Docking studies reveal a selective binding of D-penicillamine to the transactivator protein of human immunodeficiency virus type 1☆

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Received 14 November 2001; revised 14 February 2002; accepted 14 February 2002

First published online 4 March 2002

Edited by Hans-Dieter Klenk

Abstract ***DOCK*** **and** ***Affinity*** **studies were carried out to study the binding of D- and L-penicillamine to the transactivator protein (tat) of human immunodeficiency virus type 1 (HIV-1). These studies reveal a selective binding of D-penicillamine to the cysteine-rich region covering amino acid residues 20–38 of the tat protein. A careful analysis of the components of the binding energy of the D- and L-isomers reveals that the D-isomer has a more favorable van der Waals interaction resulting from an optimal placement of the dimethylthiomethyl side chain in the binding site. This observation matches the experimental data that D-penicillamine is a more potent inhibitor of tat-mediated transactivation than the L-isomer. The docking and experimental data offer an interesting approach to design structural molecules with potential application to block signal functions of the tat protein in HIV-1 pathogenesis.**

Key words: D-Penicillamine; Human immunodeficiency virus type 1 transactivation; tat protein; Docking

1. Introduction

The tat protein encoded by the human immunodeficiency virus type 1 (HIV-1) is a potent transactivator of gene expression from the viral long terminal repeat (LTR). The domains that are essential for transactivation, a Pro-xaa$_3$-Pro triad, a cysteine-rich metal binding sequence motif, and a cluster of basic residues, are present within the N-terminal 57 residues of tat protein. In recent years, additional functions for tat have been detected which are attributed to its extracellular release from infected cells. Signalling from the extracellular tat protein can activate a range of cellular genes in uninfected cells leading to immune suppression and T-cell apoptosis, a condition commonly found in patients with HIV-1 infection. The approaches leading to abolish the intracellular and intercellular signalling mechanisms of tat protein, in recent years, have become a focus of experimentation [1–10]. The first reported inhibitor of tat-mediated transactivation possessing anti-HIV activity was D-penicillamine, a structural analog of cysteine [2]. The present communication is an evidence, based on *DOCK* and *Affinity* studies, that D-penicillamine fits in the tat sequence comprising residues 20–38, whereas the L-isomer does not exhibit this selectivity. These observations are in good agreement with the experimental data reported here.

2. Materials and methods

2.1. Materials

The culture medium (RPMI 1640) and fetal calf serum were supplied by Gibco (Eggenstein, Germany). [^{14}C]Acetyl-CoA (4 mCi/mmol) was obtained from NEN/Dupont (Dreieich, Germany). All other reagents were analytic-grade obtained from Merck (Darmstadt, Germany) or from Serva (Heidelberg, Germany). Inhibitors were dissolved or diluted in water and stored as stock solutions at −20°C.

2.2. Plasmids

The tat gene (clone 1) was cloned by way of cDNA cloning, using poly(A)-selected RNA from HIV-1 (BH 10) infected cells [2,11]. The HIV-1 LTR-CAT construct was obtained by inserting clone 15 DNA into pSV0-CAT at the *Hin*dIII site [11,12] and the resulting plasmid was termed pC15CAT. The plasmid pCV1 was obtained by inserting viral cDNA containing the tat gene (clone 1) into the mammalian expression vector pCV which contains duplicated SV40 origins of replication, adenovirus major late promoter, splice sites from adenovirus and mouse immunoglobulin genes, mouse dihydrofolate reductase cDNA, and SV40 polyadenylation signal [11,12]. Plasmids were isolated according to the alkaline lysis method [13,14] and purified by anion-exchange chromatography using Qiagen columns (Qiagen, Hilden, Germany).

2.3. Transfection and CAT assay

Jurkat cells (1×10^7) were washed with serum-free medium and incubated at 37°C for 1 h in 1 ml serum-free medium containing 50 mM Tris–HCl (pH 7.3), DEAE-dextran (M_r 500 000, 250 μl/ml) and 15 μg of each plasmid DNA, pCV1 and pC15CAT. The cells were then washed with growth medium (without serum) and incubated in 10 ml serum-containing medium at 37°C. At this time, the test compounds were added at the desired concentrations in parallel batches. All transfections were performed in triplicate for each set of experiments. The processing of cell cultures and the procedure of CAT assays are described in detail elsewhere [7]. Briefly, 44 h after the incubation, cells were washed with phosphate-buffered saline and suspended in 80 μl of 0.25 M Tris–HCl (pH 7.8), and cellular extracts were prepared by three cycles of freezing (in liquid nitrogen) and thawing (37°C). All cellular extracts were adjusted to equal protein concentration and heated for 15 min at 65°C to inactivate deacetylases in the extract. For CAT assays, 30 μl of the cell extract was mixed with 20 μl of 100 mM Tris–HCl (pH 7.8) in a 6 ml glass scintillation vial. The vial was transferred to a water bath set at 37°C and 200 μl of freshly prepared CAT reaction mixture (100 μl of 0.25 M Tris–HCl,

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☆ Dedicated to Dr. Robert C. Gallo on his 65th birthday.

0014-5793/02/$22.00
PII: S0014-5793(02)02468-7

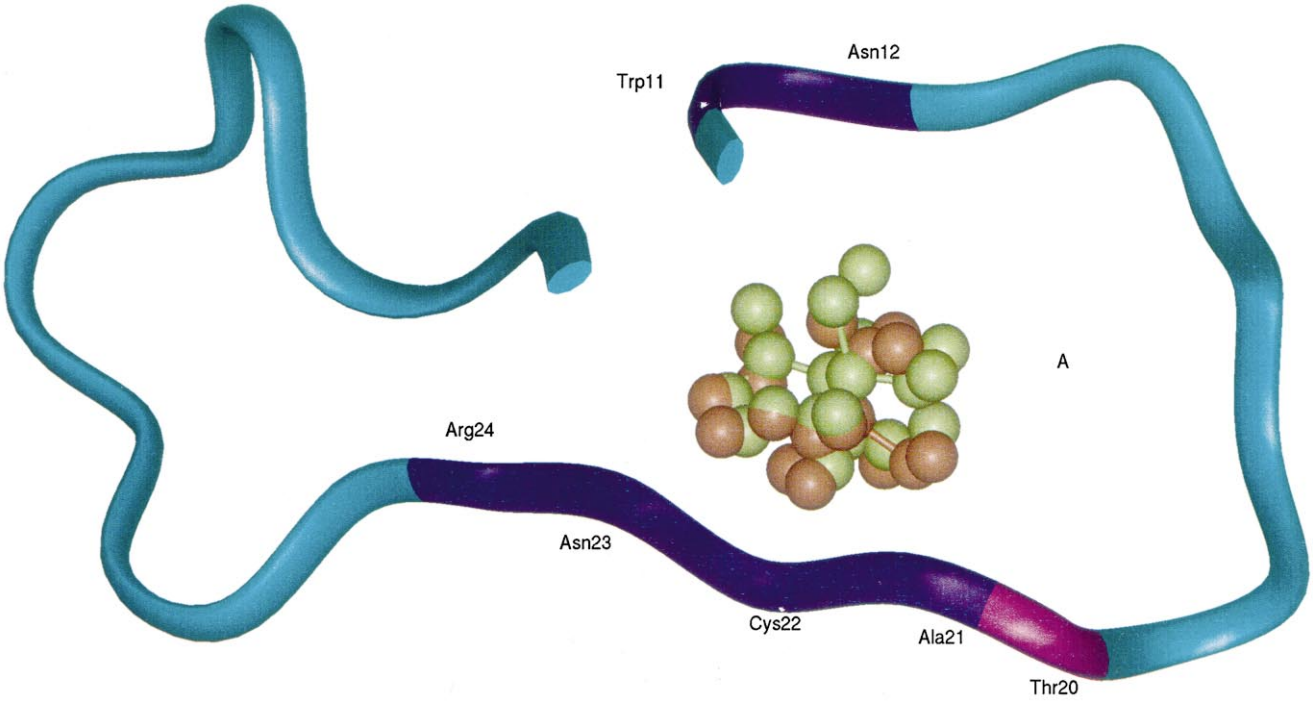

Fig. 1. Binding orientation of D-penicillamine (green) and L-penicillamine (brown) in region A of HIV-1 tat protein. The cysteine-rich region of the protein has been drawn as a ribbon, the residues of region A in the protein are also indicated.

pH 7.8, 50 μl of 5 mM chloramphenicol, 10 μl [^{14}C]acetyl-CoA, 0.1 μCi, and 40 μl water) was added. To this mixture, 5 ml of a water-immiscible scintillation fluid (Econofluor, NEN/Dupont) was carefully overlain and incubated at 37°C for 4 h.

2.4. Docking studies

The structure of HIV-1 (Zaire-2 isolate) tat determined by nuclear magnetic resonance and deposited in the Protein Data Bank was used for docking studies [15]. The protein structure was minimized with the CVFF force field [16] with the backbone atoms tethered by a force constant of 100 kcal/mol/Å^2 to a gradient of 0.01 kcal/mol/Å. Docking of both D- and L-penicillamine was carried out using *DOCK* 4.0 [17], where rough possible binding sites were discovered, which were refined by *Affinity* (MSI, USA). This strategy was followed for reasons mentioned below.

To balance between computational time and accuracy, *DOCK* uses a very simplified and approximate scoring function [18]. The program has been successfully used to generate starting rough binding modes, which can be further evaluated by other more accurate methods [19]. *Affinity* uses molecular mechanics force field in both docking and scoring, besides incorporating solvation effects [20]. This makes *Affinity* accurate at determining and evaluating final docking conformations [20]. On the other hand, it is not very efficient in sampling a large conformational space, so rough binding modes should be provided by a method like *DOCK* [20].

For *DOCK* studies, the molecular surface (Connolly Surface) of the binding site comprising residues 20–38 was prepared by the *SYBYL* program *MOLCAD*, with a probe radius of 1.4 Å. After refinement, a cluster of 46 spheres was enclosed in a grid of dimensions 34×34×29 Å^3 [17] with 0.3 Å spacing. This grid stores the steric and electrostatic information of the receptor atoms used for 'force field scoring' of the orientations determined by *DOCK*. Both 'rigid' and 'flexible' docking of the molecules was carried out with the protocol described in the *DOCK* manual.

The docking protocol in *Affinity* is based on Monte Carlo and Simulated Annealing techniques. Ligand–receptor interactions are evaluated either by the GRID/MM method of Luty et al. [21], which also incorporates the solvation model of Stouten et al. [22]. The docking procedure followed in *Affinity* was as prescribed in the manual.

3. Results and discussion

All possible binding orientations for D- and L-penicillamine clustered in two main regions, which we label as A and B. The amino acid residues composing these sites are listed in Table 1. A 6 Å radius from the center of mass of the inhibitor was used to define these sites.

The results given in Table 2 indicate that *DOCK* is unable to really distinguish between the two isomers, their estimation of binding energies is very nearly the same. This is not surprising, since as mentioned earlier, *DOCK* does not use a very

Table 1
Composition of sites A and B and H-bonds between inhibitor[a] and residues in the binding site

Isomer of penicillamine	Site A residues/H-bonds	Site B residues/H-bonds
D	Trp^{11}, Asn^{12}, Ala^{21}, Cys^{22}, Asn^{23}, Arg^{24} N^8...H-N(Cys^{22}) H^5...O=C(Cys^{22})	Pro^{10}, Trp^{11}, Asn^{12}, Ala^{21}, Cys^{22}, Asn^{23}, Arg^{24}, Gln^{35}, Val^{36} N^8...$^{\xi}$H-N(Arg^{24}) H^5...O=C(Cys^{22})
L	Asn^{12}, Thr^{20}, Ala^{21}, Cys^{22}, Asn^{23}, Arg^{24} N^8...H-N(Cys^{22}) H^5...O=C(Cys^{22})	Pro^{10}, Trp^{11}, Asn^{12}, Cys^{22}, Asn^{23}, Arg^{24}, Gln^{35}, Val^{36} N^8...H-N(Val^{36}) H^{82}...$^{\xi}$H-N(Arg^{24}) O^7...$^{\varepsilon}$H-N(Gln^{35})

[a]The atoms of penicillamine have been labelled in Fig. 3.

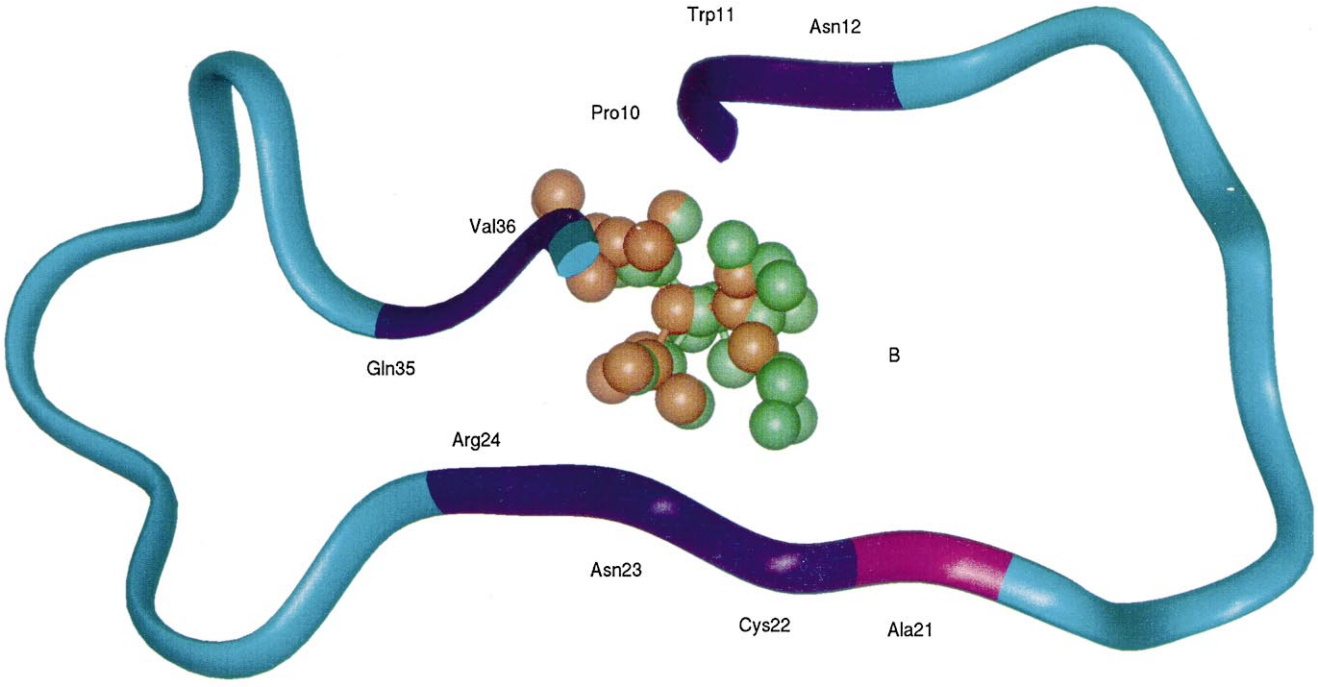

Fig. 2. Binding orientation of D-penicillamine (green) and L-penicillamine (brown) in region B of HIV-1 tat protein. The cysteine-rich region of the protein has been drawn as a ribbon, the residues of region B in the protein are also indicated.

accurate force field for the energy estimation. The two '*DOCK* best scored structures' for both the isomers were then subjected to more accurate binding studies by *Affinity*.

The results of *Affinity* studies are summed up in Table 2. It is evident from this table that the true binding energies of the D- and L-isomers in both regions A and B are clearly differentiated, with the D-isomer predicted to bind more firmly than the L-form. It can also be seen that, for both isomers, site A is preferred over B for binding. At site A, the D-isomer is seen to bind more firmly by about 3.7 kcal/mol, while the differentiation extends to almost 9.7 kcal/mol at site B. A break-up of the components of the binding energy for the D- and L-isomers shows that it is mainly the van der Waals interaction which accounts for the differences in their binding energies. A careful inspection reveals that the D-isomer has a more favorable van der Waals interaction resulting from an optimal placement of the dimethylthiomethyl side chain in the binding site. This is visible in Figs. 1 and 2, where the binding orientations for both D- and L-penicillamine in the tat protein binding sites A and B have been depicted.

The H-bonds made by the inhibitors with transactivator protein are also mentioned in Table 1. H-bonding with

Table 2
Binding energies of D- and L-penicillamine by *DOCK* and *Affinity*

Region	Energy (kcal/mol)											
	DOCK 4.0						*Affinity*					
	D-Penicillamine			L-Penicillamine			D-Penicillamine			L-Penicillamine		
	VDW	Ele	Total	VDW	Ele	Total	VDW	Ele	Total	VDW	Ele	Total
A	−12.1	−2.3	−14.4	−11.7	−2.9	−14.6	−5.4	−6.1	−11.5	−1.8	−6.0	−7.8
B	−10.6	−3.2	−13.8	−10.7	−3.1	−13.8	3.3	−3.2	0.1	12.7	−2.9	9.8

Table 3
Effects of D- and L-penicillamine on LTR (HIV-1)-directed CAT expression catalyzed by the tat protein

Experiment		pg CAT/10 μg protein	
		D-Penicillamine	L-Penicillamine
Without penicillamine		8200 ± 905	
With penicillamine (mM)	0.066	1025 ± 117	7896 ± 817
	0.132	688 ± 64	5305 ± 590
	0.198	516 ± 57	3977 ± 305
	0.264	344 ± 29	2665 ± 246

The values are means ± S.E.M. from three experiments, each experiment involving three individual transfections (total number of values = 9). Experimental details are described under Section 2.

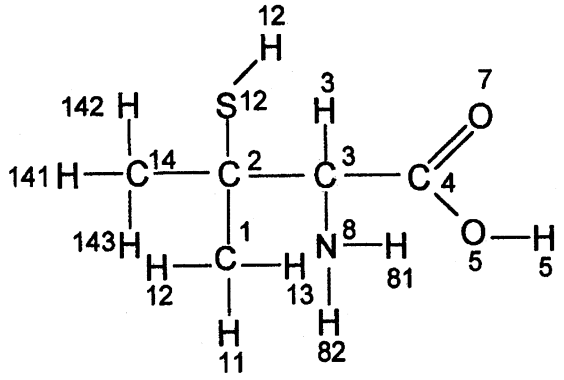

Fig. 3. Atom labels for penicillamine.

Cys^{22} is predominant, suggesting reaction with this residue through covalent linkages may very likely be contributing to the means of inhibition of these compounds.

The identification of the putative binding modes for both the D- and L-forms of penicillamine using *DOCK* and *Affinity* is in favor of the experimental observations. The transactivation of LTR (HIV-1) by the tat protein was studied in Jurkat cells in the presence of D- and L-penicillamine. The expression of CAT gene linked to LTR (HIV-1) was a measure of transactivation. As follows from Table 3, both the isomers were able to inhibit the tat-mediated transactivation; however, D-penicillamine, especially at low concentrations, has a far better inhibitory potential. The selectivity of the penicillamine effect on the transactivation is documented by the cell-toxicity studies, reported earlier [23]. Both the isomers of penicillamine had no effect on the growth of H9 cells, a human T-cell line (ATCC HTB 176), at a concentration as high as 1 mM; only at very high concentrations inhibition of cell growth was observed. Based on these [23] cytotoxic data, we have calculated the selectivity index (SI). SI is the quotient obtained by dividing the concentration (mM) exhibiting a 50% cytotoxicity (CD_{50}) by the concentration inhibiting gene transactivation to 50% (ID_{50}); the SI (CD_{50}/ID_{50}) for D-penicillamine was calculated to be 117.77 and for L-penicillamine was 21.96. Based on these calculations one can conclude that the D-penicillamine effect on transactivation is, indeed, due to its selective interaction with the tat molecule.

The observations reported here help to understand the behavior of the two isomers towards the receptor and their activities. It matches the experimental data that D-penicillamine is a more potent inhibitor of tat-mediated transactivation than the L-isomer. The L-isomer, even in traces, is highly toxic due to its incorporation into cellular proteins [24]. The neurotoxicity of L-penicillamine is well documented in the literature [25–27]. On the other hand, D-penicillamine is being used since 1956 as a therapeutical agent in the long-term treatment of Wilson's disease, cystinuria, rheumatoid arthritis, primary biliary cirrhosis and heavy metal poisoning [28]. The docking studies could be of great help in predicting structures which could be applicable to block transactivation, leading to the search for better inhibitors of HIV-1 replication.

Acknowledgements: We acknowledge with thanks the computational facilities provided by the All-India council of Technical Education (AICTE) and the Ministry of Human Resource and Development (MHRD) at Bombay College of Pharmacy. Transactivation studies were financially supported by grants (P.C.) from the American Foundation of AIDS Research (USA), the Bundesgesundheitsamt (Berlin, Germany) and the Ministry of Science and Culture of the State of Hessen. We thank the council of Scientific and Industrial Research (New Delhi, India) for support under Emeritus Scientist's scheme (A.S.) and a Senior Research Fellowship to M.K. We are grateful to Dr. S.K. Arya (National Cancer Institute, Bethesda, MD, USA) and Dr. F. Wong-Staal (University Medical School, San Diego, CA, USA) for the plasmids pCV1 and pC15CAT.

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