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D-Penicillamine inhibits transactivation of human immunodeficiency virus type-1 (HIV-1) LTR by transactivator protein

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D-Penicillamine, an amino acid analogue of cysteine, has been shown to inhibit the transactivation of HIV-1 LTR by the transactivator protein, tat protein. The transactivation was studied in Jurkat cells co-transfected with plasmids containing HIV-LTR sequences fused to the bacterial chloramphenicol acetyltransferase (CAT) gene and HIV *tat* gene. The expression of CAT activity was a measure of transactivation of LTR by the tat protein. Incubation of transfected Jurkat cells with D-penicillamine led to inhibition of CAT activity. This inhibition was found to be concentration-dependent; more than 90% inhibition of chloramphenicol acetylation was seen in extracts prepared from cultures incubated with 40 μg/ml of D-penicillamine. Earlier experiments have shown that D-penicillamine at 40 μg/ml can completely inhibit HIV-1 (HTLV-III B) replication in H9 cells [(1986) Drug Res. 36, 184–186]. These results suggest that inhibition of transactivation may be the molecular mechanism involved in the inhibition of HIV-1 replication by D-penicillamine.

D-Penicillamine; HIV-1; Transactivation; tat protein: Azidothymidine; AIDS

1. INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is the major cause of acquired immunodeficiency syndrome (AIDS). It is a retrovirus but differs from most known animal retroviruses in the complexity of its genomic organization. In addition to the known *gag*, *pol* and *env* genes, HIV-1 has at least five more genes that contribute to a system regulating replication and infectivity of the virus. One of these genes, *tat*, appears to play a central role in viral gene expression and replication [1–11]. This suggests that transactivation may be an ideal target for potential anti-HIV agents in AIDS therapy.

Transactivator is the protein product of the *tat* gene, which has been identified as a 15 kDa polypeptide in HIV-infected cells requiring only 56 amino acids for its activity. The domain essential for transactivation has some characteristic features which are suggestive of a potential nucleic acid-binding protein. Firstly, the functional domain in tat protein has an abundance of basic over acidic residues (16:3), which could mediate binding to the negatively charged backbone of a DNA segment, designated as the TAR sequence located within the HIV-1 LTR. Secondly, this region also contains a cluster of seven cysteine residues which may comprise a metal-binding domain important to nucleic acid binding. The differential ability of HIV-1 (tat) and HIV-2 (tat) to transactivate some of the same LTRs [10] supports the binding of tat protein to specific sequences in LTR.

Here, we investigated the effect of D-penicillamine on the transactivation of HIV-1 (LTR) by the tat protein. The studies were carried out in Jurkat cells which were co-transfected with plasmids containing HIV-1 (LTR) and *tat* gene sequences. For measuring transactivation, the HIV-1 (LTR) was fused to the indicator gene, chloramphenicol acetyltransferase (CAT). The transactivation of the LTR-linked CAT gene was measured by

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conversion of chloramphenicol to its acetylated forms by the enzyme synthesized in transfected cells.

2. MATERIALS AND METHODS

D-Penicillamine was a synthetic product obtained from ASTA Pharma (Frankfurt). Azidothymidine (3′-azido-3′-deoxythymidine, AzT) and diethylaminoethyl-dextran (DEAE-dextran, M_r 500000) were purchased from Sigma (Deisenhofen). The culture medium (RPMI 1640) and fetal calf serum were supplied by Gibco (Eggenstein). ^{14}C-labeled chloramphenicol (40–60 mCi/mmol) was obtained from NEN/Dupont (Dreieich). Thin-layer chromatography sheets were from Merck (DC plastikfolien no. 5748, Kieselgel 60; Darmstadt).

2.1. *Plasmids*

The HIV-1 LTR (clone C15) and *tat* gene (clone 1) were cloned by way of cDNA cloning, using poly(A)-selected RNA from HIV-1 (BH 10) infected H9 cells. The C15-CAT was obtained by inserting clone 15 DNA into pSV0-CAT at the *Hin*dIII site [12,13], and 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 [14] which contains duplicated SV40 replication origin (*ori*), adenovirus major late promotor, splice sites from adenovirus and mouse immunoglobulin genes, mouse dihydrofolate reductase cDNA, and SV40 polyadenylation signal. The plasmid pSV2CAT contains SV40 early promotor 5′ to the CAT gene [15].

2.2. *Transfection and CAT assays*

About 1×10^7 Jurkat cells 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 (250 μg/ml) and 15 μg of each plasmid DNA, pCV1 and pC15CAT or pSV2CAT. 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, D-penicillamine or AzT, were added at the desired concentrations in parallel batches. 48 h after the incubations, 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 ethanol and dry ice) and thawing (37°C). All cellular extracts were adjusted to equal protein concentration. The activity of CAT was measured by incubating 20-μl aliquots of extracts with ^{14}C-labeled chloramphenicol (Cm) and 2.5 mM acetyl-CoA at 37°C for 1 h, and separating the acetylated chloramphenicol (AcCm) from the unacetylated form by ascending thin-layer chromatography. The chromatogram was autoradiographed and spots cut from the plate were quantitated by scintillation counting. All transfections were performed in triplicate for each set of experiments.

3. RESULTS

Potential metal-binding domains are known to exist in proteins which interact with nucleic acids.

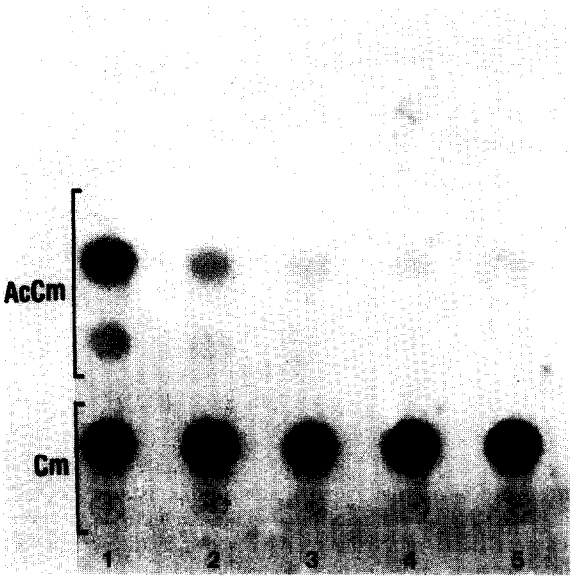

Fig.1. Effect of D-penicillamine on LTR (HIV-1)-directed CAT expression catalyzed by tat protein. About 1×10^7 Jurkat cells were transfected by the DEAE-dextran protocol with 15 μg each of pC15 (LTR-CAT) and pCV1 (tat) plasmids. Transfected cells were cultivated for 48 h without D-penicillamine (lane 1), or with the following concentrations of D-penicillamine: (lanes) 2, 10 μg/ml; 3, 20 μg/ml; 4, 30 μg/ml; 5, 40 μg/ml. After 48 h, cell extracts were analyzed for CAT activity by the conversion of labeled chloramphenicol (Cm) to acetylated chloramphenicol (AcCm). Acetylated and unacetylated forms were separated by ascending thin-layer chromatography. Shown is a 20 h exposure of the thin-layer plate to X-ray film (Kodak X-Omat S) at −80°C.

These proteins have a characteristic feature in possessing cysteine-rich regions at the binding site [16]. HIV-1 has two proteins which exhibit this character, and are known to bind to nucleic acids. Firstly, the nucleic-acid binding protein encoded by the *gag* gene, and secondly, the transactivator protein encoded by the *tat* gene [17]. Since tat is essential for HIV-1 replication, it provides an attractive target for drug design. D-Penicillamine, an amino acid analog of cysteine, is known to interact with cysteine-rich proteins [18–20]. This interaction occurs by the formation of interdisulfide bonds between D-penicillamine and cysteine or cystine, and such penicillamine-protein complexes are highly stable as suggested by the fact that cysteine or cystine added after D-penicillamine cannot remove the drug from proteins [21]. The chelation of metals by D-penicillamine is the basis of its ap-

Table 1

Effect of D-penicillamine on the LTR (HIV-1)-directed expression catalyzed by the tat protein

Experiment	Percent conversion of chloramphenicol to acetylated forms
Without D-penicillamine (control)	23.33 ± 3.5 (100)
With D-penicillamine (μg/ml)	
10	2.9 ± 0.33 (12.4)
20	1.97 ± 0.29 (8.4)
30	1.46 ± 0.22 (6.25)
40	0.97 ± 0.15 (4.15)

Percent conversion of chloramphenicol to acetylated forms was calculated by dividing the total number of counts in the AcCm region (cf. fig.1) with that found in the Cm and AcCm regions. This coefficient multiplied by 100 is shown as the percent value for the acetylated product. Values are means ± SD for 3 independent transfection experiments. Experimental procedures were the same as described in fig.1 and section 2. Values in parentheses indicate the percent of control, with the control being taken as 100

plication in the treatment of Wilson's disease [22]. The interaction of D-penicillamine with cysteine-rich proteins and its chelating potential motivated us to examine its effect on the replication of HIV-1. D-Penicillamine has been shown to block HIV replication in vitro [23] and it suppresses virus replication in AIDS patients [24–26]. To investigate the mechanism of anti-HIV activity we have studied the effect of D-penicillamine on HIV-LTR transactivation by the tat protein.

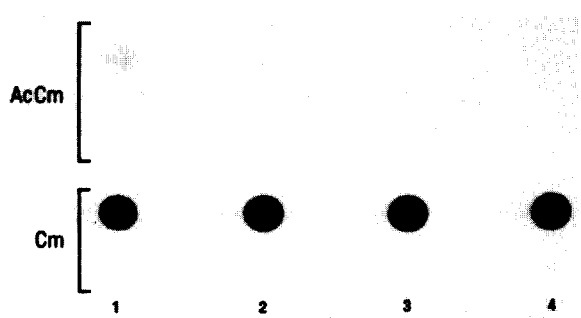

Fig.2. Measurement of CAT expression in Jurkat cells transfected with pCV1(tat) + pSV0CAT (lane 1), pSV2-CAT alone (lane 2), pCV1(tat) + pSV2CAT (lane 3); lane 4 has the same experimental set up as lane 3, except that cells were cultivated with D-penicillamine (40 μg/ml). Experimental procedures were the same as described in fig.1 and section 2.

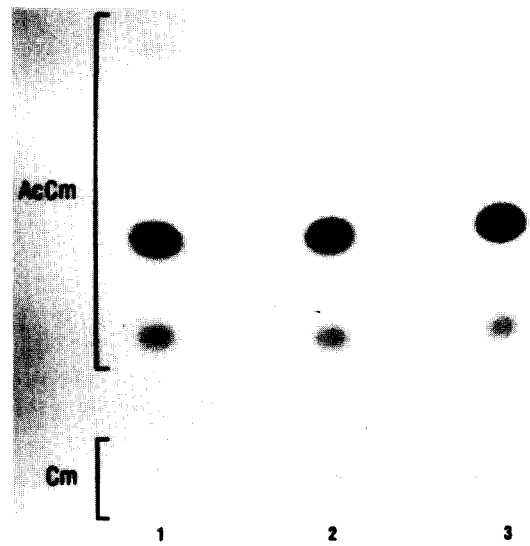

Fig.3. Effect of azidothymidine (3′-azido-3′-deoxythymidine: AzT) on LTR (HIV-1)-directed CAT expression catalyzed by tat protein. Transfected Jurkat cells were cultivated without AzT (lane 1), or with the following concentrations of AzT: lane 2, 10 μM; lane 3, 25 μM. All other procedures were as described in fig.1 and section 2.

Jurkat cells transfected with plasmids pC15CAT and pCV1 express CAT, as shown in fig.1 (lane 1). Cells incubated with different concentrations of D-penicillamine (10–40 μg/ml) exhibit concentration-dependent inhibition of CAT expression (lanes 2–5). The quantitative values for the amounts of acetylated chloramphenicol in each set of experiments are listed in table 1. As follows from these results, more than 90% inhibition of CAT expression is shown by D-penicillamine at 40 μg/ml. Considering the fact that the mean deviation between three sets of experiments for each probe was in the range 12–15%, we could consider almost a total inhibition of CAT production at 40 μg/ml.

In an earlier communication [23] we reported the cytotoxicity of D-penicillamine towards H-9 cells. We found that concentrations up to 200 μg/ml of D-penicillamine have no effect on the growth of H-9 cells; at 500 μg/ml 32% inhibition of cell growth was observed. Thus, the concentration which totally inhibits transactivation has no effect on the growth of Jurkat cells.

To document the specificity of our co-transfection assay, we designed experiments where the cells were transfected with the tat clone (pCV1) and other plasmids without the HIV-LTR region, such as pSV0-CAT and pSV2CAT. As follows from fig.2, there is no CAT activity in cells transfected with pSV0CAT and pCV1 (lane 1), pSV2CAT alone (lane 2), pSV2CAT and pCV1 (lane 3). The experiment depicted in lane 4 had the same set-up as in lane 3, except that these cells were treated with D-penicillamine (40 μg/ml). These results confirm that LTR sequences of HIV-1 are necessary for the transactivating function of tat protein.

Recently, Felber and Pavlakis [27] have reported a quantitative bioassay for HIV-1 based on transactivation, using human indicator lymphoid cell lines that contain integrated copies of HIV (LTR)-CAT. This system has been suggested by them for monitoring anti-HIV drugs, and one of the drugs which responded inhibitorily in this system was azidothymidine (AzT). It was suggested that anti-HIV activity of AzT [28,29] may not be due only to inhibition of reverse transcription [30], and that other effects may also be involved. For this reason, we have tested AzT in our system to document that AzT does not influence the transactivation process directly or indirectly. As follows from fig.3, there was no effect of AzT on the expression of CAT activity in our experiments (lanes 2,3). Quantitative measurements for the acetylation of chloramphenicol showed 16% inhibition at 10 μM and 9% inhibition at 25 μM AzT.

4. DISCUSSION

We have shown that D-penicillamine inhibits the transactivation of HIV-1 LTR by tat protein. Since D-penicillamine was added to the cells after the transfection protocol, the possibility of its intervention in plasmid DNA uptake is ruled out. The control experiments with plasmids without HIV-1 LTR sequences and with AzT further support this statement. Thus, the effects reported here clearly show that D-penicillamine blocks the interaction between tat protein and HIV-1 LTR which is a requisite for the expression of the CAT gene.

We can consider several possible models to explain how D-penicillamine blocks this interaction. Frankel et al. [17] have recently shown that the transactivating protein from HIV-1 forms a metal-linked dimer with metal ions bridging cysteine-rich regions from each monomer. Ultraviolet absorption spectra showed that tat protein binds two Zn^{2+} or two Cd^{2+} per monomer. Based on the chemical reactivities of D-penicillamine, we can postulate a bimodal function of D-penicillamine to block the transactivation process in HIV-1. The chelation of metal ions will affect the formation of tat dimer, if it turns out to be the requirement for in vivo binding to DNA. Even if we consider the monomer model of Patarca and Haseltine [31], the metal binding is a requisite for nucleic acid binding of tat protein. On the other hand, D-penicillamine could form stable interdisulfide bonds with the cysteine residues at the functional binding domain. This will affect the secondary structure of tat protein and also occupy the sites needed for metal binding. Even with taking into account that metal binding is not important for the transactivating event, the latter reaction will completely abolish the biochemical functions of tat protein.

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