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Antiviral Research 49 (2001) 101–114

Antiviral Research

www.elsevier.com/locate/antiviral

Inhibition of the early phase of HIV replication by an isothiazolone, PD 161374

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Received 15 June 2000; accepted 30 November 2000

Abstract

A new class of substituted 2′-benzisothiazolone represented by PD 161374 was discovered with antiviral activity against retroviruses similar to previously described nucleocapsid inhibitor PD 159206 (DIBA-4). In T cell culture, the 50% inhibitory concentrations (EC_{50}) of PD 161374 and PD 159206 were on average 2.5 μM (ranges of 1.2–13.5 μM) without any cytotoxic effect up to 100 μM. PD 161374 inhibited acute HIV infection and it was effective when added during the early phase of HIV infection. However, very modest effects were observed in chronically infected H9 cells and the HIV latency model line OM-10.1. Direct PCR analysis of infected cells demonstrated that PD 161374 delayed the appearance of completed HIV–cDNA products including 2LTR circles. Together all these results suggest that PD 161374 exerts its antiviral effect at pre-integration steps in the early phase of the virus life cycle. When combined with a protease inhibitor, PD 161374 did not show any antagonism and combination with a reverse transcriptase inhibitor (AZT) resulted in a synergistic effect.

Keywords: HIV; Nucleocapsid protein; 3′azido-3′-deoxythymidine; Antivirals

1. Introduction

Human immunodeficiency virus (HIV-1) is a pathogenic retrovirus associated with symptoms of the disease known as AIDS (Barre-Sinoussi et al., 1983; Gallo et al., 1984; Blattner et al., 1988). Replication of HIV occurs continuously with a high turnover rate and 10^7–10^9 virions are produced per day throughout the course of HIV infection (Perelson et al., 1996). In multiple clinical trials, administration of different combinations of *anti*-HIV compounds to infected individuals has shown remarkable efficacy followed by improved clinical status (Wei et al.,

Abbreviations: HIV-1, human immunodeficiency virus; NCp7, nucleocapsid protein; PBMCs, peripheral blood mononuclear cell.

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0166-3542/01/$ - see front matter
PII: S0166-3542(00)00143-1

1995). However, one of the challenges of current antiretroviral therapy is outgrowth of drug resistant viruses leading to failures in therapy (Coffin, 1995; Condra et al., 1995). A combination of multiple HIV inhibitors directed against different viral targets during early phases of HIV infection could be beneficial for long term suppression of viral load in infected individuals.

Current *anti*-HIV therapies include inhibitors of retroviral reverse transcriptase (Mitsuya et al., 1985) and protease enzymes (Craig et al., 1991; Rice et al., 1995; Kempf et al., 1995). In addition to retroviral enzymes, structural proteins with conserved amino acid motifs could be a potential target for developing *anti*-retroviral compounds. One such protein is the retroviral nucleocapsid (NCp7), which has been proposed as an HIV target accessible to several small molecule inhibitors (Rice et al., 1993a,b). The NCp7 of HIV-1, contains two zinc finger motifs, which is highly conserved among retroviruses (Aldovini and Young, 1990; Zhang and Barklis, 1995). The zinc finger motif is essential for virus replication and mutation of cysteine residues which functions as zinc binding ligands resulted in non-infectious virus particles (Gorelick et al., 1996; Ottmann et al., 1995). A class of benzamide disulfide compounds with *anti*-HIV activity was discovered through a joint collaboration between Parke-Davis pharmaceuticals and National Institute of Cancer. Rice et al. has previously described some of the early compounds from 2-2′-dithiobisbenzamide class as DIBA compounds (Rice et al., 1995). The zinc ejecting activity of DIBA compounds from HIV-Ncp7 had correlation with the *anti*-HIV activity (Rice et al., 1995).

Poor biopharmaceutical properties of the reported compound (Rice et al., 1995) led to synthesis of few hundred additional compounds with substitutions in different positions to identify the essential pharmacophore responsible for antiviral activity (Tummino et al., 1997). From this series, 3-methyl-2-(3-oxo-3H-benzo[d]isothiazol-2-yl)

-pentanoic acid (PD 161374) was selected for advanced preclinical evaluation. PD 161374 is a distinct chemical structure (2′-isothiazolone) which can also form in solution following reduction of PD 159206 and its subsequent cyclization to isothiazolone (Fig. 1). Both of the compounds have inhibitory activity against HIV-1 (Domagala et al., 1997; Prasad et al., 1998).

In this paper we describe the mechanism of action of 2′-isothiazolone (PD 161374) and compare it to PD 159206 (DIBA-4). After our initial observation that the syncytium formation and gene expression of the integrated provirus was not impaired by PD 161374 and PD 159206, we focused on events prior to integration of viral DNA. Using single cycle growth conditions, we monitored initiation and completion of cDNA products and formation of two long terminal repeats (2LTR) in infected cells. We conclude that PD 161374 like PD 159206, inhibits early steps of the virus life cycle and exerts antiviral activity during the first few hours of HIV infection.

2. Materials and methods

2.1. Compounds

PD 161374, PD 159206 (Fig. 1), 148310 (analog of Roche protease inhibitor, RO-3102), AZT (Glaxo Wellcome), Bicyclam (JM 2763), were dissolved in dimethyl sulfoxide (DMSO) at 10mM concentration. Frozen aliquots were thawed just prior to each assay. All dilutions of drugs were made in RPMI 1640 or DMEM medium.

2.2. Cells, virus strains and reagents

The following cells and virus strains were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: H9, HeLa-CD4-LTR-β-gal, OM-10.1 cells, virus strains of HIV-III_B and HIV-1_{LAI} /PBMC. Clinical isolate, ROJO was isolated from a pediatric patient (B. Buckheit, SRI, personal communication), AZT resistant isolates, Protease resistant isolates (AIDS reference reagent program) were analyzed by Southern Research Institute (SRI).

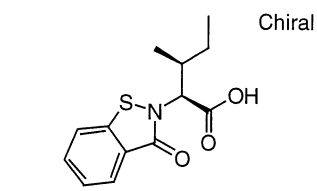
Cell culture medium, RPMI 1640, DME and calf serum were obtained from Gibco BRL. ELISA kits for p24 assay were purchased from Coulter Immunology, Fl. The chemicals used in

staining solution, *X*-galactoside, glutaradehyde, potassium ferricyanide and potassium ferrocyanide, were purchased from Sigma Chemical Company. The RNA extraction solution, RNAzol was purchased from Biotecx, Texas, T7 RNA polymerase from Ambion, and TNF-α used for activation of OM-10.1 cells were purchased from Genzyme Corp. Boston, MA.

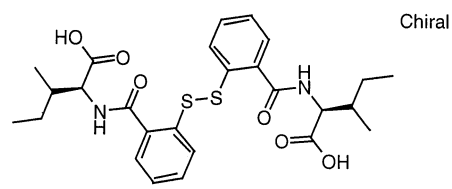
2.3. Antiviral assays

All test compounds were prepared in DMSO as 10 mM stock solutions and dilutions were carried out in culture medium with final DMSO concentration not to exceed 0.2%.

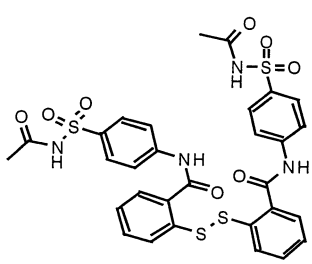
The susceptibility of HIV-1_{IIIB} to PD 161374 and 159206 in H9 and MT4 cells was determined at multiplicity of infection of 0.01 unless specifically mentioned. Infected cells were washed and seeded at 2×10^5 cells/ml RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml of penicillin and 100 μg/ml containing PD 161374, PD 159206 or DMSO and allowed to grow at 37°C for 5 days, at which time culture supernatants were withdrawn for quantitative RT or p24 antigen determination. The susceptibility of HIV-RF against PD 161374 or PD 159206 in CEM cells was determined by XTT dye reduction assay (Weislow et al., 1996). The susceptibility of HIV-1_{LAI} or clinical isolates in PBMC was determined following infection of PHA- stimulated normal donor PBMC at a multiplicity of infection of 0.01. Following a 2 h of incubation of virus infected PBMCs at 37°C, non-adsorbed virus was removed by washing cells twice with cold PBS. PBMCs were seeded at 3×10^5 cells/ml in RPMI complete medium containing 100 U of interleukin-2 per ml. After growth for 5 days at 37°C, cell free culture supernatants were assayed for virus replication by p24 antigen assay.



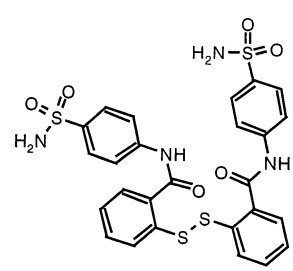
PD161374



PD159206
DIBA-4



PD156202
DIBA-2



PD022551
DIBA-1

Fig. 1. Structure of benzamide sulfides. PD 159206 is a disulfide and PD 161374 is an isothiazolone derivative of PD 159206. DIBA-1 and DIBA-2 are also derivatives of benzamide sulfides.

To determine selectivity indices (TI = therapeutic index) in either H9, MT4 or PBMC, 50% cell culture toxicity doses ($CCTD_{50}$) determined in the corresponding culture system was divided by the EC_{50} of test compounds.

2.4. Single cycle Infectivity assay

The number of infectious virus in one round of infection was measured by the Magi Assay (Kimpton and Emerman, 1992). HeLa-CD4-LTR-β-gal cells was infected with HIV-1_{IIIB} at high multiplicity of infection 0.01(optimized for detection of 200 infected blue cells from 5000 cells per well). After 48 h, medium was removed and cells were fixed in a 1% formaldehyde-0.2% glutaraldehyde solution followed by staining with solution of 4 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 0.4 mg of X-gal/ml. The number of blue cells was counted under a light microscope.

In experiment described as time of addition studies, virus stock (HIV-1_{IIIB}) was added to HeLa cells (time '0') followed by addition of compounds at 15 min, 30 min, 1, 2, 4, 6, 12 and 24 h. Infected HeLa cells were grown for a total of 48 h and the staining procedure was followed as described above.

The cytotoxicities (TC_{50}) of test compounds in uninfected H9, MT4 or PBMC culture were evaluated by measuring the formation of a formazan, a tetrazolium dye (XTT; Sigma) as described previously (Mosman, 1983).

2.5. Antiviral activity of PD 161374 and PD 159206 in chronically HIV-1 infected cells

A chronically HIV-1 infected H9 cell line was established essentially as described earlier (Sato et al., 1992). OM10.1 cells were also used as chronic model of HIV infected cell line. Cells grown in log phase in RPMI 1640 medium were activated with 2U of TNF-alpha/ml of media (0.5×10^6 cells) for 2 h. Production of virus following activation of cells at 48 h was measured by RT and p24 determinations of cell free supernatant.

For RNA analysis, virions were pelleted from cell free supernatant by ultra centrifugation at 100 000 rpm for 1 h. RNAzol (Ambion Inc., Austin, TX) was added directly to virion pellets followed by the extraction procedure suggested by the manufacturer. Total RNA was extracted from equivalent of 2×10^6 cells and hybridized overnight with antisense RNA (HIV + 261-415) radiolabelled with ^{32}[P]. Products were digested in RNAse cocktail buffer (Ambion) with RNAse T1 and RNAse A at 37°C for 15 min. Samples were precipitated with ethanol, dried, resuspended in formamide buffer and loaded on a 6% polyacrylamide gel. Protected bands were quantified by densitometric scanning.

2.6. DNA-PCR analysis of infected cells

Cell lysates from H9 (1×10^6) cells at different time points after HIV infection were prepared in lysis buffer (10 mM Tris–HCl [pH 8.3], 1 mM EDTA, 0.5% Triton X-100, 0.001% SDS, 300 μg of proteinase K/ml). To detect the HIV sequence, one of the oligonucleotide pairs was radiolabelled and 20 ng (1×10^6–5×10^6 cpm) was included in each reaction. The second oligonucleotide was not end labeled and 20 ng was included in each reaction. Each reaction contained 0.2 mM concentration of each dNTP, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$ and 1 U of Taq polymerase (Perkin-Elmer). The reaction mixture was overlaid with paraffin beads and subjected to 25 cycles of 1 min denaturation, 30 s of annealing at 60°C, and 30 s of extension at 72°C. Amplified products were analyzed by electrophoresis on 6 and 8% non-denaturing polyacrylamide gels. Oligonucleotide sequences used for early products, primer 1 = CAGATATCC ACTGACCTTTGG (U3, 8790–8810), primer 2 = GAGGCTTAAGCA GT GGGTTC (R, 9190–9204); for late products, primer 1 = GCTTAATA CTGACGCCTCTCTCGCA (gag, 231–253); primer 2 = CTCGACGCAGGACTCGGCTTGC (gag 342–362); For 2LTR circles, primer 1 = CCT TTTAGTCAGTGTGGAAAATCTCTAGCA (U5, 152–179) primer 2 = CAGTGGGTTCCCTAGTT AGC (R, 9175–9194); for globin gene, primer 1 = ACACAACTGTGTTCACTAGC, primer 2 = CAACTTCATCCACGTTCACC.

Table 1
Antiviral acivity of PD 161374 and PD 159206 in cell culture

Virus strain	Cell type	EC_{50} [a] (μM) PD 161374	EC_{50} (μM) PD 159206	TC_{50}[b](μM) PD 161374	TC_{50} (μM) PD 159206
HTLV-III_B	H9	1.3	1.2	>100	>100
LAV-1_{Bru}	MT4	3.2	2.5	>100	>100
HTLV-III_{RF}	CEM-SS	9.0	6.5	>100	>100
LAV-1_{Bru}	PBMC	8.5	7	>100	>100
Ba-L	Macrophages	6.5	24	>100	>100
ROJO	PBMC	7.2	13.5	>100	>100
BR/93/020	PBMC	9.5	7.8	>100	>100
HIV-2	CEM	5.8	6.4	>100	>100
SIV	CEM	8.5	10.2	>100	>100
RT and PR resistant clinical isolates					
AZT-R	CEM	10.5	14.5	>100	>100
A17	MT-2	ND	5.6	>100	>100
HIV-1L10R/M46I/L63P/V82T/I84V	MT-4	2.2	1.6	>100	>100

[a] EC_{50} = Concentration required to inhibit HIV-1 p24 or RT production by 50% relative to that in no-drug controls.
[b] TC_{50} = Concentration required to inhibit cell viability by 50% relative to that in no-drug controls.

2.7. Analysis of drug combination effects

The inhibitory effects of combination of compounds on HIV replication in MT4 cells with HIV-1_{IIIB} were measured by P24 Ag determination. Dilutions were made in half-log steps for both individual drugs and constant-ratio drug combinations. Drug ratios based on 50% effective concentrations (EC_{50s}) of the individual drugs were chosen. To assess the antiviral effects of different combination drug treatments, CIs (combination index values) were calculated according to the method described by Chou and Talalay (1984). For calculations of CI values, drugs were diluted in selected fixed ratio and three ratios were analyzed. Dose-response curves were determined for each individual drug and each combination by the median-effect equation. The equation was fit by using the non linear regression in PC SAS version of 6.08.

Numerically, CIs of $<1, 1$, or >1 indicate synergism, an additive effect or antagonism, respectively.

3. Results

3.1. Suppression of HIV replication by PD 161374 and PD 159206

A systematic effort for synthesis of more potent and selective HIV inhibitors was initiated, based on the earlier observation that the in vitro zinc ejecting activity of some of the disulfides was associated with *anti*-HIV-1 activity (Domagala et al., 1997; Tummino et al., 1997).

PD 161374 was selected for advanced development for its simple synthetic structure and improved biopharmaceutical properties. PD 161374 and its disulfide analog PD 159206, suppressed virus replication of laboratory and clinical isolates as summarized in Table 1. In transformed T cell lines (CEM, MT4, H9), the effective concentrations of PD 161374 and PD 159206 required for 50% inhibition of virus replication was between 1 and 9.0 μM. In primary blood lymphocytes relatively higher concentrations of compounds were required (7–13 μM) compared to transformed cell lines to inhibit 50% of HIV-1 replication. PD

161374 had similar inhibitory effects against HIV-2, SIV and HIV reverse transcriptase and protease drug resistant isolates (Table 1).

3.2. Time of addition studies with PD 161374 and PD 159206 in single cycle of HIV infection

To understand at which stage the isothiazolone interact with HIV replication cycle, compounds were added to HIV infected cells at different time points following virus addition in a single round of infection assay in HeLa-CD4-LTR-β-gal cells.

A dose response curve representing inhibitory effect of PD 161374 and PD 159206 is shown in Fig. 2A. Since PD 161374 and PD 159206 had inhibited 50–60% of virus replication in this assay without compromising cell viability, the result suggested that those compounds possibly interfere with an early step of the virus life cycle.

To determine the time period when addition of compounds is most effective against HIV-1 replication, a time of addition experiment was carried out in HeLa-CD4-LTR-βgal cells (Fig. 2B). Cells were infected with HIV-1_{IIIB} at MOI

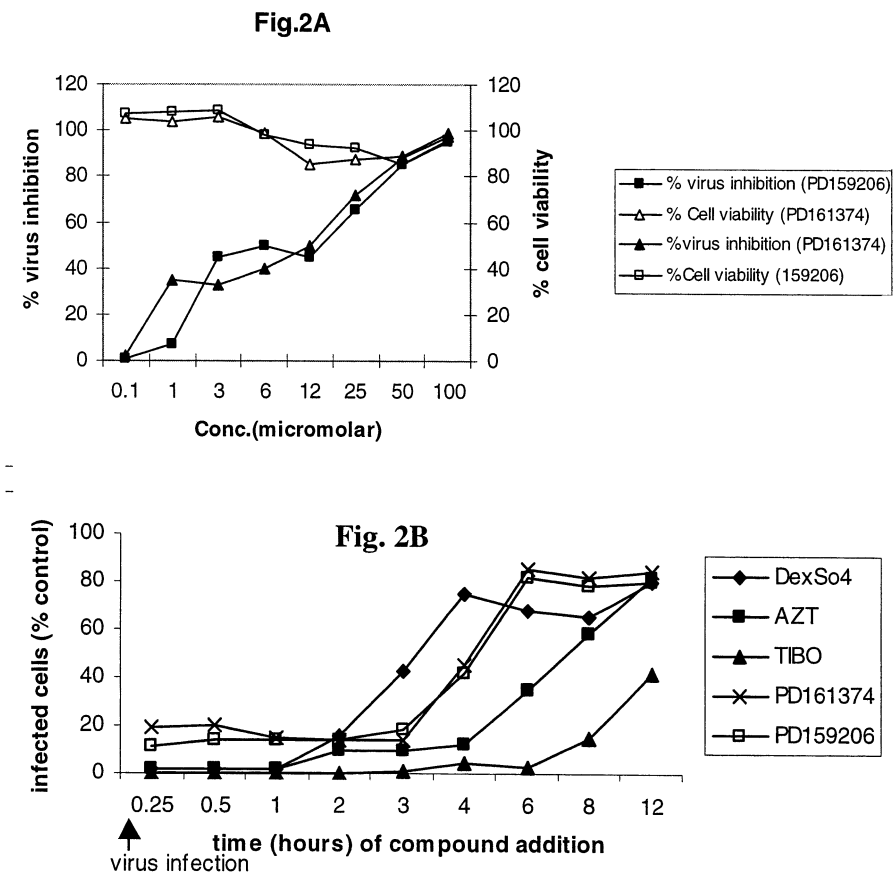


Fig. 2. (A) Dose dependent inhibition of HIV replication in HeLa Magi cells. 2×10^4 HeLa Magi cells were seeded in 48-well plates, 12 h before infection. HIV-1_{IIIB} at TCID50 of 10^4 was added to each well for 4 h. Dilutions of test compounds were added at the same time. Unabsorbed virus was removed by washes with PBS and fresh media was added with same concentrations of compounds. After 48 h cells were washed and stained as described in Method section. Numbers of blue cells were counted and results are plotted as% control (infected cells without any compound). (B) Time of addition studies in single cycle of HIV infection. 2×10^4 HeLa Magi cells were seeded in 48-well plate 12 h before infection. HIV-III_B at TCID50 of 10^4 was added to each well. Twenty micrometer of PD 161374 or PD 159206, 1 μM of AZT, 1 μM TIBO, 20 μM DexS04 were added to respective wells at different time points after addition of virus. After 48 h, cells were washed and stained and blue cells were counted under microscope.

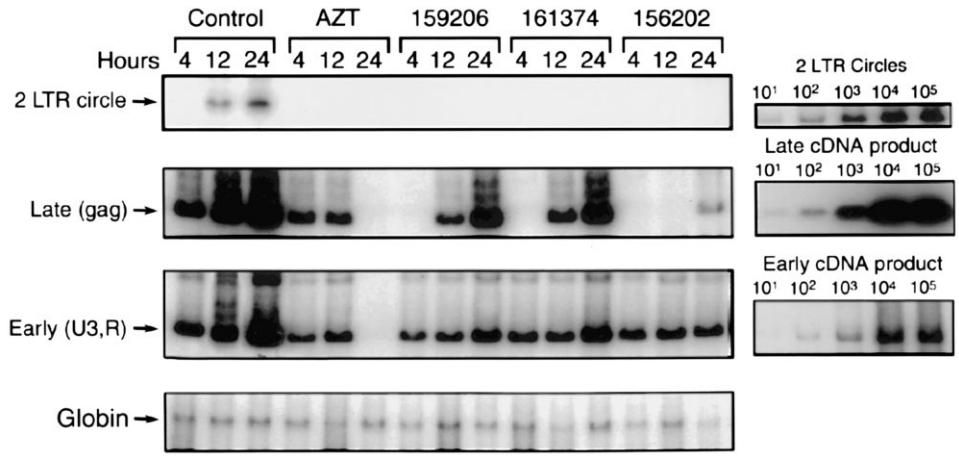


Fig. 3. Semiquantitative PCR analysis of early (U3, R primers), late (gag primers) and 2LTR circle junctions (U5, R primers) of H9 cells infected with HIV-III_B. Untreated control, AZT, PD 159206, PD 161374, and PD 156202 (DIBA-2) treated cells were analyzed in same experiment. Equal amount of cell lysate products normalized by globin DNA was loaded on the gel.

of 0.01 and compounds were added at different time points after addition of virus to cells. An inhibitor of virus attachment, dextran sulfate and a reverse transcriptase (RT) inhibitor, TIBO were included in these experiments. All infections were allowed to proceed for 48 h, after which cells were stained and blue cells were counted. Number of blue cells formed in untreated cells represented the total number of infections obtained in the control assay. If dextran sulfate was added after 2 h of infection, it became less effective as indicated by an increased number of infected foci in each well. This result supports that the virus binding to target cells was complete during first 2 h of infection. In the same experiment, when reverse transcriptase inhibitors, AZT and TIBO were added 6 h after virus infection, they lost their effectiveness. If PD 161374 and PD 159206 were added after 4 h of infection, a loss in antiviral effect was observed indicated by an increased number of infected blue cells. The antiviral effect was completely lost when PD 161374 and PD 159206 were added 6 h after infection. These results suggest that both PD 161374 and PD 159206 inhibited step(s) during first few hours (up to 6 h) of HIV life cycle.

3.3. Progression of reverse transcription products in benzamide sulfide treated cells

In several studies, it was demonstrated that structures of newly reverse transcribed DNA species exist in HIV infected cells (Zack et al., 1990; Burkinsky et al., 1993). Therefore, in a single round of infection, products related to various stages of reverse transcription processes can be detected in time dependent studies. To investigate the effects of PD 161374, two other 2′-2-dithiobisbenzamides, PD 159206 and PD 156202 were also included. Although PD 156202 (DIBA-2) was not selected for development, it was included in this experiment because it represents a prototypic nucleocapsid inhibitor (Tummino et al., 1996). Total DNA extracted from cell lysates of de novo infected H9 cells were analyzed by PCR with three sets of primers (Fig. 3). To detect early steps in reverse transcription, LTR specific primer pairs were used. This primer pair flanks the first region of DNA synthesized from RNA of the virus. A primer pair specific for the gag region was chosen (between LTR and gag) to detect only full length of nearly complete late viral DNA products. Translocation of viral cDNAs from cytoplasmic to nuclear compartment of the cell was monitored

by using primers which span the junction between the 5′LTR R and 3′LTR U5 regions as created during formation of the 2LTR.

To establish the kinetics of DNA synthesis, H9 cells in log phase were infected with 1×10^4 $TCID_{50}$ of HIV-1_{IIIB}/ml. Cell lysates were prepared after 4, 12 and 24 h as described under Section 2. Late products in untreated control cells (normalized for the same amount of DNA, PCR was linear between 10^2 and 10^5 copies of plasmid HIV DNA), were detected after 4 h of infection. After 12 h, the completed reverse transcribed product was detected indicated by 2LTR circles in untreated cells. Therefore, under the experimental conditions, completion of the reverse transcription process leading to nuclear uptake of proviral DNA was completed in 24 h.

Early products (Fig. 3) which represent DNA sequence encompassing regions from first strand transfer reaction, was detected in all samples at 4 h, which reached a maximum level at 12 h. In AZT treated cells, there was less cDNA synthesis indicated by $< 10\%$ product detected after 12 h which disappeared completely by 24 h. One possible explanation for the early cDNA products detected in AZT treated cells at 4 and 12 h following infection, were unstable. In PD 161374, PD 159206, PD 156202 treated cells, early products were detected as early as 4 h, albeit at a lower level than control cells. However, it was noted that unlike AZT treated cells, these early products could be detected in PD 161374, PD 159206 and PD 156202 treated cells as late as 24 h after infection. These products could represent DNA copies, which were relatively more stable and proceeded further in the reverse transcription reaction.

Late products which represents DNA sequences from gag regions were detected in untreated cells after 4 h of infection. In AZT treated cells, a similar level of late products like early products was detected at 12 h, which was absent after 24 h. Under these experimental conditions, some cDNA products proceeded to late products even in AZT treated cells. However, the cDNA products were still undetectable after 24 h suggesting, that those products were unstable like early products. In contrast to AZT treated cells, PD 161374, PD 159206 and PD 156202 treated cells had no late products ($< 2\%$) after 4 h. Late products in PD 161374, PD 159206 and PD 156202 treated cells were detected after 12 h which had further increased after 24 h. Interestingly in PD 156202 treated cells, late products were detected only after 24 h which suggests that there was a delay in progression from early to late products. However, unlike AZT treated cells, late products in PD 161374, PD 159206 treated cells were detected at late (after 12 and 24 h) time points.

In untreated cells, products representing junctions of 2LTR circles were detected as early as 12 and 24 h after infection (Fig. 3). In AZT, PD 161374, PD 159206 or PD 156202 treated cells, products related to 2LTR circles were absent. This result again suggest that in AZT treated cells, the products detected at 4 h and 12 h were unstable, therefore those cDNA products failed to complete reverse transcription process and generate 2LTR junctions. In PD 161374, PD 159206 or PD 156202 treated cells, the late products which were slow to appear (detected at 12 and 24 h) failed to complete reverse transcription indicated by 2LTR junction at 24 h. Altogether above results demonstrated that reverse transcription reaction did not proceed efficiently in PD 161374, PD 159206 or PD 156202 treated cells and delayed the formation of pre-integration precursors.

3.4. Effect of PD 161374 and PD 159206 in chronically HIV infected cells

The experiments described under acute and single round of infections assays, (Fig. 2A, B) supported that PD 161374 and PD 159206 inhibited early steps of virus life cycle. However, if these compounds effect assembly of virus particles, the new virus particles generated from PD 161374 and PD 159206 treated cells should be non-infectious. It was reported earlier that compounds which ejected zinc from HIV-NCp7, generated non-infectious virus particles (Turpin et al., 1996).

To address the question of assembly of non-infectious virus particles in treated cells, studies were conducted in H9 cell cultures, chronically infected with of HIV-1_{IIIB}. First, a chronically infected cell line was established by infecting H9 cell with HIV-1_{IIIB} and maintaining the culture for 8 weeks.

Fresh uninfected H9 cells were added every 2 weeks to replace dead cells to allow new cycles of infection. These cells, continuously produced infectious virus and titer of the virus in cell free superntant was 2×10^5 $TCID_{50}$/ml. Exposure of these chronically infected cells to 20 μM PD 161374 or PD 159206 did not inhibit release of virus particles indicated by equal amount of virus in cell free supernatant (Fig. 4A). When HeLa Magi cells were infected with equal amounts of released virus particles (from untreated, PD 161374 or PD 159206 treated cells, normalized by 1×10^4 RT activity and 20 ng/ml of p24), there was no difference indicated by similar number of infected blue cells in the absence (minus) of either PD 161374 or PD 159206 in the culture medium. However, the inhibitory effect was observed only when PD 161374 or PD 159206 was included (plus) in the culture medium during infection. Therefore in a HIV producer cell line in absence of new infections, the antiviral effect of PD 161374 or PD 159206 could not be demonstrated. Furthermore for antiviral effect, continuous presence of compounds were necessary during infection (Fig. 4B).

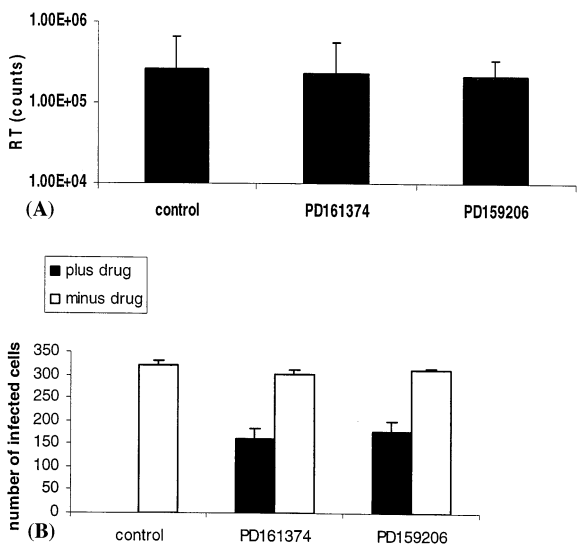


Fig. 4. Effect of PD 161374 and PD 159206 on chronically infected H9/HIV-III_B. (A) Cells were seeded at 5×10^5 cells/ml medium and 20 μM of PD 161374 and PD 159206 were added at the same time. After 48 h, RT activity of cell free supernatant was measured. (B) Cell free virus supernatant from untreated control and treated cells (from A) were collected after centrifugation, washed, and virus with equal amount of RT activity was used to infect HeLa Magi cells in presence and absence of compounds.

Next, PD 161374 and PD 159206 were tested in OM-10.1 cells, which represent a model system for HIV-latency (Butera et al., 1991; Folks et al., 1987). This cell line is also considered as a transcriptional model for viral latency since activation of these cell lines causes an increase in viral transcripts leading to an increase in all viral gene products (Adams et al., 1994). For this purpose, OM-10.1 cell was treated with TNF-α which activated HIV-1 gene expression marked by a 30–50 fold increase in virus production measured by both virus associated p24 and RT activity in cell free supernatant.

PD 161374 had a modest, dose dependent inhibitory effect on virus released in culture medium (Fig. 5A). The inhibitory effects of PD 161374, PD 159206 were compared with AZT (RT inhibitor) and PD 148310 (protease inhibitor). AZT did not have any effect because virus was already integrated in this cell line which was further downstream of reverse transcription step inhibited by AZT. The protease inhibitor was very effective in this cell line because it inhibited HIV-protease enzyme critical for processing of new virus particles. The amount of RNA packaged within virus particles was analyzed by RNAse protection analysis. The protease inhibitor (PD 148310) produced a demonstratable higher ratio ($\sim$200–250%) of RNA to p24 due to relatively low amount of P24 antigen (Fig. 5B). Treatment with PD 161374 caused a 40% decrease in p24 while decreasing RT activity by less than 10%. Treatment with PD 159206 caused a 20% decrease in p24 and a 30% decrease in RT activity. The protease inhibitor, PD 148310, caused greater than 90% inhibition of virus particles. When the amount of RNA packaged in shed virus particles was measured, the ratio of RNA/p24 was between 100 and130% for PD 161374 and PD 159206 treated cells. Thus virus particles treated with PD 161374 and PD 159206 did not have any defect in RNA packaging, because if less viral RNA was packaged, a decrease in RNA per particle would be expected. Similar results were also obtained in U1 cells (data not shown). To investigate if PD 161374, PD 159206 had any inhibitory effect on proteolysis, virus particles col-

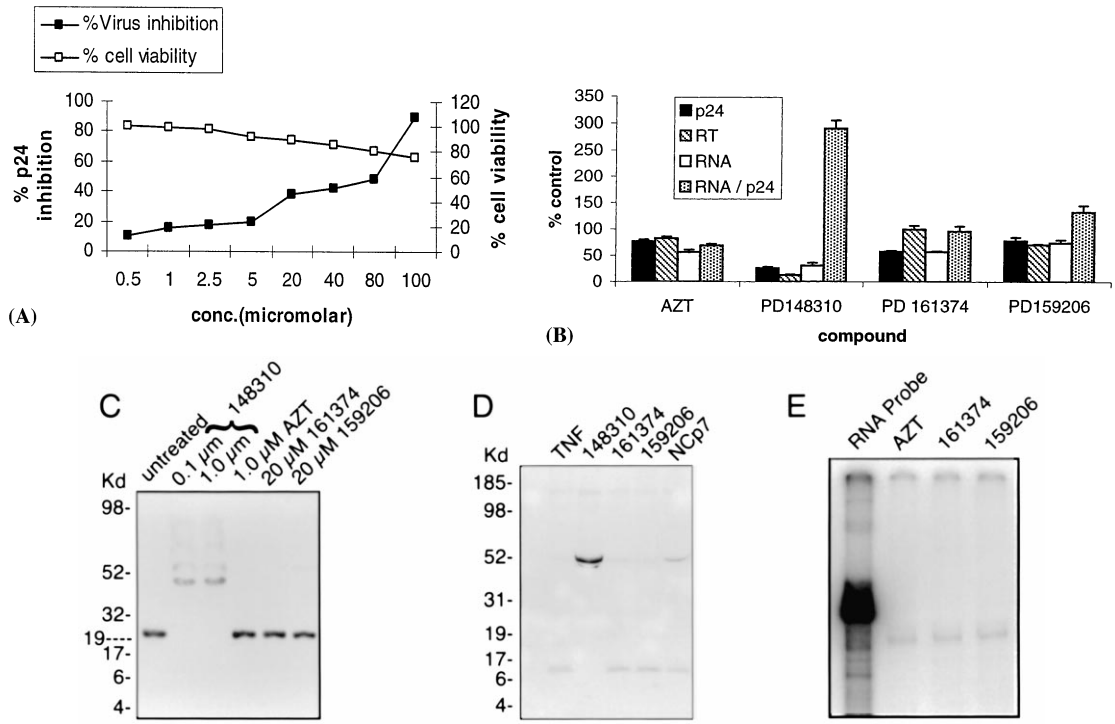


Fig. 5. (A) Dose dependent virus inhibition by PD161374 in cell free supernatant of OM-10.1 cells. (B) Virus associated RT, p24, and RNA was measured in cell free virus particles released into the medium after TNF-α activation of OM-10.1 cells. Results are plotted as percentage of control activity; control being cells which were untreated with any compounds. (C) Virus particles treated with compounds were separated on a gel and analyzed by western blot with anti p24 monoclonal antibody as described under materials and methods. (D) Virus particles analyzed by western blot with anti NCp7 monoclonal antibody as described under materials and methods. (E) Gel analysis of RNAse protected bands in virus particles treated with compounds.

lected from treated and untreated cells were separated on a gel followed by western analysis with capsid (p24 Ag) specific monoclonal and nucleocapsid specific polyclonal antibodies. There was no difference between virus particles generated from untreated or treated cells indicated by the unchanged cleavage products (Fig. 5C, D). Viral RNA packaged within virus particles were also unaffected indicated by similar size and quantity of protected RNA bands (Fig. 5E). Therefore, the observed modest suppression of virus in chronic cell line was independent of any obvious defect in virus assembly.

3.5. Antiviral activity of PD 161374 in combination with RT and protease inhibitor

The antiviral effects of combining the RT inhibitors or protease inhibitors are of considerable interest for understanding drug interactions, commonly used for HIV-therapeutic treatments. Since PD 161374 inhibited early step(s) of virus life cycle, we assessed the antiviral activity of PD 161374 in combination with another early step reverse trancriptase inhibitor, AZT. We also compared the combination of HIV protease inhibitor, Indinavir, with PD 161374. The combination index values (CIs) were calculated according to the method described by Chou and Talalay (1984), with CIs of < 1, 1 and > 1 indicating synergistic, additive and antagonistic drug effects respectively. Combinations of PD 161374 with either AZT or Indinavir in dose ranges tested (Table 2) had either synergestic or additive antiviral effect in cell culture. No cytotoxicity was evident by XTT assay with any of these drugs alone or combined at the highest two concentrations.

4. Discussion

We investigated the mechanism of HIV-1 inhibition by 2-benzisothiazolone in cell culture. The rate of zinc ejection from HIV-NCp7 by some of the analogs of dithiobenzamides was correlated with inactivation of cell free virus particles (Turpin et al., 1996). However, the relative rate of zinc ejection in vitro or cell free virus inactivation by compounds could not be used as a predicator of HIV inhibiton in cell culture (Tummino et al. 1997). Conversion of dithiobisbenzamide to 2-benzisothiazolone in neutral solution at pH 7.4, led to an initial hypotheses that 2-benzisothiazolone may be the active intermediate for this class of compounds, responsible for *anti*-HIV activity.

PD 161374 (2-benzisothiazolone) was tested along with its disulfide derivative PD 159206, in different acute and chronic HIV infection models in order to establish their time of effect and mode of action. Their effectiveness in acute infection as opposed to chronic infection and time of drug addition experiment suggested that PD 161374 target an early step in virus life-cycle. It has also been reported earlier that compounds with similar zinc ejection properties could inhibit HIV at post-integration stage (Rice et al., 1995). We conclude that PD 161374 and PD 159206 did not interfere with post-integration steps of virus life-cycle. The earlier reports on post-integration effect of some zinc ejecting compounds could be due to some other attributes of active pharmacophores generated from specific compounds as a result of exposure to cell environment.

The retroviral infection cycle of a target cell can be divided into two phases. In phase one, the afferent stage, infection of target cells is initiated by virus binding followed by the uncoating of virus particles into the intracellular environment. In the cellular environment, all biological reactions essential for replication and assembly of new progeny virus particles occur in a sequential manner. In phase two, the efferent stage, newly assembled virus particles may exist in a cell free environment before binding to a new target cell or being cleared. Compounds designed against a specific target whether it is an enzyme or a regulatory protein is expected to be active at certain stages of the virus life-cycle. For example, an RT inhibitor will target polymerase function of reverse transcriptase enzyme prior to integration. On the other hand, a protease inhibitor functions at late steps of virus assembly which results into non-infectious immature virus particles. When and how, a nucleocapsid inhibitor is expected to function in the virus life-cycle? All retroviral nucleocapsids, except spumaretrovirus (Maurer et al., 1988),

Table 2
CI-values for two-drug combinations of PD 161374 with AZT, Indiniavir and 148310 assayed against acute infection of HIV-1_{IIIB} infection of MT4 cells[a]

Drug concentrations	Molar ratio	CI at the following levels of HIV inhibition (%)				Overall result
		50	75	90	95	
PD 161374-AZT	500:1	0.75	0.70	0.58	0.62	Synergy
	50:1	0.65	0.65	0.45	0.43	
	20:1	0.67	0.62	0.52	0.46	
PD 161374-Indinavir	50:1	0.78	0.58	0.63	0.67	Synergy
	20:1	0.70	0.65	0.60	0.58	
	2:1	0.65	0.68	0.48	0.45	
PD 161374–148310	50:1	1:2	0.87	1.1	0.96	Additive
	25:1	0.87	0.95	0.98	1.1	
	10:1	0.98	1.2	0.95	0.99	

[a] For each combination ratio, CI values were computed based on the mean percent inhibition of two experiments ($\neq$). Values <1, $=1$, and >1 indicate synergism, additive effect, and antagonism, respectively.

have unique but absolutely conserved zinc finger motif. A variety of functions are mediated by retroviral nucleocapsid proteins; these include RNA binding and packaging (Aldovini and Young, 1990; Zhang and Barklis, 1995), virus infectivity (Gorelick et al., 1996), reverse transcription processes (Ottmann et al., 1995; Li et al., 1996; Driscoll and Hughes, 2000; Guo et al., 2000), maturational cleavage of gag polyprotein (Allain et al., 1994), and stimulation of retrovirus integration (Carteau et al., 1999). Considering the widespread functions mediated by nucleocapsid protein, an inhibitor designed to modify retroviral nucleocapsid could inhibit the virus by interference at both pre-integration and post-integration steps. Our data demonstrated that PD 161374 and PD 159206 had primary effect against acute infection and very modest effect in chronically infected cells. Certain mutations in the NCp7 zinc fingers severely inhibit genomic RNA packaging in virus particles (Aldovini and Young, 1990; Zhang and Barklis, 1995). However, in spite of their zinc ejecting properties from HIV to NCp7, PD 161374 and PD 159206 did not inhibit the packaging of viral RNA in virus particles. Recently, one of the analogs of Dithiobisbenzamide, DIBA-4 was reported to alter virus morphology (Berthoux et al., 1999). There was no obvious difference in virus morphology examined by electron microscopy following treatment of virus producer cell lines with PD 161374 or PD 159206 (data not shown).

The time of addition studies indicated that PD 161374 and PD 159206 inhibited early steps of virus life-cycle. Analysis of cDNA products demonstrated that sequential progression of reverse transcription products was delayed in PD 161374 and PD 159206 treated cells (Fig. 3). It was more evident in 2LTR junction analysis in treated cells. Although the effect of PD 161374 on integration reaction alone cannot be separated from in vivo cDNA analysis, its contribution to integration reaction cannot be ruled out completely. However, if PD 161374 was only inhibiting integration reaction, an accumulation of 2LTR junctions over a period of time would be expected in treated cells. Instead, a delay in late reverse transcription products following early products and prior to completion of 2LTR junctions was observed (Fig. 3). Overall the experimental evidence suggest a suppressive effect of PD 161374 on sequential progression of reverse transcription process.

The mechanism for reverse transcription inhibition by nucleocapsid inhibitors is distinct from reverse transcriptase inhibitors. Inhibitors that differ mechanistically for same reaction may function synergistically in vivo. This observation is supported by the results from the combination studies of PD 161374 and AZT in antiviral assays, which resulted in synergistic effect (Table 2). Therefore, compounds that effect reverse transcription at different stages of the process could add to overall synergy in virus inhibition.

The effective concentration required for PD 161374 to suppress HIV replication in cell culture is considerably higher than many potent *anti*-HIV compounds that are currently used. Considering the early effect of PD 161374 on virus replication, a synergistic effect could be explored with other RT inhibitors. PD 161374 was evaluated in phase1 clinical trial; however due to dose limiting serum toxicity, it was discontinued from further development. In future, selection of more potent and selective compounds will be necessary to evaluate if nucleocapsid inhibitors hold any potential as effective *anti*-HIV therapeutics.

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