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A Tat antagonist inhibits HIV-1 induction in naturally infected and experimentally infected T cells

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SUMMARY: Ro 5-3335, a novel antagonist of human immunodeficiency virus type 1 (HIV-1) Tat activity, inhibits acute and chronic HIV-1 infection in T lymphocytes. Here we describe the effects of Ro 5-3335 on the accumulation of viral DNA during primary infection, the induction of virus from a latently infected cell line, and the expression of virus upon activation of naturally infected T cells. Ro 5-3335 permitted initial DNA synthesis during primary infection, but inhibited the subsequent increase in viral DNA copy number. The induction of HIV-1, as determined by the synthesis of p24 core antigen, was inhibited by 99% by Ro 5-3335 in both the model cell line and naturally infected T cells.

A new category of antiviral agents, exemplified by Ro 5-3335 (1), antagonises the activity of the HIV-1 transactivator of transcription, Tat. HIV-1 expression, both in newly infected T cells and in T cells already infected and producing virus, is inhibited by Ro 5-3335 (1). The majority of HIV-1 infected peripheral blood lymphocytes (PBL) appear to carry viral DNA which is largely unexpressed *in vivo* (2,3). However upon stimulation in culture, such PBL produce virus, suggesting that their viral DNA is

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Abbreviations: HIV-1, human immunodeficiency virus type 1; IL-2, interleukin-2; PBL, peripheral blood lymphocytes; PHA, phytohemagglutinin; PMA, phorbol-12-myristate-13-acetate.

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expressed upon induction. Because Ro 5-3335 presumably acts at the level of the transcription of viral DNA to RNA (1), it may be able to block the induction of HIV-1 production from DNA. Here we report studies of the effects of Ro 5-3335 on the synthesis and induction of expression of HIV-1 DNA. The accumulation HIV-1 DNA was evaluated during primary infection of CEM cells in the presence of 5-3335. To investigate the induction of HIV-1 production and the effects of Ro 5-3335, we have used the ACH-2 cell line which expresses its integrated provirus upon induction with phorbol 12-myristate 13-acetate (PMA) (4). Finally, to determine the action of Ro 5-3335 on the virus culture system closest to natural infection, we have employed mitogenic stimulation and evaluation of HIV-1 induction from PBL taken from AIDS patients. We show that Ro 5-3335 acts after the synthesis of HIV-1 DNA to block the induction of virus from the proviral form.

MATERIALS AND METHODS

Cells and viruses. The human T cell line CEM, was obtained from the CDC (Atlanta, GA), the chronically/latently HIV-1 infected derivative of CEM, ACH-2 (4) was obtained from T. Folks (CDC). CEM and ACH-2 were grown as stationary cultures in RPMI 1640 supplemented with 5% fetal bovine serum and antibiotics. Blood samples were obtained from AIDS patients enrolled in the St. Lukes/Roosevelt AIDS Treatment and Evaluation Unit, and PBL were isolated from blood using Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient centrifugation. HIV-1/N1T was isolated (5), maintained (6), and prepared as described (7).

HIV-1 infection and treatment with antiviral agents. In the studies reported here we have employed concentrations of 2-5μM Ro 5-3335 (obtained from M.-C. Hsu, Hoffmann-La Roche, Nutley, NJ), based on our previous studies of drug efficacy and toxicity. CEM cells were infected with HIV-1/N1T at a multiplicity of infection of one and cultured in the presence or absence of 2μM Ro 5-3335. ACH-2 cells were cultured in the presence or absence of 5μM Ro 5-3335 for two days, washed, and cultured with 10 ng/ml PMA (Sigma, St. Louis, MO) and in the continuing presence or absence of Ro 5-3335. PBL were cultured overnight in the presence or absence of 2 μM Ro 5-3335 and with 1-5μg/ml phytohemagglutinin (PHA) (Sigma, St. Louis, MO) and 10u/ml interleukin-2 (IL-2) (Biotest, Denville, NJ). They were then washed and cultured with PHA-IL-2 and no drug.

Hirt extraction and analysis of extrachromosomal HIV-1 DNA. CEM cells were harvested at the indicated time points and extrachromosomal DNA was extracted by the Hirt procedure (8) as modified by Chinsky and Soeiro (9). Total DNA recovered from 4-5x

10^6 cells was loaded onto a 0.9% agarose gel for electrophoresis (the same cell number was assayed in all samples at a particular time point). Following electrophoresis, the DNA was transferred to a Nytran nylon membrane (Schleicher & Schuell, Keene, NH), and analyzed for HIV-1 specific sequences by hybridization using the [^{32}P]-dCTP-labeled 8.9kb *Sac*I-*Sac*I fragment derived from the HIV-1/N1G-G clone (10) as probe. Autoradiography was performed over 1-5 days using Kodak Blue-brand film and intensifying screens.

Assays of HIV-1 expression. HIV-1 p24 capsid protein in cell supernatants or cell lysates was measured by an enzyme linked immunosorbent assay using the Coulter HIV Ag Assay (Coulter Immunology, Hialeah, FL) following the manufacturer's instructions. Lysates of 10^7 cells per ml were prepared using the lysis buffer supplied by the manufacturer.

RESULTS AND DISCUSSION

These experiments were designed to evaluate the effects of Ro 5-3335 on the induction of HIV-1 proviral DNA. It was first necessary to exclude that Ro 5-3335 acts on stages of replication prior to or including the synthesis of viral DNA. We have shown previously that Ro 5-3335 blocks transient HIV-1 replication following transfection of viral DNA, suggesting that the initial synthesis of viral DNA from input RNA is not inhibited by Ro 5-3335 (1). However direct proof is lacking. To obtain information on the effects of Ro 5-3335 on HIV-1 DNA production, we infected and cultured CEM cells in the presence of Ro 5-3335 and monitored the kinetics of accumulation of extrachromosomal viral DNA in infected cells (Figure 1). Viral DNA was present at 6h, regardless of treatment with Ro 5-3335, however further DNA accumulation was blocked by exposure to the drug, as shown in viral DNA 48 hours after infection. This finding indicates that Ro 5-3335 interrupts the HIV-1 life cycle after viral DNA synthesis and raises the possibility that the compound blocks any HIV-1 production from provirus.

To test whether Ro 5-3335 can inhibit HIV-1 expression from provirus, we employed the chronically infected cell line ACH-2, which expresses its provirus upon induction with PMA (4). We incubated ACH-2 cells with 5μM Ro 5-3335 for two days, and then

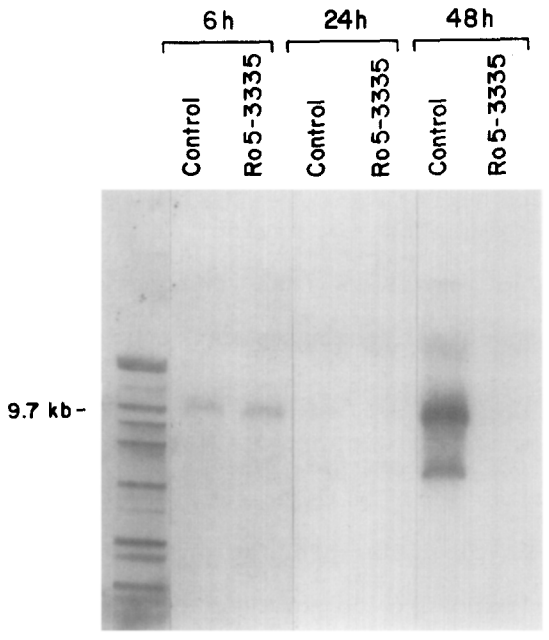

Figure 1. Kinetics of HIV-1 DNA accumulation during infection in the presence of Ro 5-3335. CEM cells were infected and cultured as described in Materials and Methods. At the indicated time points, cells were harvested for extraction of extrachromosomal DNA by the Hirt method and analysed by Southern blotting and hybridization with an HIV-1 DNA specific probe. The migration of a 9.7kb marker is indicated.

cultured the cells with the inducer PMA and Ro 5-3335 for an additional two days (Table 1). 99% of p24 production induced by exposure of ACH-2 to PMA was sensitive to the effects of Ro 5-3335.

Table 1. Inhibition of HIV-1 induction in chronically infected ACH-2 cells by Ro 5-3335

Ro 5-3335 (μM)	HIV-1 expression (pg p24/ml supernatant)
0	170,000
5	1,400

ACH-2 cells were cultured in the presence or absence of Ro 5-3335 and PMA and p24 was assayed as described in Materials and Methods.

Thus the expression of integrated provirus is inhibited by Ro 5-3335. This stands in contrast to the resistance of this induction to 3'-azido, 3'-deoxythymidine (11).

PBL infected by HIV-1 *in vivo* do not generally express their provirus until stimulated to divide (2,12). To test directly whether Ro 5-3335 inhibits the mitogen induction of HIV-1 expression and consequently its spread in naturally infected PBL, we isolated PBL from three AIDS patients, cultured them with mitogens either with or without 5-3335 overnight, prior to two weeks cocultivation with mitogen activated HIV-1 negative PBL (Table 2). 98-99% of virus production from each cell population was inhibited by exposure to Ro 5-3335. This finding indicates that the activation route in mitogenesis in T cells includes elements targeted by Ro 5-3335 and that primary isolates of HIV-1 are sensitive to the drug.

The studies presented here extend our earlier work on the mode of action of Ro 5-3335 (1). The prediction that Ro 5-3335 acts

Table 2. Inhibition of the spread of primary HIV-1 isolates by Ro 5-3335

Donor cells[a]	Expression of HIV-1 p24 core antigen (pg /10^6 cells)[b]		% Inhibition[c]
	No drug	2μM Ro 5-3335	
AIDS patient 1	209,000	1900	>99
AIDS patient 2	12,700	240	96
AIDS patient 3	320,000	1160	>99

[a] PBL from AIDS patients were cultured with PHA-IL2 with or without Ro 5-3335 for 24h prior to washing and addition of a tenfold excess of HIV-1 negative PBL.

[b] Intracellular p24 was determined after 14 days of culture as described in Materials and Methods.

[c] Calculated as the p24 in cells treated with Ro 5-3335 divided by the p24 in cells not treated with the drug.

after reverse transcription of incoming virion RNA was directly verified by showing both that viral DNA is synthesized in the presence of Ro 5-3335 and that the later expansion of infection and increase in DNA copy number (13) is inhibited by the drug (Figure 1). The induction of expression of such viral DNA arising from primary infection is sensitive to Ro 5-3335, as shown in the inhibition of HIV-1 production from ACH-2 cells (Table 1). This suggests that the complex of viral products and cellular factors induced by PMA contains targets for Ro 5-3335 which are required to increase viral transcription. Transient exposure to Ro 5-3335 blocked the HIV-1 expression from naturally infected PBL which accompanied cellular stimulation (Table 2). This suggests that the factors arising in non-transformed cells upon mitosis, as may be expected during antigenic stimulation of T cells *in vivo*, cannot overide the block imposed by Ro 5-3335 on viral transcription.

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