Our results raise the possibility that the activity of mutant CFTRs in epithelial cells might, by appropriate pharmacological intervention, be increased sufficiently to ameliorate disease symptoms that appear to be largely related to insufficient $Cl^-$ secretion. It is estimated that 92% of CF patients have at least one ΔF508 allele (*10*); therefore pharmacologic activation of this mutant protein would be of benefit in the management of CF. The demonstration of $Cl^-$ conductance in association with the defective CFTR variants also sets the stage for detailed structure-function analysis of the mutant proteins.


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27. Mutation constructs were generated by oligonucleotide-mediated, site-specific mutagenesis of a 1.7-kb cDNA fragment containing the first third of the CFTR coding sequence. This fragment was cloned into the pSelect vector (Promega), and oligonucleotides corresponding to the desired mutation were synthesized and used for second strand priming as described by the supplier. After sequencing to verify the presence of the mutation, the fragment was transferred into a full-length cDNA construct in the vector pBluescript (Stratagene).


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# Inhibition of HIV Replication in Acute and Chronic Infections in Vitro by a Tat Antagonist


MING-CHU HSU,† ANDREW D. SCHUTT, MAUREEN HOLLY, LEE W. SLICE, MICHAEL I. SHERMAN,* DOUGLAS D. RICHMAN, MARY JANE POTASH, DAVID J. VOLSKY



**The human immunodeficiency virus-1 (HIV-1) trans-activator Tat is an attractive target for the development of antiviral drugs because inhibition of Tat would arrest the virus at an early stage. The drug Ro 5-3335 [7-chloro-5-(2-pyrryl)-3*H*-1,4-benzodiazepin-2(*H*)-one], inhibited gene expression by HIV-1 at the level of transcriptional trans-activation by Tat. The compound did not inhibit the basal activity of the promoter. Both Tat and its target sequence TAR were required for the observed inhibitory activity. Ro 5-3335 reduced the amount of cell-associated viral RNA and antigen in acutely, as well as in chronically infected cells in vitro (median inhibition concentration 0.1 to 1 micromolar). Effective inhibition of viral replication was also observed 24 hours after cells were transfected with infectious recombinant HIV-1 DNA. The compound was active against both HIV-1 and HIV-2 and against 3′-azido-3′-deoxythymidine (AZT)–resistant clinical isolates.**


AMONG THE REGULATORY GENE products encoded by HIV-1 is a strong positive regulator, Tat, that is required for HIV-1 replication (*1*) and has no apparent cellular homologs. Deleting the gene or disabling its initiation codon renders a provirus nonreplicative (*2*). Tat increases gene expression directed by the HIV-1 long terminal repeat (LTR) promoter. Stimulation of gene expression is due to an increase in steady-state mRNA and also possibly to an increase in the efficiency of mRNA use (*3*). Tat functions through the cis-acting Tat-responsive sequence (TAR) immediately downstream of the transcription start site (*4, 5*). The protein stabilizes elongation of transcription and may also increase transcription initiation (*6*).

To screen for Tat inhibitors, we put an indicator gene encoding secreted alkaline phosphatase (SeAP) under the control of the HIV-1 LTR promoter in the plasmid construct pBC12/HIV/SeAP (7). The *tat* gene was similarly controlled in a second plasmid pBC12/HIV/Tat. Cotransfection of COS cells with the two plasmids led to production of alkaline phosphatase, which was secreted into the culture medium. Alkaline phosphatase was quantitated by a colorimetric assay (7) that is amenable to automation and has a high throughput. Quantities of the two plasmids used in the transfection assay were adjusted so that SeAP production was linear in response to input pBC12/HIV/Tat (*8*). To minimize the inherent variability of transfecting cells in monolayers, we transfected COS cells in bulk suspension before plating them on culture dishes for drug testing.

One of the compounds in the repository of Hoffmann–La Roche, Ro 5-3335, reduced the alkaline phosphatase activity in the culture media of COS cells transfected with the plasmids pBC12/HIV/SeAP and pBC12/HIV/Tat (Fig. 1A). Reduction of enzyme activity was due to inhibition of SeAP expression from the HIV-1 LTR promoter because the compound did not inhibit the expression of SeAP driven by the Tat-insensitive LTR promoter of Rous sarcoma virus (RSV). Inhibition of SeAP expression was not 100% in these assays because the compound was added 24 hours after transfection; at this point, SeAP mRNA and enzyme activity could already be detected, and the enzyme was stable up to


M.-C. Hsu, A. D. Schutt, M. Holly, L. W. Slice, M. I. Sherman, Department of Virology, Hoffmann–La Roche, Nutley, NJ 07110.
D. D. Richman, Infectious Diseases Section, Veterans Administration Medical Center, University of California, San Diego, CA 92161.
M. J. Potash and D. J. Volsky, Molecular Virology Laboratory, St. Luke's–Roosevelt Hospital Center, Columbia University, New York, NY 10019.

*Present address: PharmaGenics, Allendale, NJ 07401.
†To whom correspondence should be addressed.

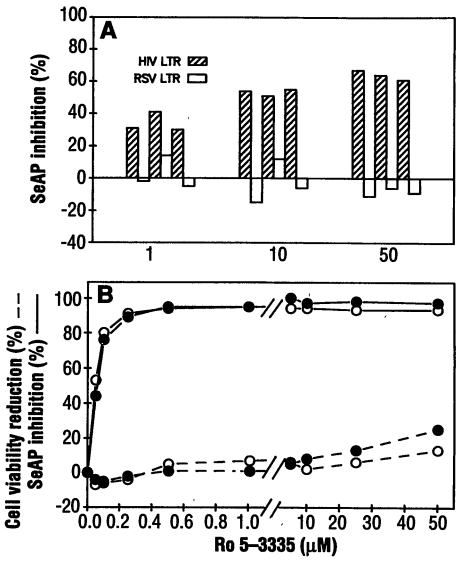


**Fig. 1.** Effect of Ro 5-3335 on the expression of SeAP. (**A**) SeAP expression from plasmid DNA. Plasmids pBC12/HIV/SeAP (16 μg) and pBC12/HIV/Tat (8 μg) were transfected into $2 \times 10^7$ COS cells in 20 ml of suspension by the DEAE-dextran procedure. Transfected cells were plated in 96-well microtiter plates at $1 \times 10^4$ cells per well. Ro 5-3335 was added to the culture media at a final DMSO concentration of 0.5% 24 hours after the cells were plated. Forty-eight hours after addition of the compound, 40 μl of medium was drawn from each well and assayed for alkaline phosphatase activity. Conversion of the substrate, *p*-nitrophenyl phosphate, to *p*-nitrophenol by SeAP was measured by absorbance at 405 nm ($A_{405}$) (7). Reduction in $A_{405}$ for drug-treated samples versus untreated samples ($A_{405} \simeq 1.5$) was expressed as percent inhibition after subtraction of background readings obtained from samples not expressing SeAP. Each bar represents an average of duplicate samples in one experiment; three separate experiments are shown. (**B**) SeAP expression by constitutively expressing cell lines CHO/19-3C (●) and 19-1F (○). Ro 5-3335 was added to culture media 24 hours after cells were plated (~30% confluency). Media and drug were removed and replenished 24 hours later. Alkaline phosphatase activity in the culture media was assayed 48 hours after the second addition of the drug as described in (A). Cytotoxicity was measured for the same cell cultures by a tetrazolium-based cell viability assay (*9*). Each data point is the average of triplicate culture wells. Two separate experiments for the Ro 5-3335 concentration ranges of 0.05 to 1 μM and 5 to 50 μM are shown.

several days at 37°C. To see the maximum amount of inhibition, we used the cloned cell lines CHO/19-3C and 19-1F, each of which contains integrated pBC12/HIV/SeAP and pBC12/HIV/Tat sequences and express SeAP constitutively. Ro 5-3335 inhibited SeAP production by 100% at concentrations of ~1 μM in these two cell lines (Fig. 1B). As we demonstrated with the tetrazolium-based assay for cell viability (*9*), the compound inhibited the expression of SeAP at noncytotoxic concentrations (median cytotoxic concentration is > 50 μM). Northern (RNA) blot analysis showed that Ro 5-3335 specifically reduced the amount of steady-state mRNA from the SeAP gene constitutively driven by the HIV-1 LTR and did not affect the accumulation of mRNA of the cellular gene actin (*10*). In the transient transfection assays, the background SeAP mRNA and protein at the time of drug addition account for the apparent lower activity.

The inhibition of gene expression by Ro 5-3335 was found to be at the level of Tat trans-activation, and the compound did not affect the basal activity (governed by cellular transcription factors) of the HIV-1 LTR promoter (Fig. 2). To measure the basal activity of the HIV-1 LTR promoter, we used the indicator gene chloramphenicol acetyltransferase (CAT) in COS cells, which support a measurable basal activity of the promoter. In a transient transfection assay, Ro 5-3335 inhibited CAT gene expression in the presence but not in the absence of Tat. The compound did not affect expression of the CAT gene under control of a mutant HIV-1 LTR promoter lacking the TAR sequence, either in the presence or absence of Tat (Fig. 2A) (*4*). In contrast, when expression of CAT was governed by an HIV-1 LTR that was mutated in the TATA sequence or NF-κB enhancer sequence or had the Sp-1 upstream activation sequences deleted (*11*), Ro 5-3335 inhibition of CAT gene expression was similar to that observed with the wild-type promoter (Fig. 2B). The action of Ro 5-3335 was thus not related to cellular transcription factors recognized by these regulatory sequences.

Consistent with its mode of action, Ro 5-3335 inhibited HIV-1 replication in chronically infected cells (Fig. 3). CR10 cells resistant to the cytopathic effect of HIV-1 (*12*) were infected with HIV-1 virus strain N1T (*13*) and maintained in the laboratory as virus producers. Parallel reductions in cell-associated viral RNA and p24 antigen were observed in these chronically infected cells after treatment with Ro 5-3335 for 3 days (the earliest time of assay) with a median inhibition concentration ($IC_{50}$) of 0.1 to 1 μM. As expected, zidovudine (AZT), a reverse transcriptase (RT) inhibitor, did not affect viral replication in these chronically infected cells.

To demonstrate further that inhibition of HIV replication by Ro 5-3335 is the consequence of inhibiting replication from proviral DNA, we transfected Jurkat cells with a recombinant HIV-1 DNA (*13*). Twenty-four hours after transfection, reduction of cell-associated p24 antigen was greater in cells treated with 2 μM Ro 5-3335 (4,398 pg of p24 per $10^6$ cells versus 59,000 pg for $10^6$ untreated cells) than in cells treated with either soluble, recombinant CD4 (sCD4; 10 μg/ml yielded 19,250 pg per $10^6$ cells) (*14*)

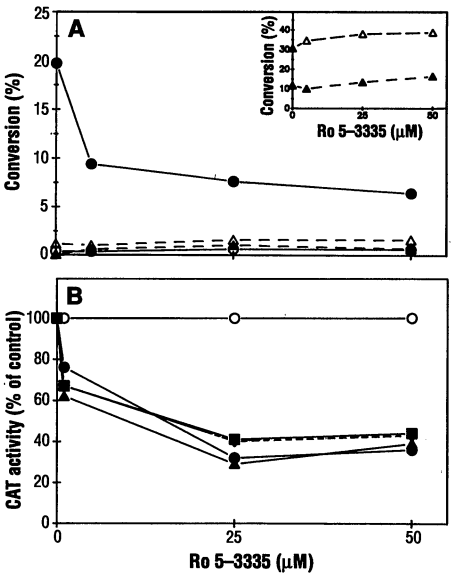


**Fig. 2.** Effect of Ro 5-3335 on mutant HIV-1 LTR promoters. (**A**) Dependence on Tat and TAR for the inhibitory activity of Ro 5-3335. COS cells ($4 \times 10^5$) were transfected with 0.6 μg of pHIV/CAT, in which the CAT gene is under the control of the wild-type HIV-1 LTR promoter (solid lines) (*11*) or a mutated HIV-1 LTR that lacks TAR (nucleotides +9 to +77) (dashed lines) (*5*), together with (●———●, ▲- - - ▲) or without (○———○, △- - -△) 0.12 μg of plasmid pBC12/HIV/Tat. Twenty-four hours after transfection, Ro 5-3335 was added to the culture media in a final DMSO concentration of 0.5%. Percent conversion was based on scintillation counts of [$^{14}$C]chloramphenicol and the acetylated derivative after resolution by thin-layer chromatography. (Inset) The effect of Ro 5-3335 on CAT gene expression from the mutant HIV-1 LTR after transfection that included a DMSO shock (10% DMSO for 2 min) to increase uptake of the plasmids (*26*). (**B**) Lack of dependence on regulatory sequences for cellular transcription factors. We used wild-type (●) and mutant HIV-1 LTR promoters with substitutions in the NF-κB (ΔNF-κB, ▲) or the TATA recognition sequences (ΔTATA, ◆ with dashed line) or with a deletion of the Sp-1 (ΔSp-1, ■) recognition sequences to drive expression of the CAT gene in plasmid constructs (*11, 27*). COS cells ($10^6$) were transfected with 0.5 μg of each plasmid together with 0.2 μg of pBC12/HIV/Tat. Ro 5-3335 was added to the culture media in a final DMSO concentration of 0.5% 24 hours after the cells were transfected, and CAT activity was assayed 48 hours after the addition of the drug. CAT gene under the control of the cytomegalovirus promoter (○) was a control for the assay. Each data point was obtained with pooled cell lysates of duplicate cell cultures. Results are representative of three independent experiments (*28*). Variabilities in percent conversions for CAT assays of duplicate samples were less than 10% of the averages.

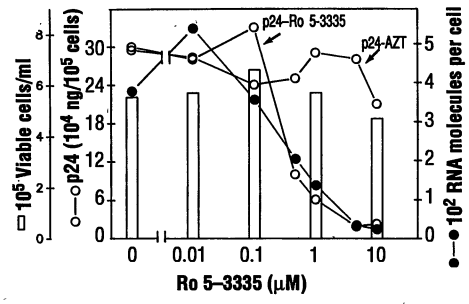


**Fig. 3.** Effects of Ro 5-3335 on HIV-1 replication in chronically infected cells. CR10 cells were infected with N1T (*13*) strain virus. Ro 5-3335 was added to the culture media of the chronically infected cells ($2 \times 10^5$ cells/ml) in 0.1% DMSO (final concentration). Three days after addition of the drug, the number of live cells were determined by Trypan blue exclusion. The viral RNA in $2 \times 10^5$ live cells was quantitated with an RNA-RNA hybridization–reversible target-capture procedure with a $^{32}$P-labeled RNA probe (*29*). Reagents were purchased from Gene-Trak Systems (Framingham, Massachusetts), and we carried out the assays according to the manufacturer's procedure. The radioactivity measured from the hybridizations was converted into the number of viral RNA molecules per cell on the basis of a standard curve constructed from a sense-strand RNA transcribed in vitro from a cloned HIV-1 *pol* gene that was used as a template for the hybridization procedure (*29*). The sensitivity of the assay was demonstrated with 0, 1, 10, 100, and 1000 pg of the in vitro–synthesized RNA mixed with $2 \times 10^5$ noninfected cell lysates that gave 0, 24, 71, 353, and 5244 cpm in the assay. Cell-associated p24 antigen was quantitated with antigen-capture ELISA plates (Coulter) with the manufacturer's procedures. For comparison, the effect of AZT on the replication of HIV-1 in these chronically infected cells (as measured by cell-associated p24) is shown.

or 2 μM AZT (39,000 pg per $10^6$ cells). Combination treatment with 2 μM Ro 5-3335 and sCD4 (10 μg/ml) had an inhibitory effect (3029 pg per $10^6$ cells) similar to treatment with Ro 5-3335 alone. These results eliminate the possibility that Ro 5-3335 inhibited HIV-1 replication before proviral DNA formation. The compound did not inhibit HIV-1 RT activity when tested with purified recombinant RT ($IC_{50}$ > 100 μM) (*15*). Ro 5-3335 therefore has a mode of action distinct from another class of benzodiazepines, TIBO, which was shown to inhibit HIV-1 RT (*16*) but did not show activity in the assay for Tat inhibitors described in Fig. 1.

The activity of Ro 5-3335 on different HIV-1 viral strains in various cell types was tested in acute infection (the drug was added at the time of infection). The compound was effective against a spectrum of HIV-1 strains [3B, Bru (now known to be Lai), BAL/85, N1T, and RF] in several cell lines (CEM, Jurkat, H9, MT2, JM, C8166, and HT4-6C) and in primary human peripheral blood lymphocytes and monocyte-derived macrophages with $IC_{50}$ of 0.1 to 1 μM and 90% inhibitory concentration ($IC_{90}$) of 1 to 3 μM (*17*). The activity of the compound was found to be consistent with different endpoint measurements [p24 enzyme-linked immunosorbent assay (ELISA), plaque reduction, syncytium inhibition, antibody staining of infected cells, and viral RNA quantitation]. The compound was also active against HIV-2 (strains ROD and UC-1, $IC_{50}$ = 0.1 to 1 μM). Ro 5-3335 was tested against two AZT-resistant viral isolates (*18*), a consideration necessary for testing potential drugs effective against acquired immunodeficiency syndrome (AIDS). Ro 5-3335 was equally effective against AZT-sensitive and AZT-resistant viral isolates ($IC_{50}$ = 0.1 to 0.5 μM) (*19*).

Ro 5-3335 is a benzodiazepine with minimal effect on the central nervous system. When compared with the therapeutic agent diazepam, the compound has <1% of the affinity for the central benzodiazepine receptors in rat cerebral cortex membranes (*20*). The compound has a high bioavailability (~85%) in dogs with a half-life of 2 hours; a peak plasma concentration of 0.8 μM was reached at an oral dose of 1 mg per kilogram of body mass (*21*). In general, the animals tolerated the drug well in a 2-week toxicity study with a dose of 125 mg per kilogram of body mass per day. However, the hydroxypyrrole metabolite gave rise to yellow discoloration in animal tissues, and nephrotoxicity was observed in rats (*22*).

Discovery of this class of compounds and continued search for an analog with a better toxicological profile raise the possibility of combination therapy for HIV infection. Distinct from currently available HIV RT or protease inhibitors that prevent new rounds of infection, a Tat antagonist inhibits HIV replication from integrated proviral DNA. In addition, in vitro studies have indicated the possibility that HIV-1 pathogenesis can be caused by viral proteins: gp120 mediates antibody-dependent cellular cytotoxicity and cytotoxic T cell killing (*23*); Tat mediates reduction of antibody-dependent T cell proliferation (*24*) and stimulates Kaposi's cell growth (*25*). A Tat antagonist that suppresses viral gene expression may eliminate these potential pathogenic effects.

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# Long-Term Improvement of Hypercholesterolemia After ex Vivo Gene Therapy in LDLR-Deficient Rabbits

J. ROY CHOWDHURY,* MARIANN GROSSMAN,* SANJEEV GUPTA, N. ROY CHOWDHURY, JAMES R. BAKER, JR., JAMES M. WILSON†

**Familial hypercholesterolemia (FH) is an inherited disorder in humans that is caused by a deficiency of low density lipoprotein receptors (LDLRs). An animal model for FH, the Watanabe Heritable Hyperlipidemic rabbit, was used to develop an approach for liver-directed gene therapy based on transplantation of autologous hepatocytes that were genetically corrected ex vivo with recombinant retroviruses. Animals transplanted with LDLR-transduced autologous hepatocytes demonstrated a 30 to 50 percent decrease in total serum cholesterol that persisted for the duration of the experiment (122 days). Recombinant-derived LDLR RNA was harvested from tissues with no diminution for up to 6.5 months after transplantation.**

MANY INBORN ERRORS OF METABOLISM are caused by inherited defects of liver function. One potential therapeutic approach for this group of diseases is the use of somatic gene transfer to permanently correct the genetic defect in hepatocytes. We used FH as a model for developing liver-directed gene therapies. FH is an autosomal dominant disorder caused by defects in the function or expression of LDLRs (*1*). Individuals with the disease have severe hypercholesterolemia and develop premature coronary heart disease. Homozygous deficient individuals are refractory to conventional therapies; however, orthotopic transplantation of a liver expressing normal amounts of LDLR has resulted in substantial metabolic improvements (*2*). The success of orthotopic liver transplantation suggests that hepatic reconstitution of LDLR expression by gene transfer would achieve therapeutic efficacy.

The existence of an animal model of FH, the Watanabe Heritable Hyperlipidemic (WHHL) rabbit, provides an experimental setting for the development of genetic therapies (*3*). One experimental approach, referred to as ex vivo gene therapy, involves the removal of a portion of the liver from an LDLR-deficient animal and the establishment of primary cultures of hepatocytes. Recombinant retroviruses are used to transduce a functional LDLR gene into the cultured hepatocytes that are then harvested and transplanted into the animal from which they were derived. The feasibility of a cell-based therapy for FH is supported by studies that demonstrated transient metabolic improvements after transplantation of allogeneic hepatocytes (for example, WHHL hepatocytes expressing human LDLR or hepatocytes derived from a wild-type rabbit) (*4, 5*).

A critical step in the development of an effective ex vivo gene therapy in the WHHL rabbit was the production of recombinant retroviruses capable of efficiently transducing a wild-type rabbit LDLR gene into primary cultures of WHHL hepatocytes. A full-length cDNA for LDLR was isolated from a λgt11 library derived from liver mRNA of a New Zealand White rabbit (*6*). The cDNA was cloned into a retroviral vector that expresses the recombinant gene from sequences of the chicken β-actin promoter, and amphotropic retroviruses were produced (*7*). Viral stocks derived from the *lacZ*-expressing vector BAG were used in control experiments (*8*).

The efficacy of ex vivo gene therapy in the WHHL rabbit was tested with the following experimental strategy (*9*). We subjected a group of 12 WHHL rabbits to 30% partial hepatectomy and perfused the excised liver lobes with collagenase to release hepatocytes. The recovered hepatocytes were placed in primary culture and exposed to either the LDLR virus or the *lacZ* virus. Retrovirally transduced hepatocytes were subsequently harvested ($2 \times 10^8$ cells or 2% of the total cells in a liver) and injected into the spleen of the animal from which the cells were originally derived. Hepatocytes were labeled with $^{111}In$ before we transferred them into a separate animal in order to assess their trafficking in vivo after intrasplenic injection; greater than 95% of the infused cells immediately entered the portal circulation and seeded into the liver. This analysis does not permit a precise determination of the cells remaining in the spleen; this number could easily vary fivefold, between 1 and 5%. The two experimental groups subjected to further analyses included WHHL rabbits transplanted with autologous hepatocytes transduced with either the LDLR virus ($n = 5$) or the *lacZ*-expressing virus ($n = 7$).

J. R. Chowdhury, S. Gupta, N. R. Chowdhury, Department of Internal Medicine and Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, NY 10461.
M. Grossman and J. M. Wilson, Howard Hughes Medical Institute, Departments of Internal Medicine and Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109.
J. R. Baker, Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109.

*Both authors made equal contributions to this work.
†To whom correspondence should be addressed.