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Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication

CHIANG J. LI*†, LIN J. ZHANG‡, BRUCE J. DEZUBE*§, CLYDE S. CRUMPACKER‡, AND ARTHUR B. PARDEE*

*Division of Cell Growth and Regulation, Dana–Farber Cancer Institute, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 44 Binney Street, Boston, MA 02115; Divisions of ‡Infectious Diseases and §Hematology/Oncology, Beth Israel Hospital, Harvard Medical School, Boston, MA 02215

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ABSTRACT **Transcription of type 1 human immunodeficiency virus (HIV-1) provirus is governed by the viral long terminal repeat (LTR). Drugs can block HIV-1 replication by inhibiting activity of its LTR. We report that topotecan, β-lapachone, and curcumin are potent and selective inhibitors of HIV-1 LTR-directed gene expression, at concentrations that have minor effects on cells. At these concentrations, each drug inhibited p24 antigen production in cells either acutely or chronically infected with HIV-1. Their target is transcriptional function of the LTR.**

The evolution of type 1 human immunodeficiency virus (HIV-1) infection and the progression of immunosuppression are associated with activation of latent provirus, which is governed by long terminal repeat (LTR) in the viral DNA (1–4). The activity of the HIV-1 LTR is determined by the complex interaction of positive and negative transcriptional regulators that bind to specific sequences within the LTR (1, 2). Changes in the quantity or quality of these factors may underlie the activation of transcription of HIV-1 latent provirus by a myriad of stimuli (3, 4). In particular, phorbol 12-myristate 13-acetate (PMA) and tumor necrosis factor α (TNF) are potent activators (1, 3, 5). TNF may play a critical role in the pathogenesis of AIDS, consistent with markedly increased levels of this cytokine in HIV-1-infected individuals (6). Compounds that block activation or suppress the activity of HIV-1 LTR are of therapeutic potential for extension of latency or inhibition of persistent progressive infection. We describe here three such compounds.

Topotecan is a semisynthetic inhibitor of DNA topoisomerase I; it has antitumor activity and is well tolerated in cancer patients (7, 8). β-Lapachone, which is isolated from plant extracts (9), has a variety of pharmacological effects (10, 11). Toxicity data in humans are not available for β-lapachone; it is well tolerated in mice at <40 mg/kg given i.v. (unpublished data). The maximum tolerated dose is about 1250 mg/kg given orally in mouse and chicken (10). Curcumin, the major active component of the food flavor tumeric, has been eaten, up to 100 mg/day, for a thousand years, and pharmacological studies demonstrate its very low toxicity. It has been used as an antiinflammatory drug (12–14). Thus, the available data suggest that these three compounds may be well tolerated *in vivo*.

MATERIALS AND METHODS

Virus. HIV-1 was obtained from the culture supernatant of HTLV-III$_B$-producing H9 cells. During the exponential phase of growth, cell-free supernatant was harvested, standardized for reverse transcriptase activity, and frozen in aliquots at −70°C.

Cells. 293.27.2 cells (15), a generous gift from L. A. Herzenberg (Stanford University), were derived from human embryonic kidney epithelial cells; they were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% (vol/vol) fetal calf serum (Sigma) plus L-glutamine. This cell clone has been stably transfected with pNAZ, which is an expression construct of the *lacZ* gene driven by the HIV-1 LTR. Expression of β-galactosidase can be greatly induced by PMA or TNF. Human peripheral blood mononuclear cells (PBMC) were prepared by Ficoll/Hypaque gradient centrifugation of blood from HIV-seronegative individuals and cultured in RPMI 1640 supplemented with 20% fetal calf serum, penicillin, streptomycin, and L-glutamine in the presence of phytohemagglutinin (3 μg/ml). The RPMI 8402 cell line, a present from Toshiwo Ando (Aichi Cancer Research Institute, Nagoya, Japan), is a human T-lymphatic leukemia cell line. It was grown in RPMI 1640 supplemented with 15% fetal calf serum and L-glutamine.

Drugs. Topotecan was a gift from M. Mattern (SmithKline Beecham). β-Lapachone was kindly provided by A. Matter (CIBA–Geigy). Curcumin was purchased from Sigma. Stock solutions (20 mM) were prepared in water (topotecan), dimethyl sulfoxide (β-lapachone), or ethanol (curcumin). Aliquots of the stock solutions were stored frozen at −20°C.

Quantitation of HIV-1 LTR-Directed Gene Expression. Exponentially growing 293.27.2 cells were plated in six-well plates at 2×10^5 cells per well in 2 ml of growth medium. After 48 h, cells were stimulated with TNF (Genzyme) at 40 units/ml or PMA (Sigma) at 2 ng/ml. Various concentrations of drugs were added to the medium at designated times after stimulation. Final concentrations of dimethyl sulfoxide or ethanol were kept to <0.1% (vol/vol). After a 6-h incubation at 37°C, cells were harvested, washed four times with phosphate-buffered saline, and lysed in *lacZ* buffer (60 mM Na_2HPO_4/40 mM NaH_2PO_4/10 mM KCl/1 mM $MgSO_4$). β-Galactosidase activities of cell lysates were quantitated (16) and standardized by protein concentration. Cell survival was determined by the colony-formation assay after cells were treated as above.

Total Cellular RNA Synthesis. 293.27.2 cells were plated at 1×10^6 cells per 150-mm plate in 20 ml of growth medium. After a 48-h incubation, cells were treated with different concentrations of drugs for 2 h. Cells were then incubated for an additional hour in the presence of [^{3}H]uridine at 1 μCi/ml (1 Ci = 37 GBq). Total cellular RNA was prepared by the guanidium/CsCl step gradient method (17). Newly synthesized RNA was quantitated by scintillation counting, which was adjusted for RNA concentration.

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Abbreviations: HIV, human immunodeficiency virus; LTR, long terminal repeat; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor α; PBMC, peripheral blood mononuclear cells.

†To whom reprint requests should be addressed.

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Northern Blot Analysis. 293.27.2 cells in logarithmic-phase growth were stimulated with TNF at 40 units/ml in the presence or absence of drugs. After 2–6 h of incubation, cells were harvested. Total cellular RNA was prepared by the guanidium/CsCl step gradient method (17). RNA was equally loaded, fractionated, transferred to membranes, and then hybridized with *lacZ*, β-actin, or c-*jun* probes.

Drug Treatment in Acute HIV-1 Infection. PBMC, after a 72-h stimulation with phytohemagglutinin at 3 μg/ml, were infected with HTLV-III$_B$ at 1 reverse transcriptase unit per 10 cells at 37°C for 2 h. Nonadsorbed virus was then removed by washing. Infected PBMC were then aliquoted at 4.5×10^6 cells per well. Various concentrations of drugs were added to cultures. After 6 days, cell viability was determined by the trypan blue exclusion method and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide metabolic assays (18). p24 core antigen in the culture supernatant was analyzed by ELISA with an HIV-1 p24 antigen kinetics assay kit (Coulter) and expressed as a percentage of the p24 concentrations of the drug-treated sample versus the untreated sample (taken as 100).

Drug Treatment in Chronic HIV-1 Infection. RPMI 8402 cells, which are resistant to the cytopathic effect of HIV-1, were chronically infected with HTLV-III$_B$. Expression of p24 antigen, which is 1000–1500 ng/ml, became constitutive and stable after five passages of the infected cell line. TNF did not further augment p24 expression. This cell line has been maintained in the laboratory, and the experiment was done after 10 passages. Different concentrations of drugs were added to these chronically infected cells. Six days after addition of drug, p24 antigen levels were analyzed as above. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide metabolic assay (18).

RESULTS

Selective Inhibition of HIV-1 LTR-Directed Gene Expression by Topotecan. To identify inhibitors of HIV-1 LTR activity, the human epithelial cell line 293.27.2, which contains an integrated HIV-1 LTR-directed *lacZ* gene (pNAZ), was used (15). Potent activators of latent HIV are PMA and TNF (1, 3, 5). Topotecan inhibited the stimulation of these cells by both TNF and PMA (IC_{50} = 0.05 μM) and did not significantly decrease survival (Fig. 1*A*). Camptothecin, the parent compound, was less inhibitory (IC_{50} for TNF stimulation was 0.125–0.5 μM). In the CD4$^+$ T-cell line RPMI 8402, which was transiently transfected with pNAZ, 0.125 μM topotecan inhibited *lacZ* expression by 62%. As a control for the effects

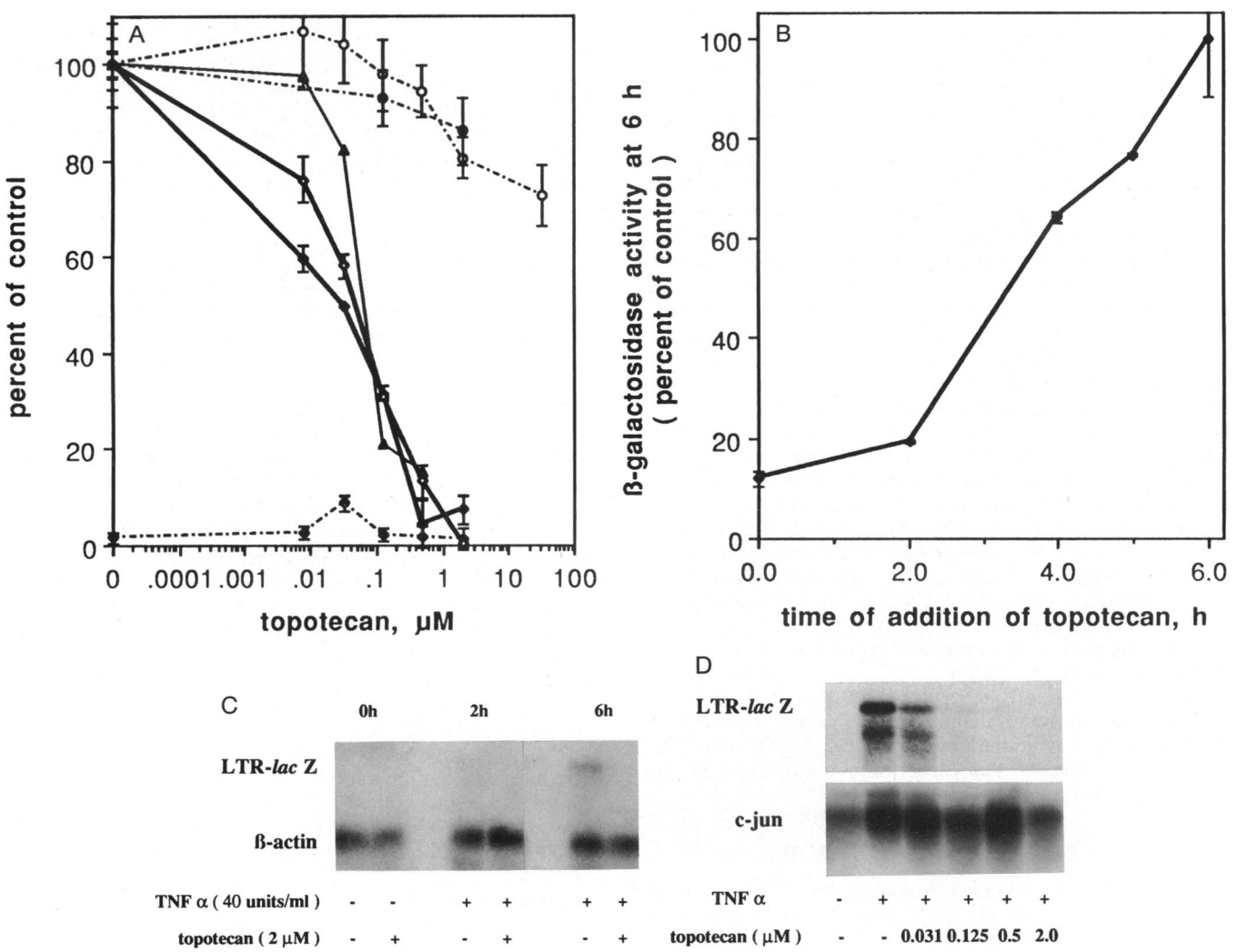

FIG. 1. Inhibition of HIV LTR activity by topotecan in 293.27.2 cells. (*A*) Dose-dependent and selective inhibition of HIV LTR-directed cytokine-enhanced *lacZ* gene expression by topotecan. ··○··, cell survival; ··●··, cellular RNA synthesis; —▲—, mRNA of β-galactosidase stimulated by TNF; —◇—, β-galactosidase activity stimulated by TNF; —◆—, β-galactosidase activity stimulated by PMA; ··◆··, basal activity of β-galactosidase. (*B*) Cells were treated with TNF to activate the HIV-1 LTR. Topotecan (0.5 μM) was added at the times indicated, and the β-galactosidase activity was determined after a total incubation period of 6 h. (*C*) Northern assays for effects of topotecan. (*D*) Differential effect of topotecan on TNF-stimulated accumulation of c-*jun* and HIV-1 LTR-*lacZ* mRNA.

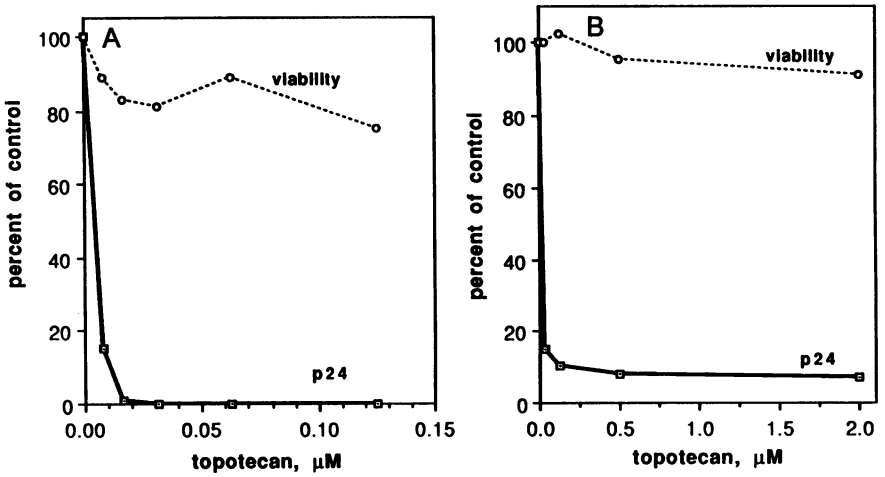

FIG. 2. Inhibition by topotecan of HIV-1 (HTLV-III$_B$) replication in acutely infected human PBMC (*A*) and the chronically infected human T-cell line RPMI 8402 (*B*).

of nonspecific DNA damage, neocarzinostatin (0.08 μM), an antineoplastic compound that breaks one DNA strand (19), was used; it did not inhibit *lacZ* expression but was lethal to 10% of the cells.

Cells stimulated with TNF showed time-dependent *lacZ* expression directed by the LTR. When topotecan was added several hours after TNF, it still inhibited the expression of β-galactosidase directed by the activated LTR (Fig. 1*B*).

Northern analysis of β-galactosidase mRNA accumulation directed by the HIV-1 LTR in cells induced by TNF showed that the accumulation of this mRNA was decreased in the presence of topotecan (IC_{50} = 0.03–0.1 μM) (Fig. 1*A*). β-Actin mRNA and total RNA synthesis (controls) were not significantly decreased by 2 μM topotecan (Fig. 1 *A* and *C*). Also, c-*jun*, a gene that is stimulated by TNF (20) but whose role in HIV replication is not clear, was much less affected by topotecan after TNF stimulation (Fig. 1*D*).

Inhibition of HIV-1 Replication by Topotecan in Acutely and Chronically Infected Cells. Virus production by acutely HIV-1-infected human PBMC was examined for effects of topotecan. Topotecan added after PBMC were infected with HIV-1 and present for the remainder of the experiment dramatically decreased HIV-1 replication as indicated by p24 antigen production by HTLV-III$_B$ (Fig. 2*A*).

In chronically infected RPMI 8402 cells, as with acutely infected cells, topotecan at 0.03 μM (IC_{50} = 0.008 μM) markedly reduced HIV-1 replication with little effect on cell viability (Fig. 2*B*). For comparison, topotecan can be tolerated in cancer patients at a plasma concentration (7) that is 200 times the concentration that inhibits 80% of p24 antigen production in chronically infected cells.

Inhibition of HIV-1 LTR-Directed Gene Expression and HIV-1 Replication by β-Lapachone and Curcumin. β-Lapachone also inhibited activity of HIV-1 LTR in cells stimulated by TNF (IC_{50} = 0.3 μM) (Fig. 3*A*) and did not decrease cell survival. 3-Allyl-β-lapachone, which is of pharmacological interest (21), exhibited similar activity (data not shown). In a dose-dependent manner, β-lapachone decreased HIV-1 replication in human PBMC acutely infected with HTLV-III$_B$ (Fig. 3*A*). In chronically infected RPMI 8402 cells, 2.5 μM β-lapachone reduced p24 antigen production by 60% on day 6, and the cell viability was >90% (data not shown).

Curcumin was inhibitory to HIV-1 LTR-directed gene expression stimulated by TNF (or PMA; data not shown) but was not as potent as the other agents. In acutely infected PBMC, curcumin decreased p24 antigen production by HIV-1 in a dose-dependent manner (Fig. 3*B*).

DISCUSSION

The molecular mechanisms of actions of these drugs remain to be elucidated. The selectivity of these compounds suggests that their major target is more critical to the virus than to the cell. That these three drugs similarly inhibit both established HIV-1 LTR activity and viral production after chronic infection with HIV-1 suggests that they affect the virus in its

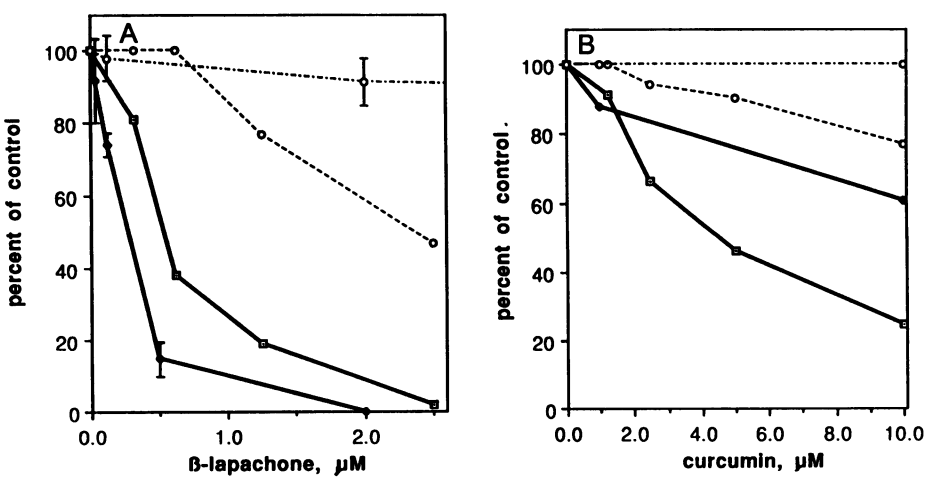

FIG. 3. Inhibition of HIV-1 LTR-directed gene expression and HIV-1 replication by β-lapachone (*A*) and curcumin (B). ··○··, survival of 293.27.2 cells; ---○---, viability of PBMC cells; —⊡—, p24 antigen in PBMC; —◆—, β-galactosidase activity stimulated by TNF in 293.27.2.

proviral stage; these findings do not support viral integration as the target. Most likely, they act directly or indirectly upon regulators of the HIV-1 LTR, since the similar results with p24 production and β-galactosidase are consistent with effects of the drugs on the viral LTR. The similar inhibitions by topotecan as measured by Northern blot and enzyme assays suggest that LTR-driven transcription or possibly posttranscription is inhibited. Our observation that topotecan did not affect stimulated c-*jun* mRNA production suggests that LTR-directed transcription is selectively inhibited rather than the signal transducers generated by PMA or TNF. This inference is supported by inhibition of chronically infected cells. Possibly, these drugs interact with a protein that binds to the LTR or one that is involved in posttranscriptional processes. It is of interest that these drugs actively block HIV-1 replication in acutely infected cells, because this suggests that effective activity of the LTR is also important in acute infection.

Priel *et al.* (22, 23) reported that camptothecin inhibits acute infection of the H9 cell line with HTLV-III_B by inhibiting HIV-associated topoisomerase I, which was assumed to be important in retroviral integration. However, a 40,000-fold higher concentration of camptothecin was required to inhibit viral topoisomerase than to decrease p24 antigen production after acute HTLV-III_B infection (22, 23). Also, our results suggest a target for β-lapachone other than oncovirus reverse transcriptase (24).

Drugs that selectively inhibit LTR activity could be effective against both acute and chronic infection and thus may provide effective therapy for patients infected with HIV-1 or other retroviruses. Screening of drugs for activity against LTR represents a useful approach for finding additional anti-HIV-1 drugs (15, 25, 26). RO 5-3335, a tat inhibitor, blocks expression from proviral DNA but not activation of HIV-LTR by TNF or PMA (25). Pentoxifylline inhibits both TNF production and activity of the HIV-1 LTR (26). A combination of LTR-directed drugs such as topotecan, β-lapachone, and curcumin with reverse transcriptase inhibitors [e.g., 3′-azido-3′-deoxythymidine or dideoxyinosine (27)] may provide synergistic inhibition of HIV.

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