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INHIBITION OF HIV-1 PROTEINASE BY NON-PEPTIDE CARBOXYLATES

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Some simple dicarboxylates are among the first reported non-peptide inhibitors of HIV-1 proteinase. Only weak inhibition ($IC_{50} \geq 10\mu M$) was observed but this may be significant since only two potential enzyme-binding groups are present. Dixon plots and preliminary kinetic data are reported and a possible mechanism for the inhibition is discussed. The dicarboxylates are long enough to engage the carboxylate side chains of Arg 8 and Arg 108 at either end of the 24Å long substrate-binding groove. This mode of binding has not been proven but other molecules with similarly separated charged ends are equally effective inhibitors, perhaps indicating a common mechanism of inhibition. There is evidence that placing other functional groups on the inhibitor enables alternative interactions with the enzyme which can reduce inhibitor potency. We propose that incorporation of ionic binding groups in more elaborate and selective non-peptides may potentiate inhibition of HIV-1 proteinase.

HIV-1 proteinase, a component processing enzyme of the AIDS virus that is crucial for viral replication, is a recognized target for anti-AIDS drugs [1,2]. It has been crystallographically characterized both in its free form [3-5] and with various peptidic inhibitors bound in the active site [6-9]. These structures have revealed that the substrate binding groove of the enzyme is hydrophobic, with the exception of two central active site residues (Asp 25,125) and several terminal charged hydrophilic residues (Asp 29,30; Arg 8) at both ends of the 24Å long active site cavity. Crystal structures of enzyme-inhibitor complexes showed that the inhibitors interact with enzyme through van der Waals and hydrogen bonds. No ionic bonds were evident although such interactions might substantially increase the inhibitor-enzyme binding energy [10].

The significance of this work is that apart from haloperidol [11] and cerulenin [12], all reported inhibitors of this enzyme are substrate-based peptides [2]. Although several active peptidic inhibitors of other aspartic proteinases (e.g. renin) are known, they have not proved pharmacologically

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useful as drugs due to inherent problems of poor bioavailability, susceptibility to hydrolysis and short lifetimes. Consequently non-peptidic inhibitors are now being actively sought as potential drugs. Based on present observations, incorporation of charged groups may prove useful in elaborating more potent and selective non-peptidic inhibitors.

Materials and Methods

HIV-1 Proteinase (SF2 sequence) was chemically synthesized by the method [13] of Kent and co-workers, who substituted cysteines 67,95,167 and 195 with 2-aminobutyric acid (Aba).

A fluorometric assay, developed by Toth and Marshall (Washington University School of Medicine, St. Louis, Missouri) [14], was used for this work. It utilizes a substrate (2-aminobenzoyl-Thr-Ile-Nle-(*p*-nitroPhe)-Gln-Arg-amide) which shows only weak fluorescence due to intramolecular quenching by the *p*-nitrophenyl moiety. Enzymatic cleavage of this substrate, confirmed by HPLC analysis of products, releases a more fluorescent product (2-aminobenzoyl-Thr-Ile-Nle) with excitation and emission peaks at 325nm and 420nm respectively. Assays were conducted in black polystyrene Fluorostrip plates consisting of 96 wells (Flow Labs, Nth. Ryde, N.S.W, Australia) and fluorescence was detected with a Titertek Fluoroskan II microtitre plate reader using excitation (355nm) and emission (460nm) filters.

Inhibitors were assayed in quadruplicate at each of a range of concentrations, using positive (with enzyme) and negative (without enzyme) controls. Typically a stock solution of each inhibitor (10mM in DMSO or buffer) was diluted with buffer (0.1M NaMES, pH 6.5) and preincubated at 37°C for 30min with enzyme (10µl, nM concentration) in a total volume of 180µl. Substrate (20µl, final concentration = 20-50µM) was then added and after 10min the enzymatic reaction was terminated by adding 100µl of 6M guanidine.HCl in buffer. The fluorescence reading for 50µM substrate alone was 22.0 ± 1.5, whereas after incubation with enzyme the reading was 35.0-40.0 equivalent to ≤ 20% cleavage of the substrate. A non-linear response of the instrument at readings > 50.0 precluded reliable studies for [substrate] > 50µM. Using this procedure Km for substrate was estimated at 37.5 ± 7.5 µM which is identical with a reported number (37 ± 8; [14]). A standard aspartyl protease inhibitor, pepstatin, yielded an IC_{50} 2.5µM (K_i 1.8µM) and serves as a reference for comparison with other assays (K_i =1.1µM, pH 5.5 [15]; 1.4µM, pH 6.0 [16]).

Light Green SF Yellowish and Pyronin B were purchased from Sigma Chem. Co. NMR spectra were recorded on a Varian Gemini 300MHz spectrometer. NaO_2C-$(CH_2)_5$-CO-$(CH_2)_8$-CO-$(CH_2)_5$-CO_2Na was made from sebacoyl chloride and 1-morpholino-1-cyclohexene according to the literature [17] and recrystallized from methanol/diethyl ether. It was converted to 7,16-dihydroxy-docosanedioic acid by reduction with $NaBH_4$ in methanol and to docosanedioic acid by reduction with hydrazine. Compounds were characterized as follows :

NaO_2C-$(CH_2)_5$-CO-$(CH_2)_8$-CO-$(CH_2)_5$-CO_2Na ^{1}H-NMR (D_2O, Dioxane) : δ2.4-2.2 (m, CH_2); 1.93 (t, CH_2), 1.32 (t, CH_2), 1.04 (br, s, CH_2). ^{13}C-NMR (D_2O,

Dioxane) : δ220.4 (CO), 184.2 (COO), 42.2, 42.1, 37.3, 28.1, 25.4, 23.2, 23.2, 23.1 (CH_2). MS: m/z = 421 (M + 2 - Na^+), 443 (M + 1), 465 (M + Na^+).
HO_2C-$(CH_2)_5$-CH(OH)-$(CH_2)_8$-CH(OH)-$(CH_2)_5$-CO_2H ^{1}H-NMR (CD_3OD, TMS : δ3.29 (br, s, OH); 2.08 (t, CH_2), 1.00-1.50 (m, CH_2). ^{13}C-NMR (CD_3OD, TMS) : δ178.1(COO), 72.4 (C-OH), 38.4, 38.19, 34.8, 30.8, 30.7, 30.2, 26.7, 26.4, 26.0 (CH_2). MS: m/z = 349 (M-COO), 403 (M +1), 425 (M + Na^+).
HO_2C-$(CH_2)_{20}$-CO_2H ^{1}H-NMR (DMSO-d_6, TMS) : δ2.08-2.20 (t, CH_2); 1.34-1.50 (m, CH_2), 1.20 (br, CH_2). ^{13}C-NMR (DMSO-d_6, TMS) : δ176.1 (COO), 34.2, 29.5, 29.4, 29.0, 25.0 (CH_2). MS: m/z = 369 (M - 1), 483 (M + TFA).

Results and Discussion

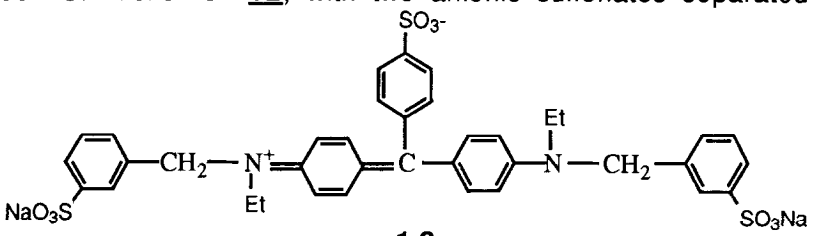
Table 1 records IC_{50} data for inhibition of synthetic HIV-1 proteinase by some compounds at pH 6.5. The 22 carbon dicarboxylic acid, docosanedioic acid **1**, was the most potent inhibitor (IC_{50} 12μM) amongst the carboxylates despite having only two potential enzyme-binding functionalities. The molecule is long enough to be able to interact with charged residues (Arg 8, Arg 108) located at either end of the substrate binding groove. The calculated binding energy for two carboxylate-guanidinium ionic interactions alone [9] accommodates the extent of observed inhibition. Placing either hydroxyl (**2**) or carbonyl (**3**) functional groups on carbons 7 & 16 significantly reduced the inhibitor potency, presumably because weaker hydrogen bonding interactions are then possible elsewhere in the enzyme so reducing the effective concentration of carboxylate ions at the major binding site. Apart from sebacic acid (**8**) the reported simple carboxylates (**4**-**11**) were not inhibitors, neither were Na_2EDTA, coumalic, caffeic or dimercaptosuccinic acids.

Table 1. Inhibition of Synthetic HIV-1 Proteinase Activity

	Compound	IC_{50} (μM)[a]
1.	HO_2C-$(CH_2)_{20}$-CO_2H	12
2.	HO_2C-$(CH_2)_5$-CH(OH)-$(CH_2)_8$-CH(OH)-$(CH_2)_5$-CO_2H	90
3.	HO_2C-$(CH_2)_5$-CO-$(CH_2)_8$-CO-$(CH_2)_5$-CO_2H	200
4.	HO_2C-$(CH_2)_5$-CO_2H	> 1000
5.	HO_2C-$(CH_2)_6$-CO_2H	> 1000
6.	HO_2C-$(CH_2)_7$-CO_2H	> 1000
7.	HO_2C-$(CH_2)_8$-CO_2H	380
8.	HO_2C-$(CH_2)_9$-CO_2H	> 1000
9.	HO_2C-$(CH_2)_8$-CH=CH-CO_2H	> 1000
10.	HO_2C-$(CH_2)_{12}$-CO_2H	> 1000
11.	HO_2C-$(CH_2)_{14}$-CO_2H	> 1000
12.	Light Green SF Yellowish	12
13	Pyronin B	17

[a] Fluorogenic assay, pH 6.5, 0.1M NaMes.

Other compounds from different chemical classes with appropriately separated charged groups capable of interacting with hydrophilic ends of the substrate binding groove were also tested. The inhibiting potencies of Light Green SF Yellowish **12**, with two anionic sulfonates separated by 22



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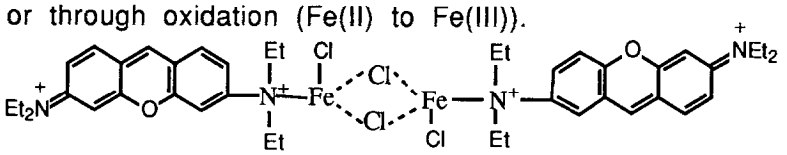
bonds, and Pyronin B **13**, with two cationic amines separated by 21 bonds, were similar to that of **1**. Pyronin B loses activity if aged in solution, presumably because it is susceptible to dimer splitting either by hydrolysis or through oxidation (Fe(II) to Fe(III)).



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Dixon plots for enzyme inhibition by compounds **1** and **12** are shown in Fig.1. The assay used for this work, although suitable for screening inhibitors, was not sufficiently sensitive to obtain more accurate kinetic data. However preliminary data collected when using multiple substrate concentrations and qualitatively examined as Lineweaver-Burk plots are more consistent with non-competitive rather than competitive inhibition for **1** and **12**. This does not necessarily mean that the inhibitor does not occupy the substrate-binding groove of the enzyme as required by the

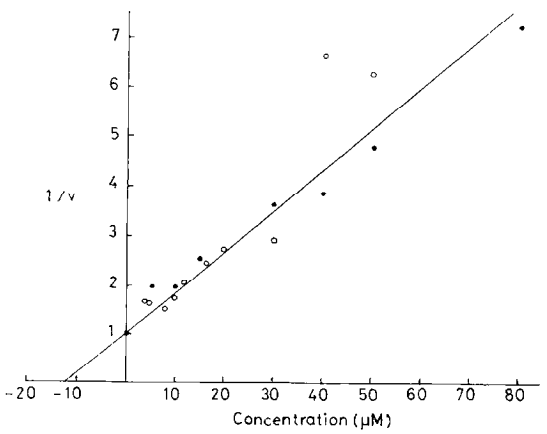

Fig. 1. Dixon plot for the inhibition of HIV-1 Proteinase by **1** (●) and **12** (○) 1/v is the reciprocal of the enzymatic rate in the presence of inhibitor relative to that in its absence.

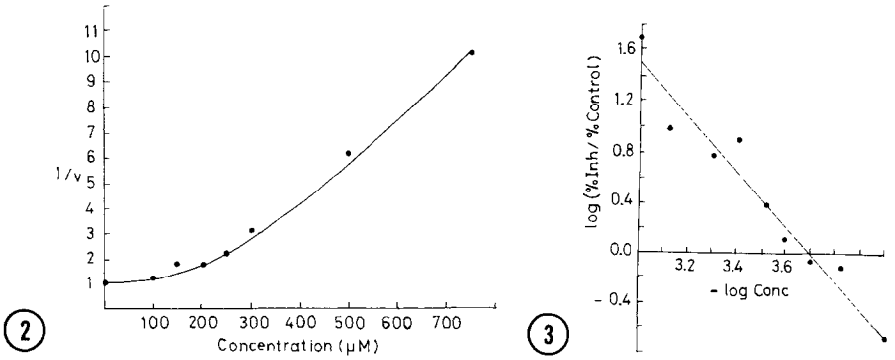

Fig. 2. Dixon plot for inhibition of HIV-1 Proteinase by **3**.

Fig. 3. Hill plot for inhibitor **3**. The ordinate axis is the log of %inhibition divided by %control activity. The abscissa axis is the log of molar concentration of inhibitor. The negative of the slope is the pseudo Hill coefficient.

proposal that the charged ends may be interacting with Arg 8 and Arg 108. This mode of inhibitor binding might be expected to block substrate access to the active site of the enzyme, thus resulting in competitive inhibition. Surprizingly, our preliminary modelling work indicates that at least for the free enzyme, where the flaps are in a more open configuration [4], **1** could simultaneously occupy the substrate binding groove with the substrate. This mode of inhibitor binding could lead to non-competitive inhibition by preventing flap motion and flap-substrate interactions thought to be necessary for enzymatic hydrolysis. Proof awaits X-ray structural identification of the enzyme/inhibitor(/ substrate) complex.

A feature of the inhibitory characteristics of the dihydroxy- (**2**) and diketo- (**3**) compounds was the non-linear nature of their Dixon plots (For **3**, see Fig.2). The curvature is caused by greater potency at high concentrations than would be expected from the degree of inhibition at low concentrations. Such parabolic plots are usually indicative of multiple inhibitor-binding sites on the enzyme. Inspection of the data in the form of a Hill plot (Fig. 3) reveals two binding sites (slope = 2.2 ± 0.2). This is consistent with these inhibitors initially saturating a weaker, and perhaps more accessible, binding site followed by a stronger binding site at higher concentrations. The plot in Fig. 2 contrasts with the linear Dixon plots for compounds **1** and **12** (Fig. 1).

In conclusion we have observed inhibition of HIV-1 proteinase by some non-peptidic inhibitors which have in common charged groups capable of binding to the enzyme. This binding is non-competitive with the substrate, it may be ionic in nature, and a mechanism for the inhibition

has been proposed. The observations may be useful in developing more potent and selective non-peptidic HIV-1 proteinase inhibitors.

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