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CHARACTERIZATION OF DYNAMIC STATE INHIBITORS OF HIV-1 PROTEASE

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The inhibitory behavior of simple N-protected amino acids containing a difunctional enol group was analyzed. Pseudo-Hill coefficients below 0.5 were obtained for the enol compounds. The active moiety of the enols described herein is involved in an equilibrium between keto and enol forms. The enol in its *E* form interacts strongly with the enzyme and we suggest that the *Z* form is expelled from the active site, as suggested by molecular modeling studies using the docking technique. Also, tetrahedral intermediate mimicry is achieved by the enol form, but not by the corresponding keto form. A pseudo-Hill coefficient of 0.25 has been determined for the Boc-phenylalanine enol, and by multiplying the concentration of inhibitor by its pseudo-Hill coefficient, a K_i of 0.82 μM has been determined for the inhibitory *E*-enol form. Boc-tyrosine enol generated a K_i of 0.39 μM. The requirements at the P1′ position to achieve good inhibition are also described.

KEY WORDS: HIV-1 protease, enol inhibitors, pseudo-Hill coefficient

INTRODUCTION

The rapid spread of Acquired Immunodeficiency Syndrome (AIDS) caused mainly by the Human Immunodeficiency Virus (HIV) type 1, represents one of the most medically important problems that social and scientific committees presently face. Only a few drugs (AZT, ddC and ddI) have been approved so far for treatment of patients. These reverse transcriptase inhibitors are limited by their toxicity as well as the appearance of resistance within a short period of time.[1,2] The need to design and synthesize novel compounds aimed to block other steps in the HIV life cycle is obvious and urgent.

During the last five years, the HIV retroviral protease has become one of the most potent targets in the design of new drugs aimed to block viral replication. The availability of high-resolution structural information of the HIV-1 protease alone[3,4] and in complexation with inhibitors[5–8] led the way to the design of new compounds with high inhibitory potential.[9–17] It is known that the retroviral protease is responsible for the processing of gag and gag-pol gene products.[18,19] The specific cleavage of the two polyproteins generates structural proteins p17 (matrix), p24 (capsid), and p9/p6 (RNA

stabilizers) and retroviral enzymes p10 (protease), p51/p66 (reverse transcriptase) and p31 (integrase). Cleavage of the gag polyprotein precursor results in condensation of the viral core, which is essential for the production of infectious viruses.[20–22] Also, other roles have been implied for the HIV protease, like the processing of NF-κB precursor[23] and possibly participation in the protein complex which migrates to the nucleus during the pre-integrational events.[24–26] Finally, HIV protease inhibitors used in combination with AZT or ddI did show synergy,[27] and such combinational therapies may prevent or delay the appearance of the viral resistance observed when single anti-viral agents are used.

To date, most of the drugs designed to inhibit HIV-1 protease show structural homologies[28] and new approaches are now sought. In a recent paper, we have analyzed the structure-activity-relationship of our new HIV-1 protease inhibitors based on the enol functionality.[29,30] Herein, we describe the dynamic kinetic between the HIV-1 protease and the two enol inhibitors, Boc-phenylalanine enol **1** and Boc-tyrosine enol **2** (Figure 1). We propose an inhibitory mechanism based on the dynamic states of the enols, i.e., the chemical equilibrium of the corresponding geometrical isomers of the enol molecule (*E* and *Z* isomers) and the corresponding ketone intermediate forms, stereoisomers with *R* and *S* configurations. Our data suggest that the enzyme is able to discriminate between the different inhibitor isomeric forms and to form a stable complex only with the *E*-enol isomer of the inhibitor.

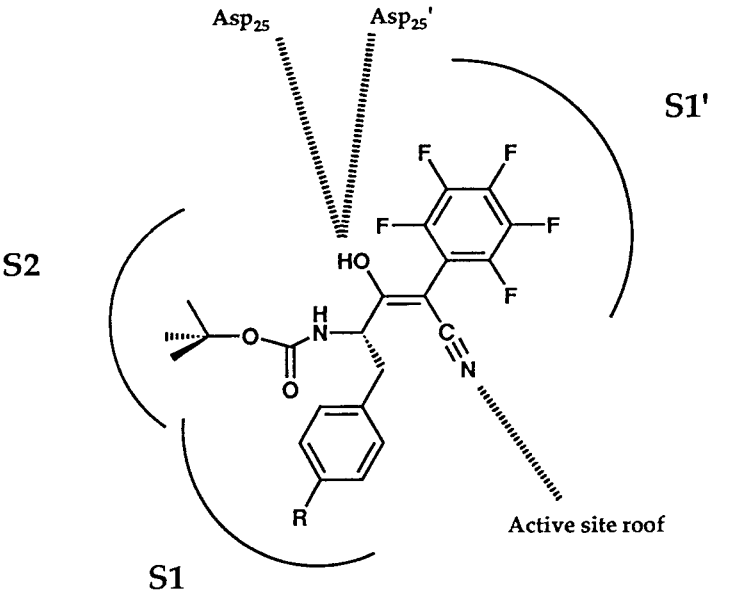
MATERIALS AND METHODS

Substrate

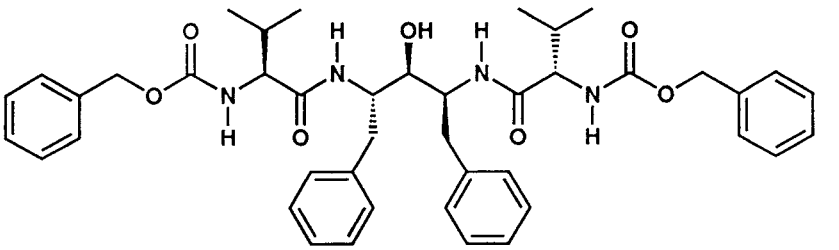
The peptide H_2N-Val-Ser-Gln-Asn-Tyr*-Pro-Ile-Val-Gln-OH (the asterix represents the cleavage site of the peptide) used as substrate for HIV-1 protease was synthesized by solid-phase method using Fmoc chemistry with an Applied Biosystem (ABI) synthesizer, Model 430A. Purification of the substrate, a nanopeptide based on the cleavage site of the gag polyprotein p17–p24, was performed on an ABI HPLC using a water-acetonitrile gradient with 0.1% TFA. Verification of the amino acid content of the pepide was performed using the Picotag derivative method of Waters.

Assays and Kinetics

The inhibitors were diluted in sodium acetate buffer 100 mM (pH 5.5), EDTA 5 mM, BSA 0.1%, NaCl 1 M containing 5% DMSO. A volume of 2 μL of the inhibitor solution was added to the substrate solutions at various concentrations for a final volume of 40 μL. The substrate was previously dissolved in the same buffer without DMSO. The $K_{i\,app}$ of inhibitors was evaluated by the Dixon plot analysis by initiating the enzymatic reaction following the addition of HIV-1 protease (final concentration 4 nM: generously provided by Dr. P.L. Darke from Merck Sharp & Dohme), unless stated otherwise. After 30 min at 37°C, 158 μL of TFA 5% were added to the reaction mixture, and the products were separated using a Brownlee 100 × 4.6 mm, C 18 reverse phase column. The progress curve experiments were performed as follows:



compound 1 R= H
compound 2 R= OH



A-74,404

FIGURE 1 Structure of our two non-peptidic lead compounds and of A-74,404 Abbott compound: Compound **1**: (*E*,*Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-2-(2,3,4,5,6-pentafluorophenyl)-5-phenyl-2-pentenenitrile. Compound **2**: (*E*,*Z*)-(4*S*)-4-*tert*-Butoxycarbonylamino-3-hydroxy-5-(4-hydroxy-phenyl)-2-(2,3,4,5,6-pentafluorophenyl)-2-pentenenitrile.

the enzyme (final concentration 4–6 nM) was added to the substrate solution, then duplicate samples of 42 μL were taken every 2.5 min and quenched with 158 μL of TFA 5%. The inhibitors were added at 10 min and samples taken for analysis after another 20 min. Separation and quantification of the products were performed as described above. When indicated, we used the Henderson equation to determine the $K_{i\,app}$ and K_i of the inhibitors based on the initial velocity (V_o) and the inhibited velocity (V_i) of the enzyme.[31]

When pepstatin was used as a control for the enzymatic assays, it generated a $K_{i\,app}$ of 28±6 nM and a K_i of 15±4 nM, which are comparable to 35 nM and 14 nM reported previously.[32]

Chemistry

The enols were prepared as described previously.[29] Briefly, the carboxyl end of the *N*-protected amino acid was activated by adding dropwise 1,1′-carbonyldiimidazole (CDI) (1.1 eq) in dry THF at 0°C. The solution was maintained at 0°C for 1 h. In parallel, sodium hydride (1.1 eq) was added to a solution of activated methylene (1.1 eq.) in dry THF at 0°C, and the reaction was allowed to proceed for 1 h. The imidazolide solution was then cooled at –78°C, and the activated methylene solution added dropwise. The solution was kept at –78°C for 1 h, and then overnight at room temperature. Separation of the product was achieved by flash chromatography.

Molecular Modeling

The crystal structure of the HIV-1 protease-A74,404 (Abbott compound) complex[33] available on the PDB CD-ROM was refined by molecular mechanics computations performed on an Indigo R4000 computer (Silicon Graphics), using the molecular mechanics package Discover (V 2.9.0, Biosym Technologies Inc., San Diego, CA), interfaced to the CVFF forcefield.

The *E*- and *Z*-enols were assumed to adopt an orientation similar to that of the A74,404 inhibitor, and so the starting docked complex was built from a superimposition of the adjacent atoms of the common hydroxyl group.

HIV-1 protease/inhibitor complexes were constructed using the Docking module of the Biosym software package, and evaluated using a molecular mechanics based energy function. Both van der Waals and electrostatic energies were evaluated continuously during docking using a 8.0 Å cutoff. The non-bonded energy between the HIV-1 protease and the inhibitor of the different docked complexes were compared, and used to determine the preferred relative orientation of the inhibitor inside the active site.

The structures were refined using the conjugate gradient energy minimizer, until the maximum derivative was less than 0.001 kcal/Å. During minimization, the following constraints were imposed: residues out of a region of 5 Å of the active site were restrained to their original positions, first by fixing the corresponding atoms until the maximum derivative was less than 0.1 kcal/Å, second by using the tethering option of Discover with a force constraint of 50 kcal/Å on the heavy atoms and then on the

backbone atoms, until the maximum derivative was less than 0.01 kcal/Å, at which point the restraints were removed.

A conformational search of the complex was performed using a combination of dynamics and minimization steps. A molecular dynamics simulation on the complex was performed in order to search for structural features that may be significant for the binding of the inhibitor.

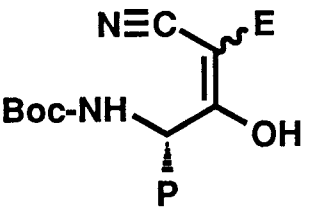
Initially, the atoms were assigned random velocities according to a Maxwell-Boltzman distribution that corresponded to the temperature of 300°K. The structure was equilibrated by running dynamics for 1 ps. Then, the simulation was allowed to proceed for 50 ps. A total of 833 configurations were sampled during the simulation. To reduce the volume of data to a more manageable level, the different conformations were clustered into similar structures. The similarity between structures was evaluated by comparing the RMS deviation for each possible pair of structures. Several distinct families of structures were encountered during the progress of the dynamics calculations. All the conformations were divided into 5 major conformational groups. One representative conformation of each family having the lowest total energy was submitted to energy-minimization and compared to the starting structure.

RESULTS AND DISCUSSION

We have recently described the inhibitory potential of a series of enolic compounds directed against the HIV-1 protease.[29] The structure-activity relationship of the enol competitive inhibitor revealed specific requirements at key positions. This study led us to synthesize highly potent non-peptidic low molecular weight HIV-1 protease inhibitors with inhibitory constants ($K_{i\,app}$) in the mid-nanomolar range. However, no detailed studies have been reported related to the inhibitory mechanism of these enol compounds with the HIV protease. Our previous studies suggested that the active moiety that interacts efficiently with the active site is the hydroxyl of the enol (Figure 1). We proposed that the highly polarized enol interacts strongly with the two aspartic acids responsible for the hydrolysis of the substrate. Evaluation of the $K_{i\,app}$ was achieved by using the Dixon plot method for competitive inhibition. We observed, however, that as the potency of these compounds increased, the $K_{i\,app}$ evaluation became more difficult and thus, less accurate (Figure 2). The inhibitory potential of compounds **1** and **2** closely fitted the tight-binding requirements, i.e. the ratio (total enzyme concentration) and K_i (inhibitory constant) $E_t/K_i > 0.01$.[34] Compounds **1** and **2** showed $K_{i\,app}$ values of 490 and 420 nM, respectively, as previously reported and evaluated by Dixon plot analysis (Table 1).[29] Because of the resulting Dixon graphs, compounds **1** and **2** were reevaluated using progressive curve analysis, in an experiment where the inhibitors were added 10 min after the beginning of the reaction. The Henderson equation was then used to evaluate the $K_{i\,app}{}^{c}$ and K_i using the progressive curve data (Table 1).

First, we evaluated the $K_{i\,app}$ of the inhibitors by using various concentrations of inhibitors and the Henderson equation. The recorded $K_{i\,app}$ are shown in Table 1 ($K_{i\,app}{}^{c}$). They varied from 2.30–8.18 μM and 1.52–2.32 μM for compounds **1**

TABLE 1
Kinetic Parameters for Enols.



1-6

Comp'd No	P[a]	E	$K^b_{i\ app}$	$K^c_{i\ app}$(SD)	$K^d_{i\ app}$	K^e_i(SD)	n[f]
1	Phenylalanine	Pentafluorophenyl	0.49	5.17 (2.10)	1.49	0.82	0.25
2	Tyrosine	Pentafluorophenyl	0.42	1.95 (0.30)	0.64	0.39	0.25[h]
3	Phenylalanine	4-nitrophenyl	2.3	n.d.[g]	n.d.[g]	n.d.[g]	0.53
4	Phenylalanine	4-(Trifluoromethyl) phenyl	n.d.[g]	62	n.d.[g]	n.d.[g]	0.53
5	Phenylalanine	3,5-Difluorophenyl	n.d.[g]	40	n.d.[g]	n.d.[g]	0.29
6	Phenylalanine	Phenyl	n.d.[g]	106	n.d.[g]	n.d.[g]	0.27
7	*N*-Acetyl pepstatin				0.028 (0.006)	0.015 (0.004)	1.04

[a]All starting amino acids are L. When not stated, standard deviations are in the range of 10–30%. [b]Evaluated using Dixon plot analysis. (μM): [c] Evaluated using progressive curves with the equation $V_o/V_i = 1 + [I]/K_{i\ app}$. ($\mu$M): [d]Evaluated using corrected values of I and the equation $V_o/V_i = 1 + [I]/K_{i\ app}$. ($\mu$M): [e]Evaluated using the Henderson equation $K_i = K_{i\ app}/(1 + [S]/K_m)$. ($\mu$M): [f]n = number of interaction sites. [g]n.d. = not determined. [h]since tyrosine does not affect the keto-enol equilibrium, it is proposed that compound **2** generates the same pseudo-Hill coefftcient value.

and **2**, respectively. These results are higher than the reported values using the Dixon evaluation (0.49 and 0.42 μM, respectively). We also performed a replot of $I(1-V_i/V_o)$ vs V_o/V_i, which determines the $K_{i\ app}$. The resulting curve generated by compound **2** is shown in Figure 3. A $K_{i\ app}$ of 2.62 μM was recorded for compound **2** while we were unable to determine the $K_{i\ app}$ of compound **1** since it generated a curve. Following these results, we proceeded to the determination of the inhibition mechanism by keeping the concentration of inhibitor **1** constant at 20 μM and by varying the substrate concentration from 0.15–1.0 mM. The resulting curves generated a positive slope indicating a competitive type of inhibition. Because of the differences in the Dixon and Henderson $K_{i\ app}$ evaluations, the kinetic behavior of the enols was further investigated.

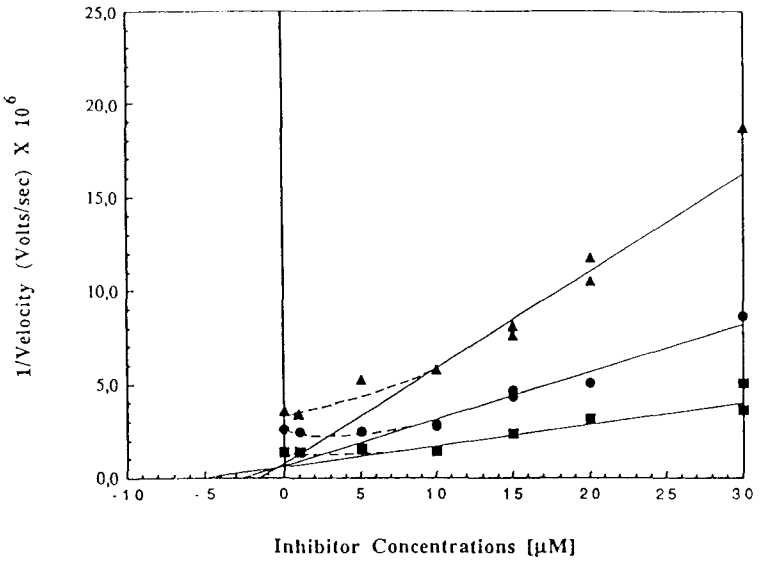


FIGURE 2 Non-linear inhibitory behavior of compound **1** analysed by Dixon plot: Enzymatic reactions were initiated by addition of the retroviral enzyme to a solution containing the substrate and the inhibitor. Reaction was allowed to proceed for 30 min and stopped by the addition of 5% TFA. Cleavage of the substrate was analysed by reverse phase HPLC using a water-acetonitrile gradient at a wavelength of 210 nm.

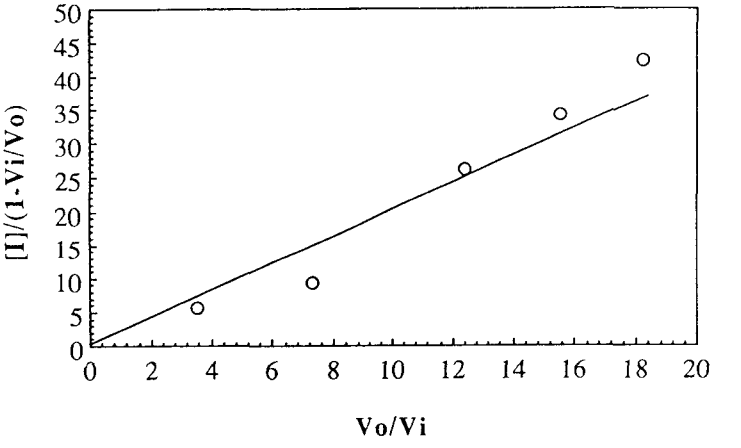


FIGURE 3 Henderson curve analysis of compound **2**: The enzymatic reactions were initiated by addition of the enzyme to the substrate solutions (0.75 mM). At 10 min, the inhibitor was added (4–40 μM) and the reaction allowed to proceed for an additional 20 min. V_o represents the initial velocity while V_i represents the velocity in presence of inhibitor **2**. The resulting slope is representative of the $K_{i\,app}$.

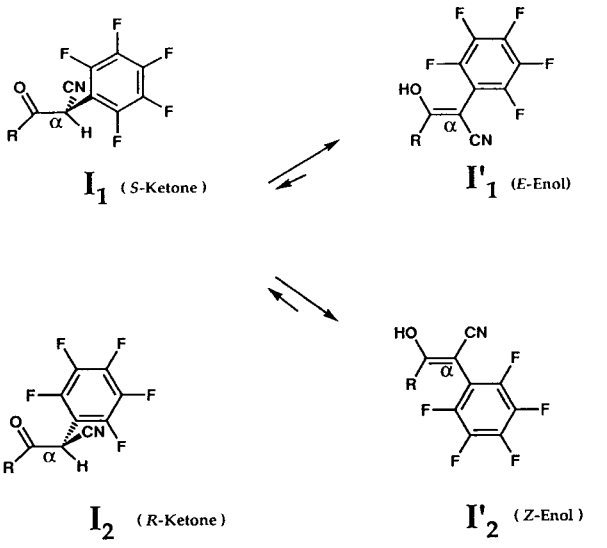


FIGURE 4 Chemical equilibrium of the enol class of molecules: The two *E* and *Z* geometrical isomers of the enol are involved in a rapid equilibrium at room temperature. Rotation at the alpha carbon is achieved by the formation of the two *R* and *S* ketone intermediates. I_1 and I_2 exist in a mixture of *R* and *S* ketone stereoisomers.

As mentioned previously, the enol chemical moiety is involved in a rapid equilibrium between two *E* and *Z* geometrical isomers. This chemical equilibrium is also composed of the corresponding *R* and *S* ketone stereoisomers (Figure 4). We propose that the physico-chemical states might be responsible for the variation of the $K_{i\,app}$ mentioned previously. Sauvé *et al.*[35] demonstrated by ^{1}H NMR that the *E* and *Z* isomers are present in very major proportion in deuterated acetone at −55°C. This suggests an equilibrium between the enol and ketone forms characterized by a rotation around the C_{alpha} carbon of the enol (Figure 4, I_2 and I_1 respectively). They also observed a rapid equilibrium between the two major *E* and *Z* geometrical isomers at room temperature. According to these data, the corresponding minor *R* and *S* ketones are thought to be intermediates.

We have previously demonstrated that as the electron-withdrawing strength of the substituents of the aromatic ring at the *E* position decreases, there is a loss of inhibitory potential (compounds **3–6**, Table 1).[29] Also, it is known that the HIV-1 protease demonstrates higher affinity for inhibitors designed with a hydroxyl group that mimics the tetrahedral intermediate when compared to the ketone as the active moiety.[36] In order to evaluate the proportion of active inhibitor isomeric form(s) (related to the capacity of the enzyme to differentiate and complex with the *E*-enol, *Z*-enol, *R*-ketone and *S*-ketone), we designed an experiment to determine the pseudo-Hill coefficients based on the modified Hill plot. Brinkworth *et al.*[37] have previously reported two binding sites on the HIV-1 protease for their non-competitive inhibitors

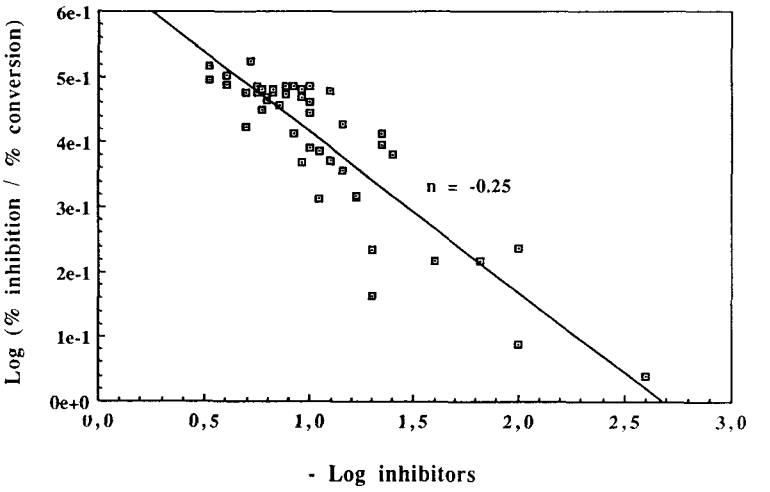


FIGURE 5 Crude data of the pseudo-Hill plot generated by compound **1**: Determination of the number of interaction sites is achieved by the modified Hill plot. Twenty-four inhibitor concentrations were used. The % conversion represents the % of substrate cleaved in the absence of inhibitor. The negative value of the slope (n) represents the pseudo-Hill coefficient.

(carboxylates) using this evaluation method. A plot of log (% inhibition/% conversion) vs -log [I] generates a negative slope which is equal to the pseudo-Hill coefficient. Figure 5 shows a representative curve for compound **1**, which generated a pseudo-Hill coefficient of 0.25. As a control, *N*-Ac-pepstatin generated a pseudo-Hill coefficient of 1.04.

The pseudo-Hill coefficient is determined by the relation that exists between the inhibitor concentration and the loss of the enzyme velocity. Any change in the presumed inhibitor concentration that interacts efficiently with the enzyme in solution will be sufficient to modulate the pseudo-Hill coefficient. The two hydroxyethylene (hydroxyl *R* and *S* configurations) diastereoisomers of peptide-based protease inhibitors can be easily separated while the two *E* and *Z* geometrical isomers of the enols **1** and **2** are in equilibrium and cannot be separated. In general, to achieve potent inhibitory potential, promising compounds have to interact in a stable fashion with the enzyme with the substituents at positions P1, P1′, P2, P2′. This is the first step in enzyme recognition of substrate or inhibitor. Parallel to this mechanism, the tetrahedral intermediate analogs have to interact strongly with the two aspartic acids to tightly maintain the complex. Side chain interactions provide high specificity for the enzyme and contribute largely to the stabilization of the inhibitors, while the enol provide mainly the interaction of the "hydroxyl" with the two aspartic acids of the catalytic site. In our case, only the *E*-enol isomer of the inhibitor appears to be able to fit into the active site as will be described in the molecular studies below. The two ketone stereoisomeric forms of the inhibitors are thought to interact weakly with the aspartic acids, as demonstrated by lower $K_{i\,app}$'s.[29]

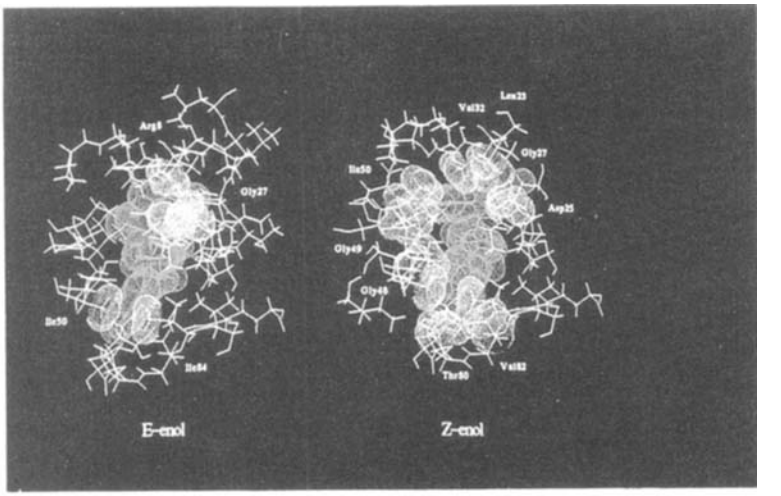


FIGURE 6A

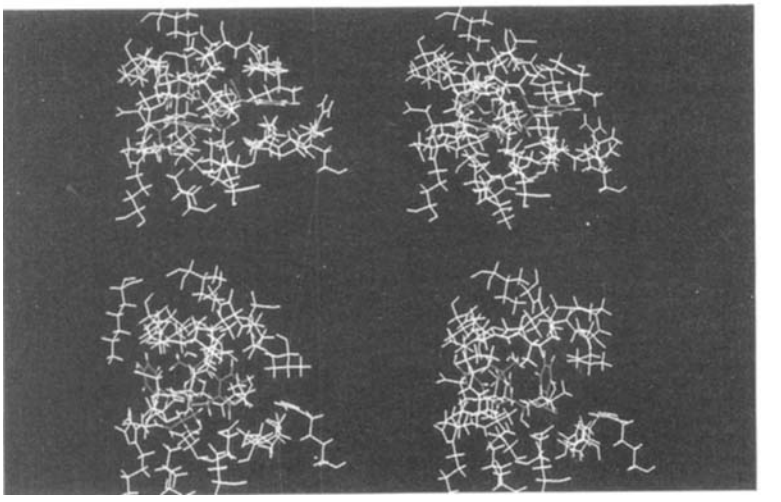


FIGURE 6B

FIGURE 6 Computer-assisted analysis of the enol docked at the active site of the retroviral protease: The analysis was performed with the InsightII and Discover programs from Biosym Technologies Inc. using the available crystallographic structure of the HIV-1 protease. (A) Repulsive interactions of the *E* and *Z*-enols of compound **1**: White shields represent the steric repulsion between the enzyme and the inhibitor. The P2 (*t*-Boc) and P1′ (pentafluorophenyl) positions of the *Z*-enol generate higher amounts of repulsive interactions. (B) Resulting energy-minimized complexes of the retroviral protease with the four isomeric forms of the enol inhibitor (compound **1**); 1) *E*-enol; 2) *R*-ketone, 3) *Z*-enol and 4) *S*-ketone. The enzyme three-dimensional structure is perturbed when complexed with the *Z*-enol compared to the *E*-enol form. See Colour Plate I.

In order to determine the type of interaction of the enolic inhibitors, we have proceeded to molecular modeling studies. Docking at the active site was performed with the two *E* and *Z* geometrical isomers of the enol. The resulting complexes are shown in Figure 6A. Regions with steric hindrance are shown in white. Steric interactions in the docked structure of the *Z*-enol resulted in a more positive non-bonded interaction energy as compared to the *E*-enol complex. The negative interactions are more evident at the active site roof position of the docked *Z*-enol.

Following the docking, the minimization procedure was performed as described earlier. The energy difference of the enzyme-inhibitor complexes following this procedure was 12 kcal/mol favoring the *E*-enol complex compared to the *Z*-enol. This procedure was also performed with the two ketone stereoisomers. All of the minimized isomers are shown in Figure 6B. From these complexes, some conclusions can be drawn. First, it was observed that the *E*-enol is in the elongated form (β-plated sheet) like the natural polypeptide substrates. Second, slight deviation is observed when the ketone form is compared with the corresponding *E*-enol form. This deviation may be caused by the less rigid structure of the ketone. Third, in the case of the *Z*-enol, large deviation is seen when compared to the *E*-enol, as the pentafluorophenyl group rotates and is oriented up in the *Z*-enol-enzyme complex. Also, for the *Z*-enol, docking did not allow efficient stabilizing interactions. Even if movements are allowed for the protease residues surrounding the inhibitor within a diameter of 5 Å, a large difference in the interaction energy favoring the *E*-enol complex is found. It is clear that the pentafluorophenyl group of the *Z*-enol which points up at the active site roof would generate an opening of it. The energy of the complexed protease with the *S*- or *R*-ketone was similar to *Z*-enol complex.

According to these results, we propose that the *E*-enol is able to efficiently complex the retroviral protease while the *Z*-enol is thought to be expelled from the active site. A closer analysis at the protease-*E*-enol complex demonstrates interactions with specific residues of the active site (Figure 7A). First, a distance of 3 Å is calculated between the oxygen of the enol's hydroxyl and the oxygens of the two aspartic acids. Second, the distance between the cyano group and the active site roof is around 2 Å. We previously suggested that the *Z*-enol opens the active site. According to Figure 7A, it is clear that the large pentafluorophenyl substituent cannot be oriented this way without substantial changes in the structure of the enzyme. Thus, the *Z*-enol is thought to be unstable at the active site.

One way to stress again on the importance of the chemical equilibrium and the presence of the polarized hyroxyl (enol form) is to calculate the pseudo-Hill coefficient of diverse electron-withdrawing groups at the P1′ position. The inhibitory potential of these compounds is shown in Table 1. Our data suggest that the presence of electron-withdrawing substituents on the aromatic ring at the P1′ position generates lower $K_{i\,app}$'s since pentafluorophenyl, difluorophenyl, *p*-trifluoromethylphenyl and *p*-nitrophenyl demonstrated higher inhibitory potential than the non-substituted phenyl group. The effect of such substituents on the pseudo-Hill coefficient was determined for compounds **3**, **4**, **5** and **6**. Even though the keto-enol equilibrium and the polarization of the enol changes as the electron-withdrawing strength varies, a general scheme can be drawn. First, it is proposed, that the evaluation of the number

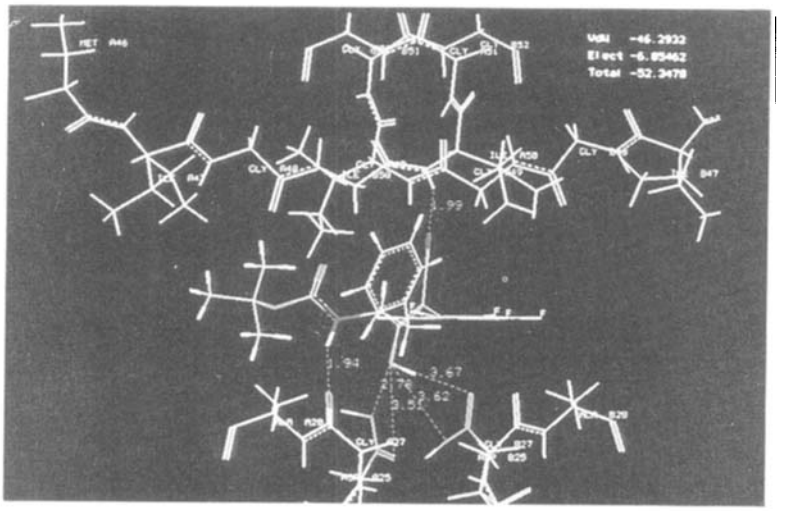


FIGURE 7A

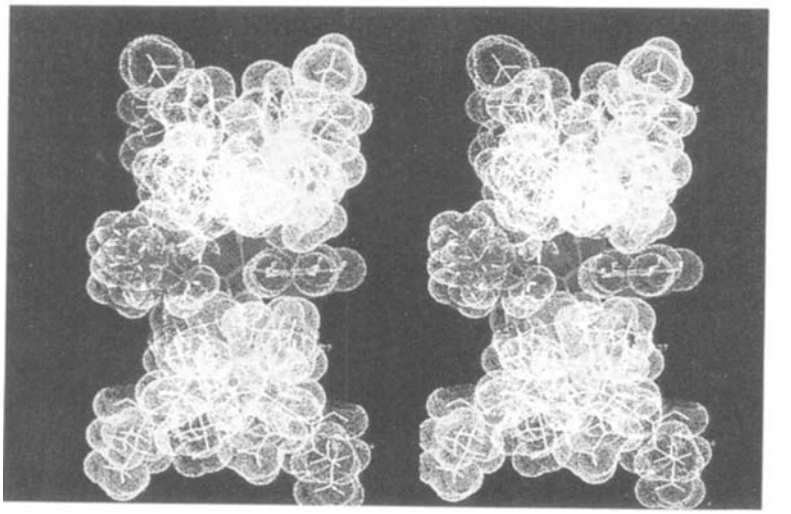


FIGURE 7B

FIGURE 7 Close view of the interaction of inhibitor **1** with the catalytic site: (A) Proposed interactions at the active site of *E*-enol inhibitor: The oxygen of the enol is at a distance of 3 Angströms from the oxygens of the two aspartic acids 25 and 25′. Also, the cyano group points up at a distance of 2 Angströms from Gly.[49] (B) Stereoview of the pentafluorophenyl group embedded at the entrance of the S1′ position: The enol inhibitors lack one carbon between the hydroxyl and the group at the P1′ position compared to peptide-based inhibitors which place the P1′ position of the *E*-enol at the entrance of the S1′ position. See Colour Plate II.

of binding sites on the enzyme will be representative of the isomeric form of the inhibitor that complexes the strongest with the enzyme. If the measured pseudo-Hill coefficients are relative to the strongest protease-inhibitor complex as we propose, it follows that the number of binding sites for this class of inhibitor will be less than 1.0 (*E*-enol fraction in solution). Second, the equilibrium involves the active moiety of the inhibitor that is thought to interact strongly with the two aspartic acids 25 and 25′ at the active site. Thus, as the electron-withdrawing strength increases at the *E* position (cyano is the other electron-withrawing group), the hydroxyl will be more polarized, which should generate a lower K_i. Third, the phenyl group at the *E* position is embedded at the active site and should not be able to rotate easily. Stabilization of the complex may be achieved by substituents at the *para* position of this aromatic ring. Since the enol lacks one carbon between the hydroxyl and the P1′ position when compared to the peptide-based HIV-1 protease inhibitors,[29] *para*-substitution at the P1′ position may stabilize the complex. The evaluation of the pseudo-Hill coefficient would have to take into account these three situations.

Compounds **1** and **2** are nearly quantitatively in the enol form and possess a highly polarized hydroxyl, respecting the second requirement. Compound **1** generated a pseudo-Hill coefficient of 0.25. It is clear that very strong interactions occur between the enol and aspartic acids 25 and 25′ of the enzyme and these interactions decrease the K_i to about 1 μM, even though the coefficient is still at 0.25. We suggest that the difference in activity between **1** and **2** is caused by stronger affinity at the P1 position by the tyrosine residue compared to the phenylalanine. Compounds **3** and **4** with coefficients of 0.53 respect the second and third requirements. The nitro an trifluoromethyl groups at the *para* position should allow a better complementarity at the P1′ position. Compounds **5** and **6** generated coefficients of 0.28. This suggests that the keto form may be more abundant (see ref. 29), but still respects the second and third requirements. Taken together, no pseudo-Hill coefficients higher than 0.5 have been measured, respecting the first requirement and also the results from the molecular modeling studies.

Our results, taken together, suggest that the enol molecule, because of its smaller backbone and the necessity of strong electron-withdrawing groups, does not have the same requirements as transition state analogs. We hypothesized that the *Z* form of the enol would force the flap region of the enzyme into the open position. This led us to suggest that only the enol in its *E* form is able to occupy instable manner the active site of the protease. The pseudo-Hill coefficients measured with compounds **3** and **4** suggest another requirement when compared to compounds **1**, **2**, **5** and **6**. Compounds **3** and **4** have substituents at the *para* position on the phenyl and generated coefficients around 0.5 (Table 1). Compounds **1**, **2** and **5** with coefficients around 0.25 also have substitutions in *ortho* and *meta* positions of the ring. A molecular model clearly shows that the pentafluorophenyl ring is embedded at the active site as seen with the stereoview of the minimized structure (Figure 7B). Since the enol lacks one or two carbons between the hydroxyl and the aromatic ring (*E* position), we propose that the *para* substituents provide additional stabilization of the inhibitor and rotation of the aromatic ring at the *E* position is limited.

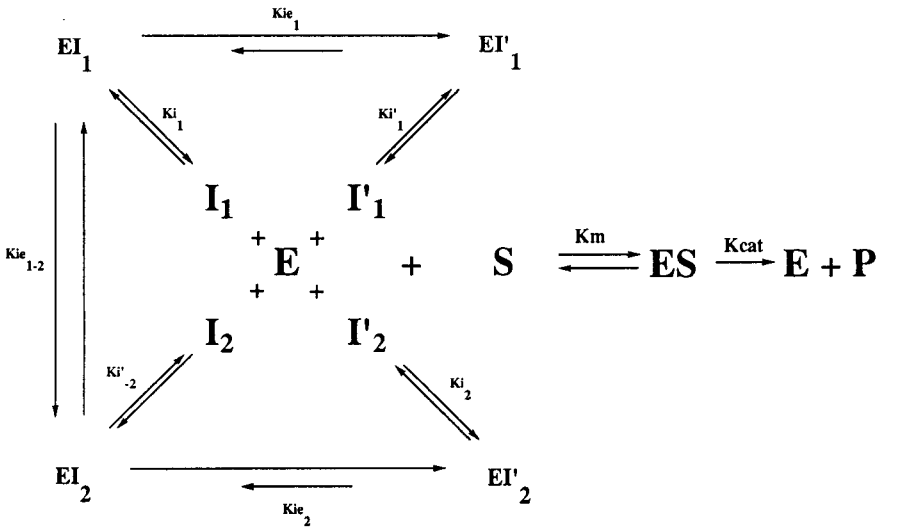


FIGURE 8 Relationship between the chemical equilibrium of the enols and the enzymatic equilibrium involved in the inhibition: This proposed mechanism takes into account the rapid equilibrium involving the geometric isomers and the inhibitory behavior of these molecules. The stable inhibitor-enzyme complex is thought to be represented by EI'_2.

This brings us to propose the following mechanism to explain why a 0.25 binding site coefficient is observed with the most potent inhibitors. The model shown in Figure 8 takes into account the dynamics of the inhibitor in solution and in complexation with the enzyme. The chemical equilibrium involves the active inhibitory moiety of the enols, and any change in the dynamic state will have a dramatic effect on their activity. Although the time elapsed for the *E* enol inhibitors at the active site can be hardly defined, it is sufficient to inhibit the activity of the protease.

According to our model, the enzyme can collide with any of the four isomeric forms of the inhibitor, form a more or less stable complex and dissociate. The equilibrium constants are represented by K_{i2}, $K_{i'2}$, K_{i1} and $K_{i'1}$ (Figure 8). Also, the inhibitor when complexed, still retains its dynamic state even though it can be stabilized by the enzyme to a greater extent in the *E* isomer. The relation between the chemical and the enzymatic equilibrium is represented by the equilibrium constants K_{ie2}, $K_{ie\ 1-2}$ and K_{ie1}. According to this model and the results obtained with the modified Hill plot, we propose that 0.25 coefficient is representative of the active proportion of the inhibitor in solution that at any time is in complex with the HIV-1 protease. A plot of $I_a/(1-V_i/V_o)$ vs V_o/V_i (Henderson equation) obtained by multiplying the total concentration of inhibitor with the pseudo-Hill coefficient (I_a = active proportion of inhibitor) generated a K_i of 0.82 μM for compound **1** and is thought to be represented by $K_{i'1}$. This is, to our knowledge, the first proposed mechanism based on data and molecular modeling of inhibitors that involves dynamic states of the active moiety. The same mathematical treatment has been applied to compound **2**, assuming that the equilibrium is not changed by the tyrosine group which replaces the phenylalanine.

This led us to calculate a K_i of 0.39 μM. These K_i values are more consistent with the IC_{50} values recorded with these inhibitors, i.e. 19 μM and 3 μM for **1** and **2**, respectively.

We have completed the characterization of the enols with our two best inhibitory compounds **1** and **2**. In this study, MT_4 cells acutely infected with a clone of HIV-IIIB called UHC-1 (provided by Dr M.A. Wainberg, Lady Davis Institute, Montréal, Canada) were treated with various concentrations of compounds **1** and **2**.[38] Both showed antiviral potential below their cytotoxic level in MT_4 cells (compound **1**: IC_{50} of 24 μM and compound **2**: IC_{50} of 40 μM). Compound **1** also inhibited the polyprocessing of the gag and gag-pol polyproteins in transfected COS cells at a concentration of 50 μM. These data clearly show the effectiveness and application of the enol in enzyme and viral inhibition even though more potent enol inhibitiors are needed for better viral inhibition. Our pioneered work on the enol inhibitors of HIV-1 protease followed by the recently published work with cyclic enols directed against the HIV-1 protease with K_i below 50 nM[39] suggest useful therapeutic applications for this class of compounds. The enols are part of many important biological processes and can be expected to be useful in the development of drug targeted to enzymes other than the HIV-1 protease.

CONCLUSION

In this study, we have shown that inhibitors with dynamic states involving the active moiety still retain their inhibitory potency. Not only does the equilibrium have a dramatic effect on such compounds, but also the size and electron-withdrawing character of the aromatic ring substituents that would displace this equilibrium. By analyzing the pseudo-Hill coefficients, a general scheme can be drawn. First, the transition state analog interacts strongly with aspartic acids 25 and 25′ of the enzyme, and this interaction greatly stabilizes the inhibitor-enzyme complex, as seen with compounds **1** and **2**. This interaction is thought to be the strongest one. Second, since the group at the *E* position of these compounds is very large (aromatic rings), the *Z* isomer of the enol is thought to be expelled from the active site. The 2 Å distance between the cyano group and Gly 49 does not allow such a rotation and large structural modifications of the enzyme are observed with the *Z*-enol complex. The *Z* isomers are thought to decrease the relative pseudo-Hill coefficients. Third, because of the shorter backbone compared to the peptide-based inhibitors, the *E* group is not perfectly located in the P1′ groove site. *Para*-substituted rings would therefore increase the coefficient as they would stabilize the inhibitors at the active site, as seen with compounds **3** and **4**. Even though determination of the pseudo Hill-coefficient does not modify the potencies of these inhibitors, we believe that the highlight of such inhibitory behavior has to be taken into account when inhibitors are designed which involve a dynamic moiety.

Finally, the enol family of compounds are found in many biological processes. We have demonstrated that the enol can be used in drug development involving aspartic proteases and possibly many other classes of enzymes with pharmaceutical interest.

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