THE MECHANISM OF ACTION OF A TRITERPENE DERIVATIVE IC5964 AGAINST HIV-1 REPLICATION

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The Mechanism of Action of a Triterpene Derivative IC5964 Against HIV-1 Replication

A Thesis

Presented for the

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Degree

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ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) and its subsequent result, acquired immunodeficiency syndrome (AIDS), are part of an epidemic that strikes and kills millions of people globally. Current treatment is limited to a small repertoire of drugs that control HIV infection. These regimens are limited in scope due to recurring problems with treatment failure and threats of viral resistance and drug toxicity in individuals. For this reason, drugs with novel modes of action are needed. Preliminary studies indicate that the betulinic acid derivative IC5964 is a potent anti-HIV compound that blocks HIV-1 envelope-mediated membrane fusion. This anti-HIV-1 agent exhibits a very unique pharmacological profile in that it appears to inhibit HIV-1 at the entry stage of the viral life cycle. Analysis of a chimeric virus derived from swapping envelope sequences between IC5964 sensitive and IC5964 resistant viruses indicated that gp120 is a key determinant for the drug sensitivity. By developing a drug resistant mutant for sequencing, two specific mutations were found within the gp120 sequence. The primary mutation involved a change from a neutral amino acid with a nonpolar uncharged side change to a basic amino acid with a polar charged side chain. To analyze this drug resistant mutant, a cell-cell fusion system was used to determine drug sensitivity of the mutant virus to IC5964, when compared with the wildtype virus. There was a five-fold increase in resistance of the mutant virus to the compound when compared with the wildtype virus. In addition, the ability of the mutant to induce syncytia appears to be compromised due to a single base change. With its potent anti-HIV activity and novel mode of action, this compound has potential to become a powerful addition to current anti-HIV combination therapies.

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INTRODUCTION

Human Immunodeficiency Virus (HIV) infection is associated with the development of Acquired Immunodeficiency Syndrome (AIDS). Once in the body, HIV invades certain cells of the immune system - including CD4, or helper, T lymphocytes - replicates inside them and spreads to other cells (1).

At least 15 gene products have been identified that are encoded by the HIV genome. The structural genes of HIV are gag, pol, and env. The mature HIV envelope glycoprotein, essential for viral entry, is composed of two proteins, gp120 and gp41. The surface glycoprotein gp120 mediates receptor binding on the CD4 lymphocyte, initiating the viral life cycle. The pol gene products, which include HIV reverse transcriptase (RT) and integrase, are essential in the early events of HIV replication. The gag protein is necessary for viral particle assembly and maturation. There are seven regulatory and accessory gene products encoded by the viral genome: tat, rev, nef, vpu, vif, tev, and tev/tnv.

The HIV life cycle, depicted in Figure 1, begins with the attachment and entry of the virus into the host CD4 T-lymphocyte cells. Binding of the gp120 envelope glycoprotein to the CD4 molecule alone is not sufficient for viral entry. A class of chemokine receptors, such as CCR5 and CXCR4, has been identified as cofactors in this envelope-mediated membrane fusion process (2-8). Subsequent to CD4/chemokine receptor binding, gp41 undergoes conformational changes to become the key protein mediating membrane fusion. This change then allows the gp41 to attack the host cell

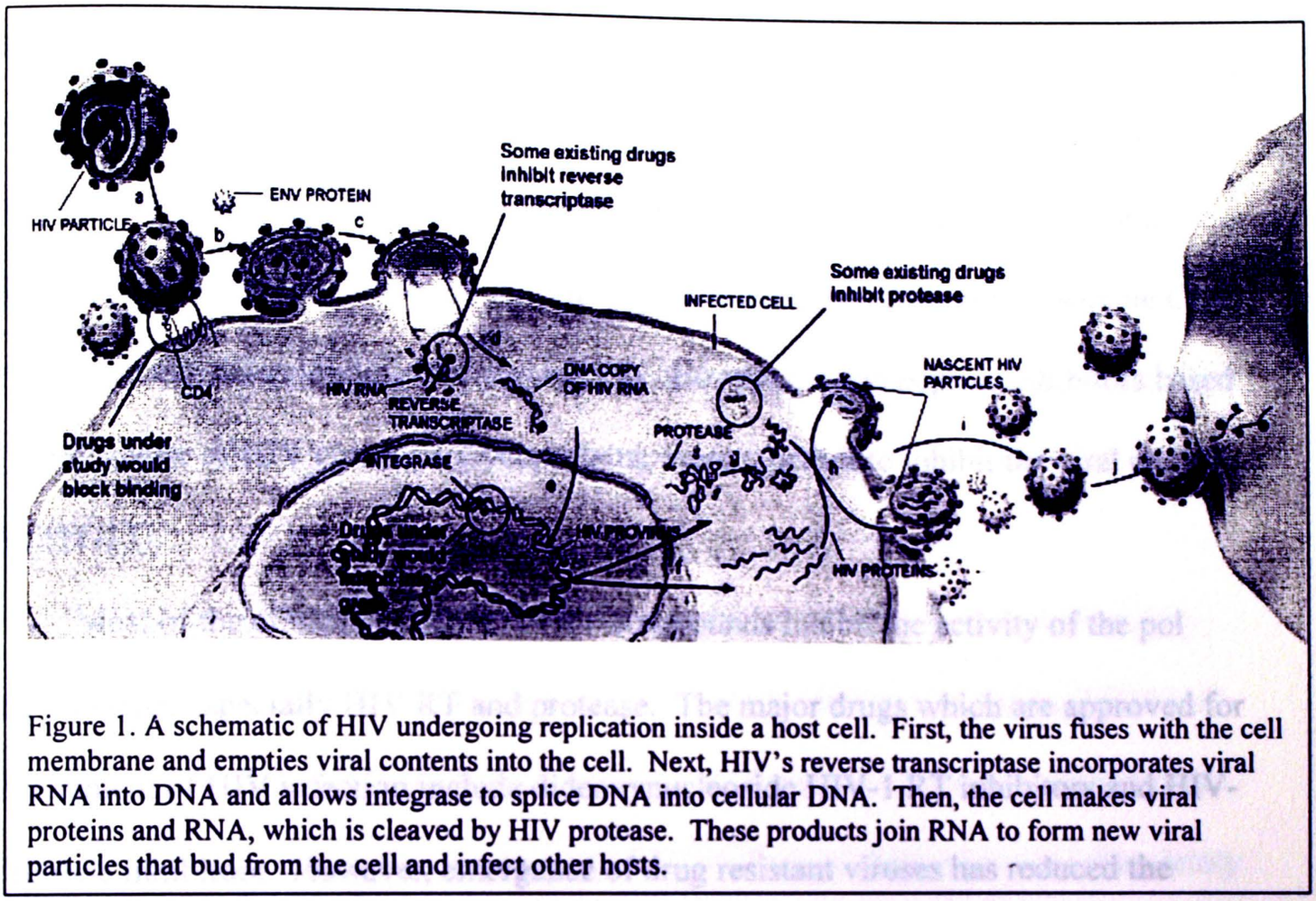

Figure 1. A schematic of HIV undergoing replication inside a host cell. First, the virus fuses with the cell membrane and empties viral contents into the cell. Next, HIV's reverse transcriptase incorporates viral RNA into DNA and allows integrase to splice DNA into cellular DNA. Then, the cell makes viral proteins and RNA, which is cleaved by HIV protease. These products join RNA to form new viral particles that bud from the cell and infect other hosts.

membrane and finish the steps of entry into the CD4 cell. Once the virus becomes fused to the cell membrane, its contents are dispersed into the cytoplasm. Those contents include HIV RT and two strands of RNA each carrying the entire HIV genome (1). Reverse transcriptase copies the RNA genetic material into a double strand of DNA, with integrase permanently integrating the HIV DNA into a host cell chromosome. Once the viral genome is integrated into the host chromosomes, HIV begins its proviral cycle. The provirus uses the cellular machinery to produce viral proteins and RNA. Protease cleaves the new proteins, enabling them to join the RNA in new viral particles that bud from the cell and infect others (1). Post-translational control is necessary to excise the gp160 precursor into functional envelope glycoproteins gp120 and gp41.

The fundamental process of HIV replication is one of the most intense research areas in recent years. Many strategies, including vaccine development and anti-HIV drug therapy, have been taken to prevent and/or control the virus infection. Soluble CD4 molecules, synthesized based on an understanding of the primary cellular receptor of HIV-1, effectively block viral entry in vitro (9). However, the efficiency of soluble CD4 in vivo has been disappointing (for review, 10). However, some peptide inhibitors based on the structure of HIV envelope glycoproteins, were reported to inhibit the viral entry in vitro (11-13).

Most of the clinically-used anti-HIV compounds inhibit the activity of the pol gene products, especially HIV RT and protease. The major drugs which are approved for the treatment of HIV infection include dideoxynucleoside HIV-1 RT inhibitors and HIV-1 protease inhibitors. However, emergence of drug resistant viruses has reduced the clinical benefit of these anti-HIV drugs in monotherapy (14). Highly active antiretroviral therapy (HAART), which involves using multiple drug combinations, has been shown to be able to reduce plasma viral load to undetectable levels in HIV infected patients (15-16). However, recent reports indicate that an effective anti-HIV therapy remains elusive. Chun et al. (17) has shown that a population of resting CD4 cells are capable of producing HIV even though patients were undergoing highly active combination therapy. Viruses recovered from HAART patients are still sensitive to the drugs. This persistent viral infection under prolonged combination therapy is likely due to the inability of current drug treatments to clear HIV from certain reservoirs, such as resting CD4 lymphocytes (17). Likewise, Wong et al. (18) and Finzi et al. (19) were able to recover replication-competent HIV from patients undergoing prolonged HAART. Although

triple-drug therapy could effectively control plasma viremia, it has become clear that the virus is suppressed rather than eradicated in HIV infected individuals.

Therefore, persistent HIV infection remains a challenge for AIDS therapy. In addition, other drawbacks, such as side effects incurred during HAART, may result in drug withdrawal. Nevertheless, it is hopeful that further improvements might be achieved for optimal anti-HIV therapy. Novel regimens, such as using drugs with unique modes of action, might be needed to further improve current combination anti-HIV therapy.

Triterpene derivative(s) (TD) are a class of potent anti-HIV agents that inhibit HIV replication at nanomolar concentrations (20). The mechanism of action (MOA) of TD is different from those of the drugs currently used for anti-HIV therapy. IC5964 is a TD that targets HIV-1 entry into the host cell (Figure 2). It has been shown to completely inhibit syncytia formation at a concentration of 0.1 μg/ml (Chen, unpublished results, Table 1).

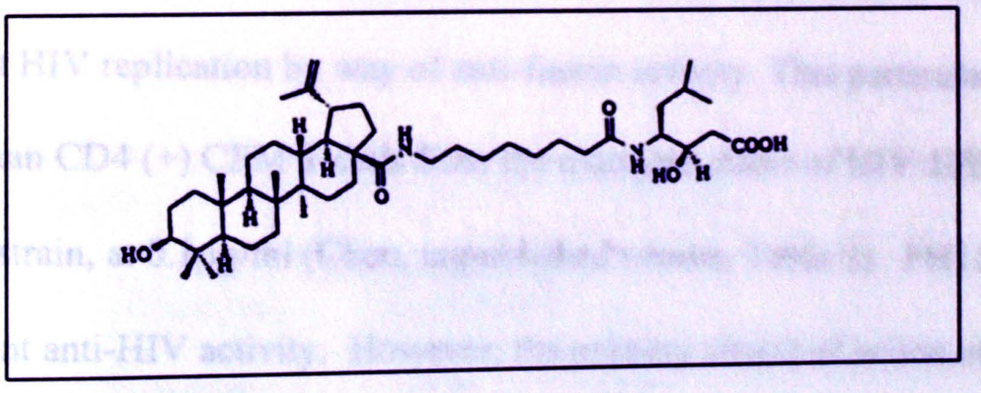

Figure 2. Chemical structure of IC5964.

Table 1. Cell infusion assay with TD inhibiting syncytia formation.

Compound	*IC 100 (μg/ml)
FH11309	20
FH11318	40
FH11328	40
FH11327	30
IC5964	0.1

*IC 100= concentration required to completely inhibit HIV-1 induced syncytia.

The discrepancy between that of IC5964 and other TD compounds may be explained in that membrane fusion is not the major site of action for the TD such as FH11328, or FH11318. These compounds are classified as N-triterpenes (N-TD) as their primary site of action is unknown. Therefore, IC5964 is classified as an E-TD due to its ability to inhibit HIV replication by way of anti-fusion activity. This particular compound can protect human CD4 (+) CEM T cells from the cytolytic effect of HIV-IIIB, a lab-adapted HIV-1 strain, at 0.1μg/ml (Chen, unpublished results, Table 2). FH11327 is an N-TD with potent anti-HIV activity. However, the primary site(s) of action of FH11327 is not at the virus entry stage as mentioned.

Table 2. Anti-HIV-IIIB activity of TD.

Compounds	*IC50(ug/ml)
IC5964	0.1
FH11327	0.006

*IC50 = concentration required to inhibit 50% of HIV-1 activity.

Both the anti-viral and anti-fusion concentration of IC5964 is 100 ng/ml. However, to determine whether there was another site of action in the anti-HIV activity of TD compounds, their effect on RT activity was examined. The results in Table 3 (Chen, unpublished results) indicate that the tested compounds, including IC5964, did not inhibit HIV RT activity at a concentration of 100 μg/ml. The nucleotide analog ddCTP is a known HIV RT inhibitor and was used as a control. Accordingly, IC5964 appears to inhibit HIV replication through its activity against HIV envelope-mediated cell-cell fusion.

Table 3. Effect of triterpene derivatives on HIV RT activity.

Compound	*IC 50 (μg/ml)
FH11318	>100
FH11328	>100
FH11327	>100
IC5964	>100
ddCTP‡	8

*IC50 = concentration required to inhibit 50% of HIV-1 RT activity.

‡ddCTP was used as a positive control.

HIV-1 envelope glycoproteins are the key viral components that mediate membrane fusion. Therefore, they could be the potential targets for anti-fusion compounds such as IC5964. There are three specific goals of this study. First: to construct a chimeric virus using IC5964 sensitive and resistant HIV strain. This chimeric virus will allow determination of the viral component that is responsible for the drug sensitivity. Second: to develop drug resistant HIV variants after determining the target for IC5964. These variants will be used to fine map the key viral sequences that are determinants of the drug sensitivity. Third: to study and identify the key amino acid residues that contribute to the drug resistant phenotype. The primary purpose is to understand how IC5964 inhibits the virus by comparing the differences between wild type and mutant viruses.

MATERIALS AND METHODS

I. NL4-3/DH012 chimeric virus

A. Construction of virus

The IC5964 sensitive strain NL4-3 and the IC5964 resistant primary isolate strain DH012 were used to construct a chimeric virus NL4-3/DH012 which contained the gp120 sequence from DH012 virus in the genetic background of NL4-3 virus. This chimeric virus was constructed by replacing the EcoRI/HgaI NL4-3 envelope fragment with the EcoRI/HgaI DH012 envelope fragment. The EcoRI/HgaI fragment contains the entire gp120 sequence of the envelope glycoproteins.

B. Virus infectivity reduction assay

To determine if the chimeric virus was sensitive or resistant to the compound, a virus infectivity reduction assay was performed. CEM cells (T-lymphoblastoid cell line) were used in this assay. A 96-well microtiter plate was used to set up the assay. After infecting the cells with the virus, samples (culture supernatants) were collected at day 5 for micro RT assay to estimate the virus infection. The details of the micro RT assay are described in the following paragraph.

C. Micro HIV-1 RT assay

Reverse transcriptase activity was determined by a modification of the published methods of Goff et al. (21) and Willey et al. (22). Briefly, harvested culture supernatants were adjusted to 1% (vol/vol) Triton X-100. A 10 µl sample of each Triton lysate was mixed with 50 µl of a reaction cocktail containing: 50 mM Tris-HCl, pH 7.8, 75 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, 5 µg/ml Poly rA, 1.5 µg/ml Oligo dT_{12-18}, 0.05% NP-

40, and 10 μCi/ml ^{32}P-TTP, and incubated at 37°C for 90 min. Aliquots (40-50 μl) of the reaction mixtures were spotted onto DE-81 paper (Whatman) in a minifold sample filtration manifold (Schleicher & Schuell), washed several times with 2X SSC (0.3M NaCl, 0.03M NaCitrate), followed by 2X SSC containing Bromophenol blue to locate spots. Autoradiography was performed, and radioactivity quantified with a Packard Matrix 9600 Direct Beta Counter.

II. Molecular cloning of the HIV resistant envelope

A. Isolating the envelope sequence

The NL4-3 virus was grown in increasing concentrations of IC5964. The mutant virus was infected into CEM cells. The cellular DNA that contained the viral insert was then extracted. A polymerase chain reaction (PCR) was used to amplify the gp120 envelope sequence using the Perkin-Elmer GeneAmp PCR system.

B. Ligation of envelope into TA vector, pCR 3.1

The gp120 envelope sequence from the mutant virus was cloned into a TA vector (Invitrogen) by ligating the PCR product directly into the TA cloning vector. By using Taq polymerase, single 3' A-overhangs are added to each end of the PCR product. The TA vector is linearized with single, 3' T-overhangs to enable direct ligation. A transformation was then performed with oneshot competent cells in order to obtain the correct colonies.

C. Restriction endonuclease digestion of DNA

To select for the appropriate vector with the gp120 insert, a restriction endonuclease digestion was done. Restriction endonucleases were chosen based on the restriction enzyme sites designed within the insert. Each enzyme, XhoI/KpnI (10 u/λ), along with 10X A Buffer (Promega), was added to each plasmid DNA according to the reaction conditions listed in the protocol (23). The final volume was adjusted using ddH_2O. Each reaction was incubated at 37°C for 4 h.

III. Mutagenesis with pBluescript II KS (+/-) phagemid vector

A. Restriction endonuclease digestion of PBK, NL4-3, and PSRHS

Due to the large size of the PSRHS plasmid (9.2Kb), there was difficulty in inserting the mutated envelope sequence into this vector. Therefore, the envelope was removed from the NL4-3 plasmid, 3'NL, since it contained DNA from an HIV-1 primary isolate. The 3'NL envelope was then ligated with PBluescript II KS (+/-), PBK, which is a commercial cloning vector (Stratagene) chosen due to its small size and cloning capabilities. Once mutagenesis was complete and the mutated envelope sequence was determined, the mutated 3'NL sequence was removed from the PBK vector and inserted into the PSRHS plasmid vector to express the mutated envelope sequence. Wild-type PSRHS was digested to removed the envelope sequence in order to ligate the 3'NL mutated sequence as well as the wild-type 3'NL sequence.

Restriction endonucleases were chosen based on the restriction enzyme sites of the PSRHS plasmid map (Figure 3), PBK plasmid map (Figure 4), and of the 3'NL sequence map. In order for the insert to be compatible with both plasmids, Kpn and XhoI

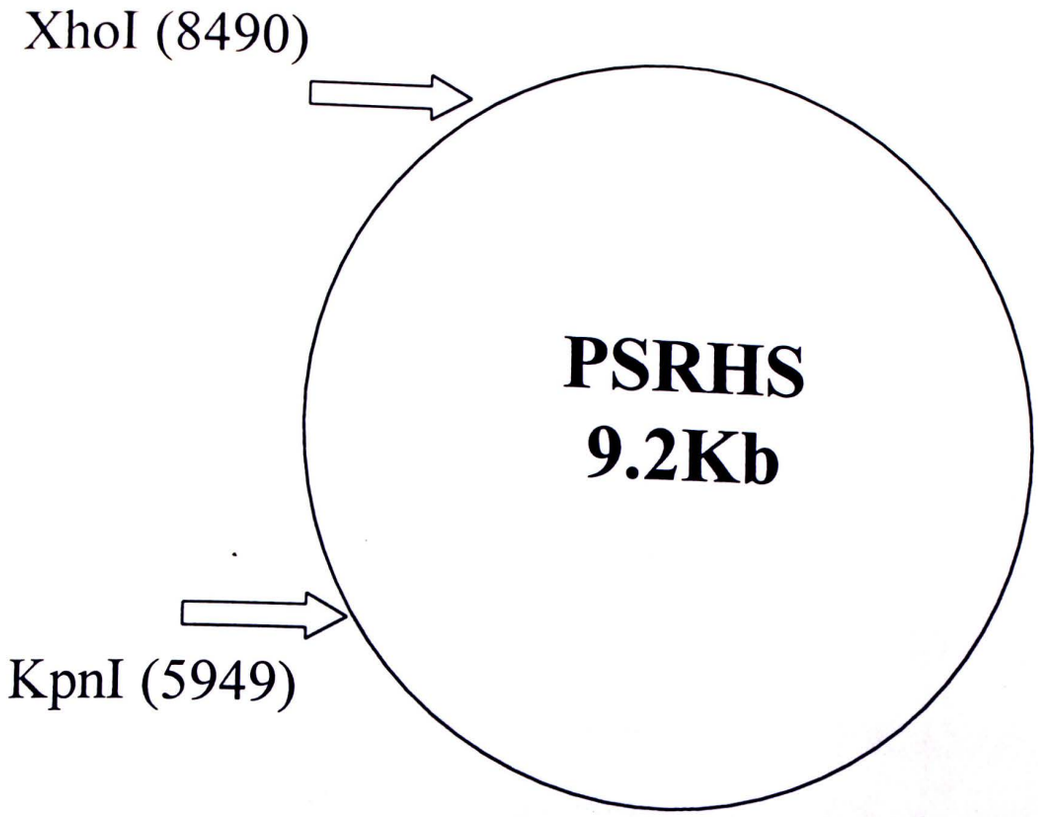

Figure 3. Restriction endonuclease map of PSRHS plasmid.

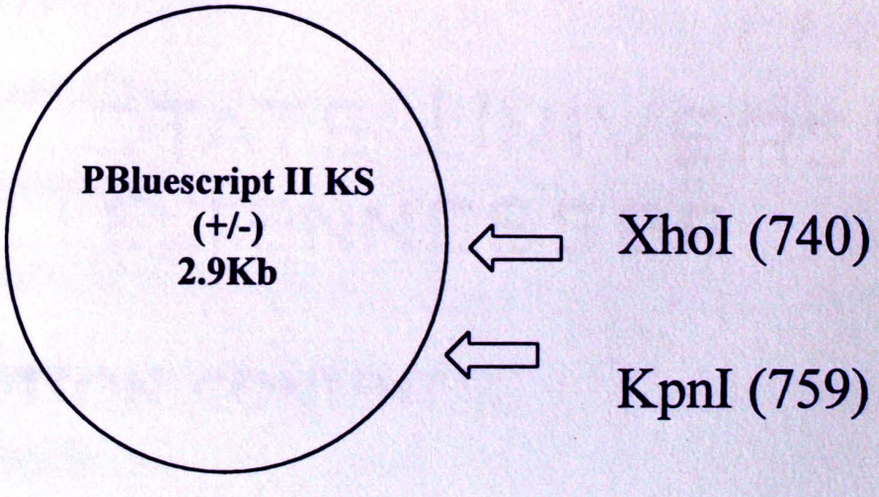

Figure 4. Restriction endonuclease map of PBK plasmid.

restriction endonucleases were chosen. Both KpnI (10 u/λ) and XhoI, along with 10X A Buffer (Promega), were added to each plasmid DNA according to the reaction conditions listed in the protocol (23). The final volume was adjusted using ddH_2O. Each reaction was incubated at 37°C for 4 h. Each reaction was loaded into a 0.8% agarose gel to confirm digestion and for purification.

B. Purification of DNA

The DNA was isolated and purified from a 0.8% agarose gel. Using an ultraviolet (UV) light source, along with a razor blade, the piece of DNA was extracted, placed into an Eppendorf tube, and froze for 1 h at -20°C. The frozen agar was then centrifuged at 14 X 1000 min^{-1} for 5 min to loosen the agar, transferred to a spin-x tube (Falcon 2059), and centrifuged for 15 min. Two-hundred microliters of TE80 buffer was added to the tube, centrifuged 15 min, and repeated a second time. The volume was adjusted to approximately 500 µl using n-butanol and chloroform.

C. Precipitating DNA

DNA was precipitated by the addition of 1/10 volume 3 M Sodium acetate, pH 5.2, and one volume isopropanol. After remaining at -20°C for one hour, the DNA formed a pellet by centrifugation at 14 X 1000 min^{-1} for 20 min (Eppendorf Centrifuge 5415 C). The DNA pellet was washed with 300 µl of 70% ethanol and centrifuged for 10 min. After drying with a vacuum, the DNA was allowed to air dry, resuspended in TE80 buffer (1 M Tris-HCl, 0.5 M EDTA, pH 8.0) according to the size of the pellet and stored at 4°C.

D. Agarose gel quantification of DNA

To quantify the amount of each DNA present to perform a ligation, a 1% agarose gel was prepared by dissolving 2 g agarose in 200 ml dH_20 by heating to 100°C. After the agarose solution cooled, 4 ml 50X TAE buffer was added along with 10 λ of a 10 mg/ml stock solution of Ethidium bromide. The entire contents were poured into an agarose gel forming apparatus (7200 electrophoresis system, Gibco BRL, Life Technologies). After solidifying, the comb was removed from the gel and the entire apparatus was placed in the electrophoresis system. A 1 X TAE buffer solution was added to the chamber until the wells of the gel were completely submerged. DNA samples and PSRHS standards were combined with 6 X loading dye (Promega), loading the wells accordingly. The agarose gel was electrophoresed at 180 V for approximately 45 min. The amount of the DNA present was quantified according to the brightness of the band in comparison with each standard.

E. Ligation of 3'NL envelope sequence / PBK plasmid vector and 3'NL envelope sequence / PSRHS plasmid

Approximately 9 μg of the envelope sequence insert and 3 μg digested PBK vector were combined with 1 unit of T_4 DNA Ligase (30 u/λ), T_4 DNA Ligase 10 X buffer (Promega) and ddH_20. The reaction was incubated overnight at room temperature. To confirm ligation, a 1 % agarose gel was run using 1 λ of the reaction.

F. Transformation of 3'NL/PBK and 3'NL/PSRHS into HB101 competent *E. coli* cells

The ligated plasmid was then transfected into competent cells in order to obtain colonies to screen for the appropriate clone. The reaction conditions were set up according to the Gibco BRL protocol (24). The ligation from above was combined with a 50 λ aliquot of HB101 competent cells (Life Technologies, Gibco BRL) into a prechilled Falcon 2059 polypropylene tube. The reaction was incubated on ice for 30 min, heat shocked for 45 s in a 42°C water bath, and incubated on ice for 2 min. A 0.5 ml aliquot of LB medium was added to the transformation reaction and placed into a shaking incubator at 225-250 rpm for 1 h at 37°C. The reaction was spread onto LB agar plates containing 100 μg / ml ampicillin, using 100 λ reaction per plate. The plates were incubated overnight at 37°C. Several isolated colonies were chosen to extract and purify the DNA using a miniprep for a small yield.

G. Miniprep DNA Purification

The plates from the transformation contained a number of colonies following incubation. Isolated colonies from certain plates were chosen and inoculated into 2 ml LB broth containing 100 μg / ml ampicillin and grown overnight at 37°C in a shaking incubator at 225-250 rpm. Each reaction was set up according to the Life Technologies protocol (25). Each bacterial culture was centrifuged for 5 min at 14 X 1000 min^{-1} to form a pellet. Cells were resuspended in 210 λ Cell Resuspension Solution. Cell Lysis Solution, 210 λ, was added along with 280 λ Neutralization Solution. The lysate was centrifuged at 14 X 1000 min^{-1} for 10 min. The cleared lysate was placed into a

minicolumn and centrifuged at the same speed for 1 min, discarding the supernant. Additional wash buffer, 500λ, was added, incubating one minute and centrifuging for another minute. Wash buffer solution, 700λ, was added, centrifuged one minute, discarding the supernant, and again centrifuged. The DNA was eluted with 75λ TE80 buffer at 68°C and stored at 4°C.

H. Restriction endonuclease digestion of colonies

To determine which colonies contained the 3'NL insert in the PBK vector and the 3'NL insert in the PSRHS vector, a digestion was performed. The same restriction endonucleases chosen to digest the 3'NL, PBK, and PSRHS plasmids (KpnI/XhoI) were used to determine the appropriate clones. A second digestion was done on the selected clones using the restriction endonucleases (MluI/XhoI) to confirm the mutation. Each reaction was loaded into a 0.8 % agarose gel to confirm digestion.

I. Site-directed mutagenesis

The mutated 3'NL sequence was created using the 3'NL/PBK plasmid as the template. The procedure was setup according to the STRATAGENE mutagenesis protocol (26) to obtain the first mutation (M1). Sample reactions, including the primers and template 3'NL/PBK, were cycled using the Perkin-Elmer GeneAmp PCR System 2400. Products were digested by using 1μl of the restriction enzyme DpnI (10 u/μl) and incubated at 37°C for 1 hr to digest the parental DNA. A 50 λ aliquot of Epicurian Coli XL-1 Blue supercompetent cells were thawed on ice and transferred to a prechilled Falcon 2059 polypropylene tube. The Dpn-1 treated DNA, 2λ, was transferred to the aliquot of cells. The reaction was swirled gently and incubated on ice for 30 min. The transformation reaction was heat pulsed for 45 s at 42°C and then placed in ice for 2 min.

LB medium, 0.5 ml at 42°C, was added to the reaction and incubated at 37°C for 1 hr at 225-250 rpm. The transformation was plated immediately on LB agar plates containing 100 μg/ml ampicillin, using 100λ per plate. The transformation plates were incubated at 37°C overnight. Isolated clones from each plate were inoculated into LB broth containing 100 μg / ml ampicillin and grown overnight at 37°C shaking incubator at 225-250 rpm to perform a DNA Miniprep (Section III: G). A restriction endonuclease digestion was also performed to confirm the correct clone (Section III: H).

IV. Ligation of mutated 3'NL envelope sequence with PSRHS plasmid

A. Restriction endonuclease digestion of mutated 3'NL/PBK

Once the mutated 3'NL/PBK sequence was determined correct, the envelope sequence was removed for placement into the PSRHS plasmid vector (Section III: A). Each reaction was loaded into a 0.8% agarose gel to confirm digestion and to purify (Section III: B-C). Each DNA sample was then quantified on a 1% agar gel (Section III: D). The mutated 3'NL envelope sequence was then ligated with the PSRHS vector (Section III: E).

V. DNA sequencing

A. Gel electrophoresis preparation

To determine if the desired mutation was present, each set of DNA samples was sequenced. The 20 X glycerol tolerant gel buffer and 5 % sequencing gel was prepared according to the "T7 sequenase quick-denature plasmid sequencing kit"(Amersham Life

Science) protocol (27). Each gel was allowed to polymerize overnight. Prior to loading each reaction, each well was cleaned well with 20 X glycerol tolerant buffer. To check for leaks, 4 µl of 6 X loading dye (Promega) was added to several wells, pre-running the gel at 1800 V, 400 mA, 80 W to warm the gel for sequencing.

B. Alkaline denaturation

Each reaction was set up according to the protocol (Amersham Life Science, 25) using the alkaline denaturing method. For the alkaline denaturation, 0.5 - 5 µg mutated plasmid DNA was added with 2 µl of 1.0 M NaOH, 1 - 3 pmole/ µl primer, and ddH_2O to adjust to a total volume of 11 µl. The reaction mixture was incubated in PCR tubes (Perkin Elmer) at 37°C for 10 min and placed on ice. Two microliters of both 1 M HCL and plasmid reaction buffer was added to the reaction, annealed for 10 min at 37°C and placed on ice. The termination mixture, 2.5 µl, (A, C, G, T) was aliquoted to labeled tubes, capped, and placed on ice.

C. Labeling reaction

The DNA reaction mix, along with 1 µl of 0.1 M dithiothreitol (DTT), 2 µl diluted labeling mix (DLM), 1 µl [α^{35}S]dATP, and 2 µl T_7 sequenase plasmid sequencing formulation were combined. The labeling reaction was incubated at room temperature for 3 min. The termination mix was preheated at 37°C for 1- 3 min. The labeling reaction, 4.5 µl, was transferred to each dideoxynucleotide (adenine-A, cytosine-C, guanine-G, thymine- T) tube, respectively, and incubated at 37°C for 5 min. A stop solution, 4 µl, was added to each tube.

D. Electrophoresis

Each sample was heated at 75°C for 2 min and immediately loaded on the gel (4 µl), using the same parameters as for heating the gel (Section VI-A). A second loading of the samples was done when the first load of samples reached the lower middle portion of the gel. When completed, the gel was cooled in cold H_2O, separating the two plates, and transferring to Whatman 3MM filter paper. The gel was dried at 80°C for 2 hrs using the Fisher Biotech FB GD 45 Gel Drying System.

E. Exposing the gel

The dried gel was placed into a Fisher Biotech Electrophoresis System Autoradiography Cassette, taken to the dark room, with a piece of double-coated film being placed on the gel. The gel was exposed at room temperature overnight.

F. Developing the film

In the dark room, under red light, the film was removed from the cartridge and placed into the developing solution for 5 min. After rinsing in H_2O, the film was fixed in fixing solution for 4 min. The film was washed well with tap H_2O and dried at room temperature. Each sequence was read using the Fisher Biotech White Light Transilluminator.

G. DNA Maxiprep Purification

For each correct clone, a larger amount of DNA was prepared in order to sequence and perform a cell infusion assay. The clones from each plate were inoculated into 200 ml LB broth containing 100 µg / ml ampicillin and grown overnight at 37°C in a shaking incubator at 225-250 rpm. Each reaction was set up according to the Gibco BRL protocol (25). Each bacterial culture was centrifuged for 5 min at 3,000 rpm to form a

pellet. Cells were resuspended in 6 ml G1 (resuspension) buffer. The same amount of G2 (lysis) buffer was added, incubating the reaction at room temperature for 5 min. G3 (neutralization) buffer was added, 8ml, and centrifuged at 3,000 rpm g for 10 min to pellet the cellular debris. The supernant was poured into a cartridge and centrifuged at 3,000 rpm for 10 min. After discarding the supernant, 15 ml GP4 (wash) buffer was added, centrifuging for 5 min at 2,000rpm. DNA was eluted with 4 ml TE80 that had been preheated to 68°C. The DNA was precipitated (Section III: C). An ultraviolet (UV) spectrophotometer was used to determine the yield.

VI. Cell Infusion Assay

A. Transfection of COS cells with wildtype and mutant DNA

To determine if each mutation present in the PSRHS plasmid was sensitive or resistant to the IC5964 compound, each was transfected into COS (monkey kidney cells) and incubated with MOLT-4 (CD4 lymphoid cells) in a cell fusion assay to determine if viral entry took place. The COS cells were trypsinized by washing with trypsin-EDTA and removing immediately. Trypsin-EDTA was added again (1-5 ml), completely covering the cells. After 1-2 minutes, the trypsin-EDTA was removed, leaving 0.2-0.3 ml in the tissue flask. The COS cells were then placed into a 37°C / 5% CO_2 incubator 5 minutes or until complete detachment of the cells. Cells were resuspended completely using 5 ml Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Gibco BRL), using a hemacytometer to calculate the total cell number as well as the amount of cells per ml. One-tenth of the cells were placed into a T_{25} tissue culture flask with 5 ml DMEM and kept in the incubator. A portion of the cells (10^6) were centrifuged in a 50

ml tube at 1,000 rpm for 5 minutes. After discarding the supernant, the pellet was resuspended in 0.4 ml DMEM. The cells were transferred into a sterilized 1.5 ml Eppendorf tube, with the addition of 2 μg of DNA. The mixture was incubated on ice for 10 minutes. Following incubation, the COS cells underwent electroporation (150V, 950μF). The electroporated cells were transferred into a T_{25} flask with 5-10 ml DMEM and placed into the incubator overnight.

B. Incubation with MOLT-4 cells

The transfected COS cells were trypsinized according to the above protocol. Cells were resuspended in 5 ml DMEM and counted. According to the cell number, the cells were centrifuged at 1,000 rpm. For cell concentrations less than 1.6×10^5 / ml, centrifugation took place for 5 minutes. The supernant was discarded. The cells were resuspended in DMEM at a concentration of 1.6×10^5 / ml and placed on ice. About 3×10^6 MOLT-4 cells were centrifuged at 1,000 rpm for 5 minutes, resuspending in RPMI Medium 1640 (Life Technologies, Gibco BRL) at 2.4×10^6 / ml. The TD compound was prepared at six different concentrations, starting at 50 mg/μl and using 3-fold dilutions thereafter. For a control, 50 μl RPMI, 25 μl transfected COS, and 25 μl MOLT-4 cells were placed into each well of the 96-well incubation plate. For evaluating the effects of the compound, 50 μl of each concentration of the compound was added to each well of the 96-well incubation plate along with 25 μl transfected COS and 25 μl MOLT-4. The plates were placed into a 5% CO_2 incubator and incubated at 37°C overnight. The COS syncytia formation was checked the following morning.

C. Enzyme linked immunoassay (ELISA)

The cells cultured on 24 well plates were treated with 100% fetal calf serum (FCS) to block the protein binding sites of the tissue culture wells. Phosphate-buffered saline (PBS) containing antibodies (Triton 100-treated sera from HIV-1 positive individuals at a 200-fold dilution) were added in the presence of 0.5 % Bovine serum albumin (BSA) in Tris-buffered saline (TBS) BS (40 mM Tris-HCl pH 7.5, 150 mM NaCl) and incubated for 1 hour. The plates were washed three times with TBS after incubation with the antibodies. Horse radish peroxidase (HRP) conjugated secondary antibodies (goat anti-human IgG, Sigma) at a 2000 fold dilution in TBS with 0.5 % BSA were added to each well and incubated at room temperature for 45 minutes. The plates were then washed four times with TBS. The peroxidase substrate o-phenylene diamine (0.4 mg/ml) and 0.03% H_2O_2 was added for color development. The supernants were collected in eppendorf tubes for the removal of cell debris. The reaction was stopped with an equal volume of 4.5 N H_2SO_4 after incubation at room temperature for 10 minutes. The optical density of the reaction mixture was measured at 490 nm with a micro plate reader (Molecular Design).

RESULTS

Mapping of viral genes responsible for IC5964 sensitivity.

In order to evaluate the mode of action of the TD compound IC5964, the envelope glycoprotein gp120/gp41 was hypothesized as the target for IC5964. To test this hypothesis and identify which envelope subunit (gp120 or gp41) could be the determinant for the drug sensitivity, a chimeric virus was constructed by using a drug sensitive HIV strain and a drug resistant HIV strain.

Construction of a chimeric virus.

An IC5964 sensitive strain, NL4-3, and the IC5964 resistant strain, DH012, was used to make a chimeric virus NL4-3/DH012 which has the gp120 sequence from the DH012 virus in the genetic background of NL4-3 virus. This chimeric virus was constructed by replacing the EcoRI/HgaI NL4-3 envelope fragment with the EcoRI/HgaI DH012 envelope fragment. The EcoRI/HgaI fragment contains the entire gp120 sequence of the envelope glycoproteins.

The results in Figure 5 show that NL4-3 is very sensitive to IC5964 and DH012 is relatively resistant to the compound. The chimeric virus behaves similarly to DH012 in the virus infectivity reduction assay in the presence of IC5964. This assay was done by infecting CEM cells with various dilutions of viruses in the presence of different concentrations of IC5964. The culture supernants were collected and assayed for HIV

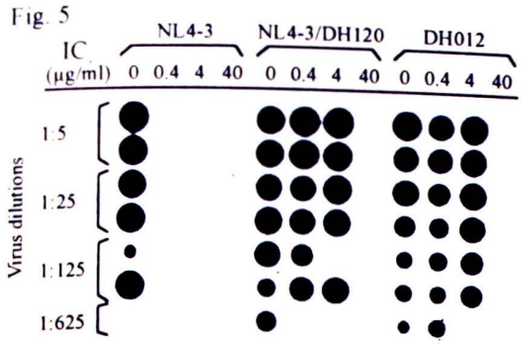

Figure 5. Virus infectivity reduction assay. Autoradiograph of micro-RT assay. Each positive dot is an indicator for active HIV replication.

RT activity with a micro-RT assay. Figure 5 is an autoradiograph of the micro-RT assay. Each positive dot in the figure is an indicator for active HIV replication. These data suggest that gp120 is the viral protein responsible for the drug sensitivity since the change in gp120 sequence is sufficient to alter the drug sensitivity of the virus. However, DH012 provides limited usefulness in further mapping the key site within the envelope sequence that is responsible for the IC5964 sensitivity due to the extensive differences between NL4-3 and DH012 throughout the gp120 sequence. Therefore, a drug resistant mutant derived from the IC5964 sensitive NL4-3 was a better candidate to further map the amino acid residues involved in the drug sensitivity. In order to do this, a drug resistant mutant was selected.

Development of drug resistant HIV variant.

The selection and analysis of the drug resistant variant was accomplished by growing the virus in increasing concentrations of the compound to develop a drug resistant mutant. A micro RT assay was performed to determine if the mutant was

actually resistant to the drug. According to Figure 6, the mutant virus continued to maintain RT activity and replicate in escalating doses of the IC5964 compound, with each dot representing active viral replication.

To obtain the envelope sequence from the drug resistant mutant, the virus was used to infect CEM cells. Following infection, cellular DNA containing the viral insert was extracted. Once the cellular DNA was isolated, a PCR was used to amplify the gp120 envelope sequence. The sequence was then cloned into a TA vector. The TA cloning system (Invitrogen) uses Taq polymerase to add a single 3' A-overhang to each end of the PCR product. The plasmid is supplied linearized with single, 3' T-overhangs to enable direct ligation of PCR products at high efficiencies. After cloning the PCR insert into the plasmid, a transformation was done for bacterial replication of the plasmid. To select a colony with the mutated gp120 sequence, a restriction endonuclease digestion was performed. Once the correct clone was identified, the DNA was extracted and amplified. The gp120 sequence of the drug resistant mutant virus was sequenced (Figure 7).

According to the gp120 envelope sequence map, there were two particular mutations of interest. The first mutation (M1), located at amino acid 237, is of major importance. This particular mutation is an amino acid change from a glycine, neutral amino acid with uncharged nonpolar side chain, to an arginine, basic amino acid with charged polar side chain (G→R). The second mutation, located at amino acid 252, is a more conserved change that remains a basic amino acid with charged polar side chains, arginine to a lysine (R→K).

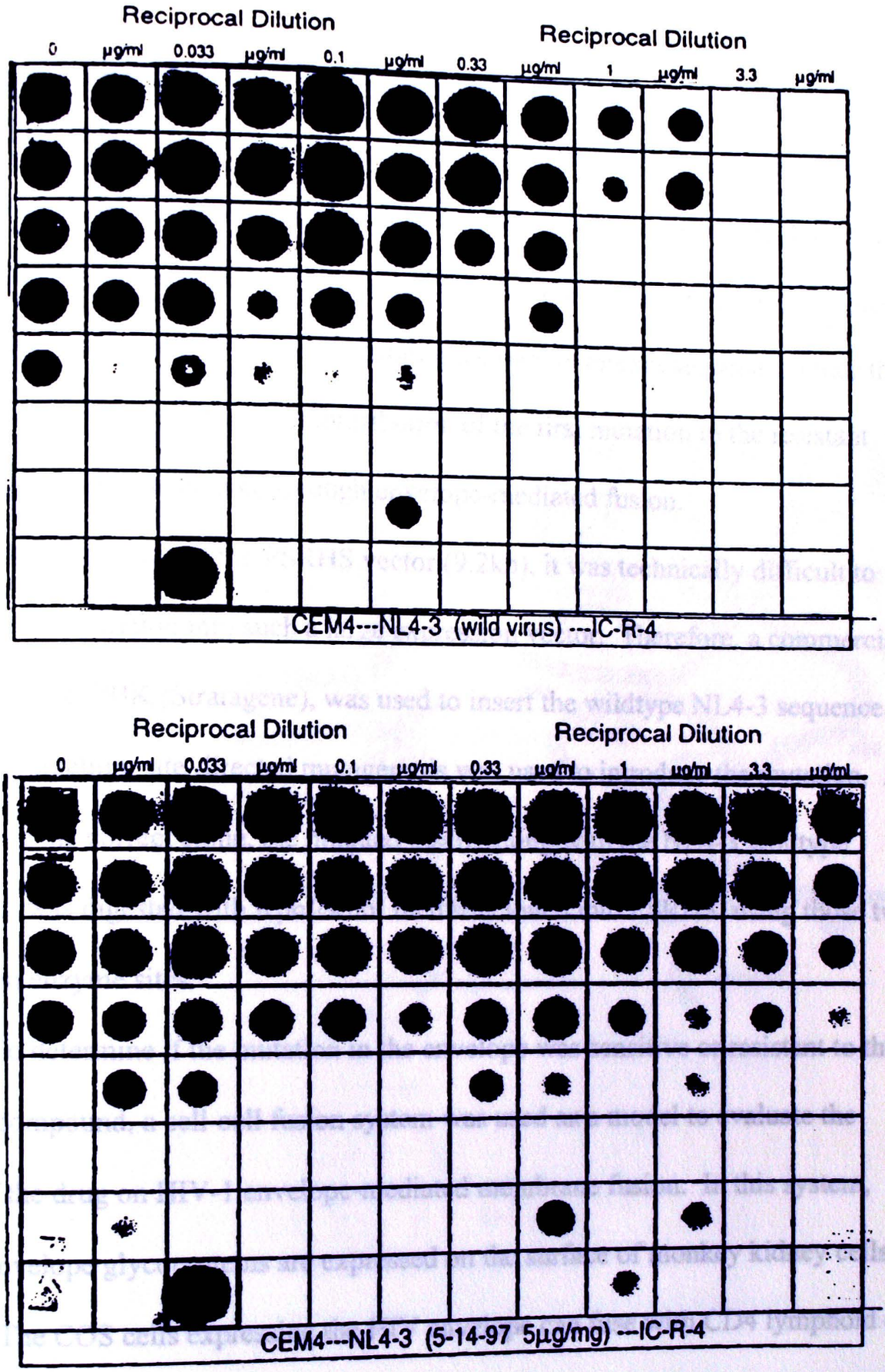

Figure 5. Autoradiograph of micro HIV-1 reverse transcriptase assay.

Analysis of each mutation to the drug resistant phenotype.

The goal of this particular part of the study was to analyze each mutation, beginning with M1. This analysis was performed in order to confirm the sequence analysis previously obtained. At times, random mutations of the virus occur. Therefore, this evaluation was important for confirming the correct mutant sequence. Once the correct mutant was obtained, the contribution of the first mutation to the resistant phenotype could be evaluated through envelope-mediated fusion.

Due to the size of the PSRHS vector (9.2kb), it was technically difficult to introduce the mutation into such a large expression vector. Therefore, a commercial cloning vector, PBK (Stratagene), was used to insert the wildtype NL4-3 sequence. Following ligation, site-directed mutagenesis was used to introduce the mutation. After amplifying the correct clone, the mutated insert, along with the NL4-3 wildtype sequence, was digested with KpnI/XhoI then subcloned into PSRHS, using those two restriction enzyme sites.

To determine if the mutation in the envelope was sensitive or resistant to the IC5964 compound, a cell-cell fusion system was used as a model to evaluate the effect of the drug on HIV-1 envelope-mediated membrane fusion. In this system, HIV-1 envelope glycoproteins are expressed on the surface of monkey kidney cells (COS). The COS cells expressing the HIV envelope can fuse with CD4 lymphoid cells (MOLT-4) to form multi-nucleated giant syncytia cells. Anti-HIV agents that can interfere with the interaction between the HIV-1 envelope and cellular receptors could inhibit syncytia formation.

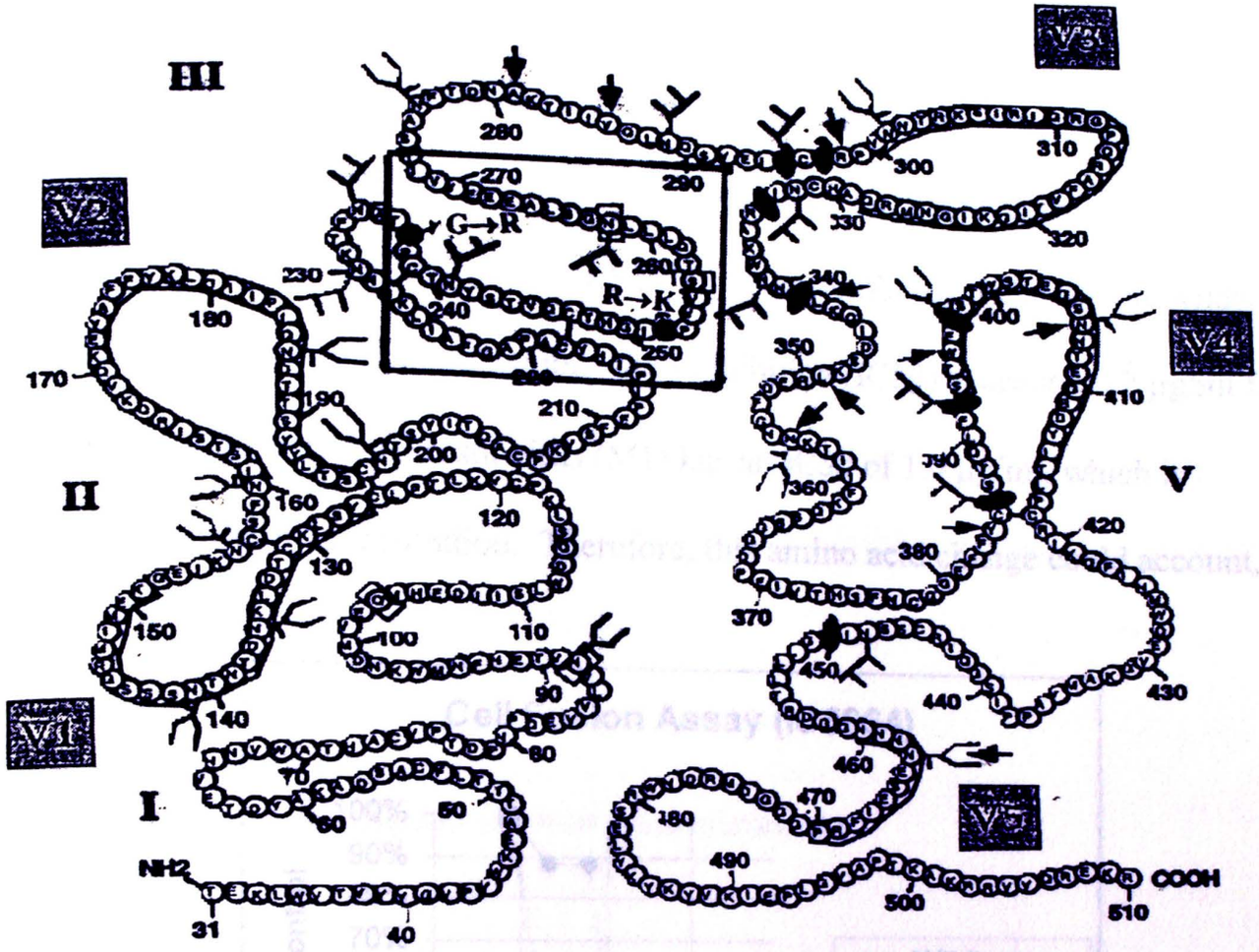

Figure 7. Amino acid map of gp120 HIV-IIIB sequence. Each mutation is located within the box. The first mutation is noted at amino acid number 237 (G→R, glycine to arginine). The second mutation is noted at 252 (R→K, arginine to lysine).

Figure 8 shows the results of this assay for the wildtype and M1 viruses with syncytia percentages of the control. Multi-nucleated syncytia cells were measured as fusion of simulated HIV viral and host cells. Controls consist of both wildtype and mutant virus without the presence of the IC5964 compound. The average number of syncytia in the wildtype control wells was 45. Figure 8 shows the percent of control that was achieved at various concentrations of IC5964 when combined with both the wildtype and mutant virus. The compound exhibits 50% inhibition (IC50) at around 0.2 μg/ml for the wildtype. However, the mutant virus (M1) has an IC50 of 1.0 μg/ml, which is a five-fold increase in concentration. Therefore, this amino acid change could account, in

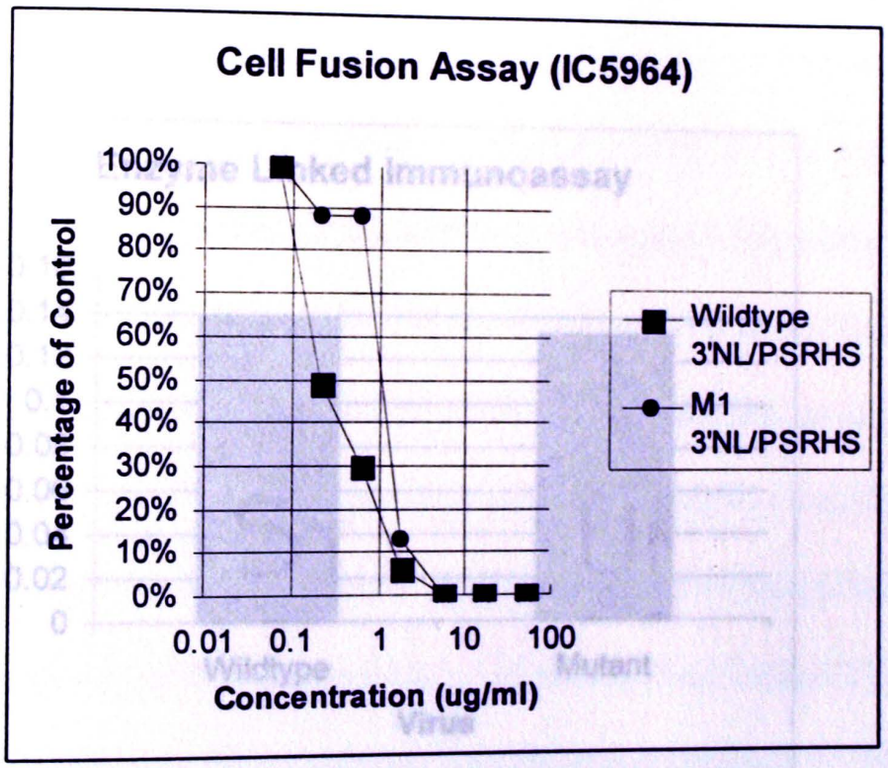

Figure 8. The results of a cell-cell fusion system that evaluated the effects of IC5964 on HIV-1 entry are shown. Multi-nucleated syncytia cells were measured as fusion of simulated HIV viral and host cells. Wells without compound were used as controls. The average number of syncytia for the wildtype control was 45, with m1 being 8. The compound exhibits IC50 at around 0.2 μg/ml for the wildtype virus and 1.0 μg/ml for the mutated virus.

part, for the virus becoming less sensitive to the IC5964 compound.

Another phenomenon observed in the fusion assay was that M1 had a compromised ability to induce syncytia. One explanation for this observation is that the amount of envelope expression of M1 is low. Therefore, an enzyme linked immunoassay (ELISA) was performed in order to determine the amount of gp120 that was expressed on the COS cell surface.

By electroporating COS cells with both wildtype and M1 DNA, the envelope would be expressed on the cell surface. By adding antibody (HIV-1 positive serum at a 200-fold dilution) to each well, the amount of gp120 expressed on the surface could be measured according to the amount of binding,

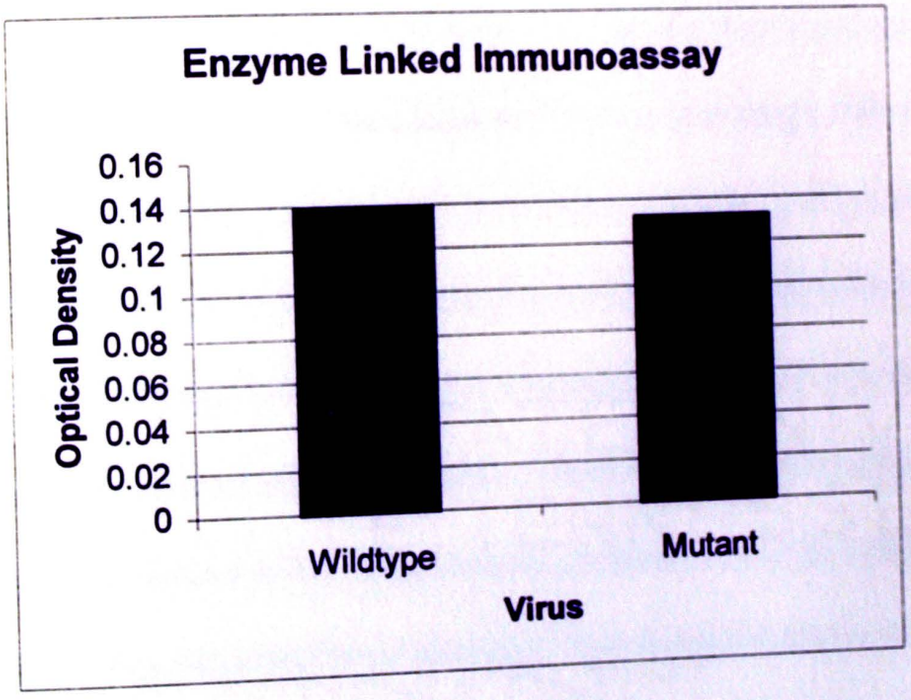

Figure 9. ELISA results, with antibody (HIV-1 positive serum at a 200-fold dilution), indicating the amount of gp120 envelope glycoprotein expressed on the surface of COS cells for both wildtype and mutant 3'NL/PSRHS. The optical density was measured at 490 nm.

compared to controls. The results in Figure 9 indicate that approximately the same amount of envelope is expressed on both the wildtype and M1 COS cells. Therefore, the reduced fusion ability of the M1 envelope most likely resulted from the glycine to arginine mutation that affected the function of the envelope glycoprotein.

DISCUSSION

The data from this study indicates that IC5964 is a HIV-1 entry inhibitor that can inhibit replication at certain concentrations. With documented potent anti-HIV activity (Chen, unpublished data), the primary site of action of IC5964 is the gp120 envelope sequence. IC5964 is one of the only compounds that can primarily block HIV-1 replication at the very early stage of virus replication. Although this compound has not been used in pharmacological studies, it has been found to inhibit both HIV-1 primary isolates as well as laboratory-adapted strains (Chen, unpublished data).

Analysis of the chimeric virus derived from IC5964 resistant and sensitive strains indicated that gp120 is the key determinant for the drug sensitivity. This was accomplished by replacing the IC5964 resistant DH012 primary isolate strain's gp120 sequence in the genetic background of the NL4-3 sensitive strain. By using a virus infectivity reduction assay, culture supernants were collected and tested for RT activity in the presence of various IC5964 concentrations with each positive dot on an autoradiograph indicating HIV replication. As expected, NL4-3 was sensitive to the compound when compared with the control. There was no active replication. The DH012 remained resistant to the compound at all concentrations, indicated by continual HIV replication. The NL4-3/DH012 chimeric virus behaved similarly to DH012 in the presence of IC5964. These data suggested that gp120 was the viral protein responsible for the drug sensitivity since the change in the gp120 sequence was sufficient to alter the drug sensitivity of the virus.

After isolating the target for drug sensitivity, a drug resistant mutant was required to analyze the key viral sequences for the drug sensitivity. A micro-RT assay was used to determine if the NL4-3 virus, grown in escalating doses of the compound, was resistant to the compound. The results in figure 6 indicated that the IC5964 escape mutant was at least 10-fold less sensitive when compared to the wildtype NL4-3 virus. Therefore, the viral sequence could be determined to evaluate which mutations had taken place.

By using the IC5964 drug resistant strain of the virus, two major mutations were found to contribute to resistance within the gp120 sequence. Of the two mutations, the primary single-base mutation at amino acid 237 involved a change from a neutral amino acid to a basic amino acid, glycine to arginine. As opposed to the second mutation located at amino acid 252 in which the amino acid remains basic with a side polar side chain (arginine to lysine), the first mutation (M1) changed from an uncharged nonpolar side chain to a basic amino acid with charged polar side chain. When considering the two mutations, M1 was more biologically dramatic in its amino acid change. Therefore, testing began with M1.

To study the role of M1, the strategy involved introducing the single base mutation into an envelope expression vector (PSRHS) and testing for IC5964 sensitivity by use of a fusion assay. To achieve this purpose, M1 was cloned into the PBK commercial vector. Because of the size of the envelope expression vector, PSRHS (9.2Kb), it was technically difficult to directly obtain M1. Therefore, PBK, a relatively small cloning vector, was used to perform site-directed mutagenesis in which the single base mutation was introduced directly. After acquiring the M1/PBK clone, a XhoI/KpnI digestion was done in order to obtain the M1 envelope insert. PSRHS, having undergone

the same digestion to remove the wildtype envelope sequence, was used to subclone the mutant sequence. Drug sensitivity could now be determined using the M1 3'NL/PSRHS plasmid.

The M1 envelope, along with wildtype 3'NL, was expressed on the surface of COS cells and incubated with MOLT-4 cells in order to evaluate the envelope mediated cell-cell fusion by comparing the amount of syncytia for each. By expressing the M1 envelope on the COS cell surface, the number of syncytia could be numerated after incubation with MOLT-4 cells. The degree of resistance to the compound could then be determined. According to the cell fusion assay, there was a five-fold increase in resistance to the compound when comparing the IC50 of the wildtype virus to M1 (0.2 μg/ml, 1.0 μg/ml), respectively. This phenomenon could be explained by the fact that a single mutation within the gp120 sequence affected the sensitivity of the envelope to IC5964.

The amount of syncytia, when comparing wildtype (45 syncytia) and M1 (8 syncytia) control wells, was low. Speculation was that the amount of M1 envelope on the surface of the COS cells was less than that of the wildtype. Therefore, an ELISA was used to determine if there was any difference in the amount of envelope expressed by COS cells expressing the wildtype and M1 virus. By expressing the wildtype and M1 envelope on COS cells, antibodies (HIV-1 positive serum at a 200-fold dilution) were added to the cells and compared to cells without antibody to evaluate the amount of binding that had taken place. After reading the optical density at 490 nm, results indicated approximately the same amount of envelope expression had occurred on both wildtype and M1 cells (0.139, 0.132), respectively.

Since the envelope expression of wildtype and M1 was equivalent, this ruled out the chance of less envelope expression by M1. This suggests that the M1 envelope was compromised in its ability to induce syncytia. This could be due to the fact that the viral envelope may evolve a means of evading fusion inhibitors such as IC5964. Although the virus is still able to invade cells, the ability of the M1 envelope to induce fusion is compromised due to the mutation that is present. The mutation alters the envelope, which in turn, effects its function.

While this study contributes to a better understanding of how IC5964 exhibits an anti-HIV activity profile, it also raises many new questions concerning the compound. Although this compound is known to block HIV entry into the cell, the site of interaction remains unknown. The gp120 sequence must bind with the CD4 molecule initially. However, this alone is not sufficient for viral entry. The envelope must also interact with chemokine receptors, such as CCR5and CXCR4, that are cofactors in the envelope-mediated membrane fusion process. What role the second mutation may play in the entire process also remains to be found. With further investigation, these results could contribute significantly in developing this promising anti-HIV compound.

The effect of IC5964 on HIV entry into the cell creates a unique anti-HIV pharmacological profile that current therapy lacks. Drugs that are currently used in combination drug therapy are either HIV-1 reverse transcriptase or protease inhibitors. An anti-HIV agent, such as IC5964, that can block the early stages of the virus life cycle might have potential to be very useful in anti-HIV therapy.

Highly active antiretroviral therapy treatments are more efficient in their purpose. In addition, a new therapeutic strategy (28) involves the combination of interleukin-2 (IL-

2) along with HAART was reported to be encouraging. Human immunodeficiency virus could lie dormant in resting cells; therefore current drug therapy is only effective against those cells that are active. With the intermittent administration of IL-2 with continuous HAART, the number of resting CD4+ cells that contain replication-competent HIV can be lowered. However, anti-HIV therapies, including HAART, have many negative effects. Problems include additional drug resistant viruses that will be more difficult to eradicate as well as multiple side effects in the individual. For these reasons, drugs with a novel mode of action such as IC5964 have the potential to add to the repertoire of anti-HIV therapy.

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VITA

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