tetrahedral amide hydrate intermediate formed during catalytic cleavage of the natural substrate by HIV-1 protease have been described over the past five years[1]. Indeed, the essential role this enzyme plays in the replicative cycle of HIV identifies its inhibition as an attractive new therapy for AIDS. In this respect, we recently reported the discovery of short and unexpectedly potent inhibitors of the difluorostatone type[2].

We have shown that derivative 1a exhibits strong inactivation of purified HIV-1 protease *in vitro* (K_i = 1 nM) as well as inhibition of HIV-1 replication in various T cell lines at micromolar concentrations (MTT assay). However this dipeptide analogue was found to be cytotoxic at concentrations approximately 5 to 10 times greater than the inhibitory concentrations. Our attempts to circumvent this problem and thereby increase the "therapeutic window" (selectivity index) are described here.

Figure 1

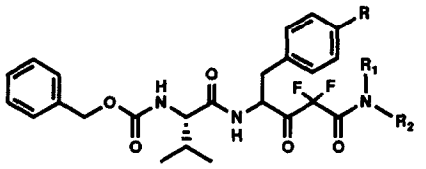


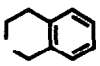
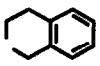
1a : R = H
2a : R = $OCH_2C_6H_5$

P_1 modification (Table I)

Cellular penetration, one of the key factors for efficient inhibition of virus replication, could hopefully be improved by making the side chains of compound 1a more lipophilic. Several groups have modified the carboxy and amino termini and the P_1' subsite[3] side chain of their inhibitors[4a,5a]. The effect of modifications to the P_1 side chain on inhibitor potency has been the subject of a very limited number of studies[5]. Modification of our phenylalanyl type difluorostatones (1a-c) to O-benzyltyrosyl analogues (2a-c)[6] was extremely beneficial as shown in table I, and resulted in an increased inhibition of replication as well as an optimized selectivity index (CC_{50}/EC_{50}).

TABLE I



Nr	R_1	R_2	R	K_i* or IC_{50} (nM)°	EC_{50} (μM)°°	CC_{50} (μM)°°°	CC_{50} / EC_{50}
1a	H	$CH_2C_6H_5$	H	1*	5	23	~5
2a	H	$CH_2C_6H_5$	$OCH_2C_6H_5$	5*	0.3	>100	>300
1b			H	70*	50	>100	>2
2b			$OCH_2C_6H_5$	5*	3.2	64	20
1c			H	50	-	-	
2c			$OCH_2C_6H_5$	2	0.36	>100	>280

° See ref (10); °° Drug concentration that inhibits cellular growth of MT_4 cells acutely infected with HIV-1 RF strain by 50%, value determined by the MTT viability assay; °°° Toxicity monitored by the MTT assay.

The inhibition of viral replication by dipeptide 2a has been demonstrated on several HIV-1 strains in different cell lines as well as on HIV-2 and SIV (Table II).

TABLE II : Antiviral activity of inhibitor 2a in acutely infected cells.

Cell line (HIV strain)	EC_{50} (μM)	CC_{50} (μM)
MT_4 (HIV-1 $_{RF}$)	0.3°°	>100°°°
C8166 (HIV-1 $_{RF}$)	0.1*	>100°°°
JM (HIV-1_{GB8})	0.14*	>100°°°
CEM (HIV-1 $_{IIIB}$)	0.72*	
C8166 (SIV $_{mac\ 251}$)	0.63§	
C8166 (HIV-2 $_{rod}$)	0.35Δ	
H9 (HIV-1 $_{IIIB}$)	0.29*	
H9 (HIV-1 $_{RF}$)	0.056*	
H9 (HIV-1 $_{U455}$)	0.42*	
PBMCs (HIV-1 $_{CCI}$)[1]	0.56*	
MT2 (HIV-1 $_{105/A}$)[2]	0.23*	
MT2 (HIV-1 $_{105/F}$)[2]	0.38*	

°° Drug concentration that inhibits cellular growth of MT_4 cells acutely infected with HIV-1 RF strain by 50%, value determined by the MTT viability assay; °°° Toxicity monitored by the MTT assay.
* p24 antigen; § p27 antigen; Δ p26 antigen.
[1] CD8 depleted PBMCs were infected with HIV-1 $_{CCI}$ (patent isolate) for one hour at room temperature. After 7 days the cell free supernatant fluid was assayed for p24 viral core antigen.
[2] MT2 cells were infected for one hour at room temperature with HIV-1_{105A} (patient isolate, AZT sensitive) or with HIV-$1_{105/F}$ (patient isolate after development of AZT resistance). After 5 days, the cell free supernatant fluid was assayed for levels of p24 antigen.

Dipeptide 2a, despite the modification to its P_1 side chain, is a "flexible" structure that can accommodate a number of harmless modifications without loss of inhibitory activity, at the P_2 site, as well as at the amino and carboxy termini.

P2 Modifications (Table III)

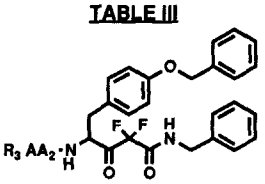
Replacement of the P_2 valine residue by a tert-Leu residue results in a slight decrease of the effective dose in infected cells whereas it dramatically increases the cytotoxicity. Five or six membered carbocyclic substitutes for valine give markedly different results. Cyclopentyl glycine[7] is a well tolerated replacement *in vitro*, both on the purified enzyme and infected cells whereas cyclohexyl glycine, in contrast to pyran urethane P_2 ligands[8], strongly affects binding to the target enzyme *in vitro* and inhibition of virus replication .

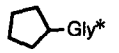
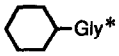
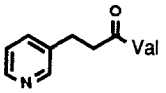
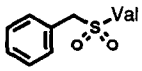
Amino terminal modifications (Table III)

In contrast to results obtained with inhibitor 1a[2], the replacement of the benzyloxycarbonyl aminoprotecting group in statone 2a by a tert-butoxycarbonyl moiety results in a complete loss of inhibitory activity. Similarly the introduction of a benzylsulfonyl side chain generates a very weak HIV-1 protease inhibitor (8; IC_{50} = 48 μM).[9]

However, a 3-pyridylpropionyl substituent, resulting in a more hydrophilic compound, does not alter the inhibitory properties, inhibitor 7 being equipotent to 2a[2].

TABLE III



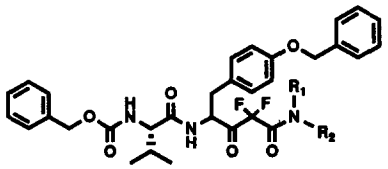
Nr	R_3	AA_2	K_i* or IC_{50} (nM)°	EC_{50} (μM)°°	CC_{50} (μM)°°°	CC_{50} / EC_{50}
2a	CBZ	Val	5*	0.3	>100	>300
3	CBZ	*tert* - Leu	15	0.19	3.2	17
4	CBZ		6	3	>100	>33
5	CBZ		70	>100	>100	-
6	BOC	Val	1000	>10	10	-
7			8	0.32	>100	>100
8			48000	-	-	-

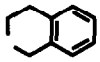
° See ref (10); °° Drug concentration that inhibits cellular growth of MT_4 cells acutely infected with HIV-1 RF strain by 50%, value determined by the MTT viability assay; °°° Toxicity monitored by the MTT assay.
* racemic mixture

Carboxy terminal modifications (Table IV)

The carboxy terminus of 2a was modified to explore the volume accessible to the inhibitor in the S'_2 subsite. Several secondary amines were coupled to the intermediate ester[11] to produce tertiary statone-amides. When compared to benzylamide 2a, the morpholine and the dihydroisoindole amides (2b and 2c) exhibit slightly lower and equivalent inhibitory potencies, respectively. The acyclic analogue of 2c, inhibitor 2e, surprisingly was 20 times less potent and 10 times more toxic giving a selectivity index (CC_{50}/EC_{50}) about 300 times smaller. Replacement of the N-ethyl substituent of 2e by a N-methyl moiety (2d), produced an inhibitor 10 times more potent on the purified enzyme than 2a, with comparable cellular potency.

TABLE IV



Nr	R_1	R_2	K_i* or IC_{50} (nM)°	EC_{50} (μM)°°	CC_{50} (μM)°°°	CC_{50} / EC_{50}
2a	H	$CH_2C_6H_5$	5*	0.3	>100	>300
2b			5*	3.2	64	20
2c			2	0.36	>100	>280
2d	CH_3	$CH_2C_6H_5$	0.4*	0.28	>100	>300
2e	CH_2CH_3	$CH_2C_6H_5$	15	8.0	10	≈1

° See ref (10); °° Drug concentration that inhibits cellular growth of MT_4 cells acutely infected with HIV-1 RF strain by 50%, value determined by the MTT viability assay; °°° Toxicity monitored by the MTT assay.

In conclusion, replacement of the "classical" phenylalanyl P_1 substituent[1,4] of inhibitors 2a-c by an O-benzyltyrosine side chain does improve the potency of our dipeptide statone analogues and optimizes their selectivity index.

Moreover, the introduction of a N-methyl benzylamine at the carboxy terminus (2d) generates an extremely potent inhibitor, *in vitro*, of relatively small size. Further investigation of the mode of binding and the antiviral effects of these ligands will be published elsewhere.

References

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3. Nomenclature of amino acid residues at the cleavage sites is according to : Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.

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5. a) C-2 Symmetric HIV-1 protease inhibitors : Dreyer, G.B.; Boehm J.C.; Chenera, B.; Desjarlais, R.L.; Hassel, A.M.; Meek, T.D.; Tomaszek Jr, T.A. *Biochemistry*, **1993**, *32*, 937.
Ghosh, A.K., McKee, S.P.; Thompson, W.J. *Tetrahedron Letters*, **1991**, *32*, 5729.
Wittenberger, S.J.; Baker, W.R.; Donner, B.G. *Tetrahedron* **1993**, *49*, 1547.
Jadhav, P.K.; Woerner, F.J., *Bioorganic and Medicinal Chemistry Letters*, **1992**, *2*, 353.
b) Non symmetrical HIV-1 protease inhibitors
Thompson, W.J.; Ball, R.G.; Darke, P.L.; Zugay, J.A.; Thies, J.E. *Tetrahedron Letters*, **1992**, *33*, 2957.
6. All new compounds were characterized by ^{1}H NMR and ^{19}F NMR, MS and/or combustion analysis.
7. Recently, 3'-tetrahydrofuranylglycine, a novel and unnatural amino acid surrogate for asparagine, has been introduced in the design of inhibitors of HIV-1 protease of the amino alcohol type.
Thompson, W.J.; Ghosh, A.K.; Holloway, M.K.; Lee, H.Y.; Munson, P.M.; Schwering, J.E.; Wai, J.; Darke, P.L.; Zugay, J.; Emini, E.A.; Schleif, W.A.; Huff, J.R.; Anderson, P.S. *J. Am . Chem. Soc.* **1993**, *115*, 801.
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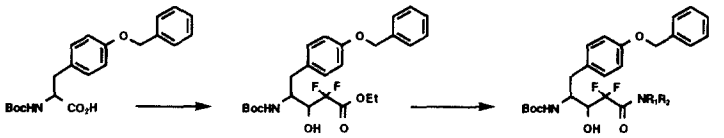
9. Although the introduction of a sulfonamide functionality increases the solubility in aqueous media, geometrical and/or physicochemical reasons may explain this dramatic loss in affinity (ionizable group).
10. HIV-1 Protease assay
Protein source : recombinant enzyme (E. Coli) ; substrate :
H-SerGlnAsnTyrProIleValNH2 ($K_m \approx 1$ mM) ; buffer : 0.1 M Mes-tri acetate, 0.2 M NaCl, pH 5.5 - 6.0 (EDTA, Phenylmethylsulfonylfluoride, DTT 1mM and 0.5% BSA), 37°C ; kinetic analysis : HPLC analysis of the two products.
11. Key intermediate ethyl ester B is obtained in 3 steps from N-Boc-O-benzyltyrosine A in 25-40 % overall yield.



A B Secondary or tertiary amides

Conversion of acid A to the corresponding aldehyde (CH_3NH-OCH_3, N-Methyl Morpholine, DCC, HOBt ; LAH)[12] and condensation with the Reformatski reagent derived from ethyl bromodifluoroacetate (EtO_2CCF_2Br, Zn, THF)[13] gives easy access to intermediate B. Coupling with secondary or primary amines in THF leads to the desired tertiary or secondary amides respectively in good yields.
12. Intermediate aldehydes were prepared according to a method described in the following reference : Fehrentz, J.A.; Castro, B. *Synthesis* , **1983**, 676.
13. As : Thaisrivongs, S.; Pals, D.T.; Kati, W.M.; Tuner, S.R.; Thomasco, L.M.; Watt, W. *J. Med. Chem.*, **1986**, *29*, 2080.

(*Received in Belgium* 17 *August* 1993; *accepted* 22 *October* 1993)