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Topotecan Inhibits Human Immunodeficiency Virus Type 1 Infection through a Topoisomerase-Independent Mechanism in a Cell Line with Altered Topoisomerase I

JIE LIN ZHANG,[1]* PREM L. SHARMA,[1] CHIANG J. LI,[2] BRUCE J. DEZUBE,[2,3] ARTHUR B. PARDEE,[2] AND CLYDE S. CRUMPACKER[1]

Division of Infectious Diseases[1] and Division of Hematology/Oncology,[3] Charles A. Dana Research Institute, Beth Israel Deaconess Medical Center, and Division of Cell Growth & Regulation, Dana-Farber Cancer Institute,[2] Boston, Massachusetts

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Topotecan (TPT), a known inhibitor of topoisomerase I, has previously been shown to inhibit the replication of several viruses. The mechanism of inhibition was proposed to be the inhibition of topoisomerase I. We report that TPT decreased replication of human immunodeficiency virus type 1 (HIV-1) in CPT-K5, a cell line with a topoisomerase I mutation. TPT inhibited production of HIV-1 RNA and p24 in CPT-K5 and wild-type cells equally effectively. The antiviral effects of TPT were observed not only in the topoisomerase-mutated CPT-K5 line but also in peripheral blood mononuclear cells (PBMC) acutely infected with clinical isolates and in OM10.1 cells latently infected with HIV and activated by tumor necrosis factor alpha. Little toxicity from TPT was noted in HIV-1-infected PBMC and in CPT-K5 and OM10.1 cells as measured by cell growth and proliferation assays. These observations suggest that TPT targets factors in virus replication other than cellular topoisomerase I and inhibits cytokine-mediated activation in latently infected cells by means other than cytotoxicity. These results suggest a potential for TPT and for other camptothecins in anti-HIV therapy alone and in combination with other antiretroviral drugs.

Topotecan (TPT), a semisynthetic analog of camptothecin (CPT), is a potent topoisomerase I (topo I) inhibitor. As an antineoplastic agent, TPT has a more favorable side-effect profile than CPT and has been approved for the treatment of ovarian cancer (8, 18, 22, 24). CPT and its analogs produce drug-induced accumulation of topo I-DNA complexes in vitro and in vivo (5, 7, 9–12). These complexes inhibit topo I activity and lead to single-strand breaks in DNA. As a result, these drugs inhibit DNA replication and terminate RNA transcription at sites of complex formation (3, 13, 14).

The antiviral activity of CPT and its analogs has been reported in several studies. These antiviral activities have been related to cellular topo I, virus-associated topo I, human immunodeficiency virus (HIV) reverse transcriptase (RT), or the HIV long terminal repeat (LTR) (17, 20, 21, 23, 25, 27). We have shown that TPT selectively inhibits HIV type 1 (HIV-1) LTR-directed *lacZ* gene expression in a human epithelial cell line (17) and also found that TPT inhibits both acute and chronic HIV-1 infections. The question of whether TPT's anti-HIV activity is related to inhibition of topo I remained. In this report we show that the anti-HIV effect of TPT may be independent of its anti-topo I activity. We examined the anti-HIV activity of TPT in CPT-K5, a chronically infected cell line carrying a topo I mutation (2, 26). The anti-HIV activity of TPT was also examined in a latently infected cell line activated by tumor necrosis factor alpha (TNF-α) and in peripheral blood mononuclear cells (PBMC) acutely infected with clinical isolates.

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* Corresponding author. Mailing address: Beth Israel Deaconess Medical Center, Division of Infectious Diseases, 330 Brookline Ave., Boston, MA 02215. Fax: 617-667-5541.

MATERIALS AND METHODS

Cells. Human T-lymphatic leukemia cell lines RPMI 8402 and CPT-K5 were gifts from T. Andoh (Aichi Cancer Research Institute, Nagaya, Japan). These cells were chronically infected with HIV-1_{IIIB} in the laboratory. Both cell lines constitutively produce the virus in culture media; p24 antigen levels were 1,000 to 1,500 ng/ml as measured by a viral antigen enzyme-linked immunosorbent assay (Coulter Corp.). The OM10.1 cell line was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. Human PBMC were processed according to the protocol of the AIDS Clinical Trial Group virology manual (1).

Viruses. HIV-1_{IIIB}/H9 was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program and processed as previously described (28). HIV-1 clinical isolates were isolated from patients with AIDS as defined by the Center for Disease Control and Prevention. The viruses were obtained from the first passage of the coculture of patients' PBMC with HIV-1 antibody-negative donor PBMC. A012D, a zidovudine (AZT)-resistant virus (15), was from the virology quality assurance program of the AIDS Clinical Trial Group. HIV-1 LTR mutants pNFA and pSPC and their wild type, pILIC, were gifts from A. Rabson. These LTR mutants and their wild type have been shown to replicate in PBMC (16).

HIV-1 acute infection. A total of 10^6 RT units of HIV-1_{IIIB} or clinical isolates per ml were used to infect 10^7 PBMC. Cells were incubated with virus for 2 h at 37°C. The infected cells were washed and resuspended in fresh medium for TPT treatment.

Compound. TPT was a gift from M. Mattern (Smith Kline Beecham) to C. Crumpacker and A. Pardee. The stock solutions of TPT were made in dimethyl sulfoxide and stored in aliquots at −20°C.

TPT treatment of HIV-1-infected PBMC and cell lines. Chronically infected RPMI 8402 and CPT-K5 cells and acutely infected PBMC were treated for 6 days with TPT; TPT was prepared as two- or fourfold dilutions ranging from 0.002 to 2 μM. All samples were tested in duplicate. The spent medium was replaced on day 3 with fresh medium containing TPT. The supernatants were collected on day 6 for a p24 enzyme-linked immunosorbent assay (Coulter Corp.). Cells were assayed for cytotoxicity, cell proliferation, and HIV-1 RNA. OM10.1 cells latently infected with HIV-1 were cultured to log growth phase, the cells were transferred to a 24-well culture plate, and 20 units of TNF-α (Endogen Inc.) per ml was added to the cells. TPT (0.004 to 0.031 μM) and pentoxifylline (PTX; 50 to 500 μM) were added to the cell suspensions. Levels of p24 antigen and cell viability were analyzed at 36 h poststimulation. The 50% inhibition dose (ID_{50}) was measured by p24 titration and calculated by the median-effect equation (6) with Systat version 5.1 software.

RNA hybridization assay. HIV-1 RNAs from 2×10^4 live cells under each experimental condition were tested in duplicate by an RNA-RNA hybridization assay (Gene-Trak System) (28) with a pGAP probe specific for HIV-1 RT.

Cell viability assay. Three different assays were carried out to examine cell viability and cell proliferation after TPT treatment. In the trypan blue exclusion test, the number of viable cells for each TPT treatment condition was determined after 120 cells were counted in duplicate. Cell viability was calculated as the ratio of the number of viable cells after each TPT treatment to the number of viable cells without TPT treatment. In the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, 10^4 cells from each experimental condition were transferred to a 200-μl-well microculture plate in duplicate. The assay was performed according to the procedure of Mosmann (19). Median toxic dose (TD_{50}) was calculated by the median-effect equation (6) with Systat version 5.1 software. For the cell proliferation analysis, the cell samples were collected in duplicate before and after TPT treatment and counted with a Coulter counter (ZM).

RESULTS

TPT decreased production of HIV-1 p24 and RNA in CPT-K5 cells. CPT-K5 is a CPT-resistant human leukemia cell line. The purified topo I from these cells exhibits greater than 125-fold resistance to CPT compared with wild-type topo I from RPMI 8402 cells (2, 26). Single amino acid changes from aspartic acid to glycine at residues 533 and 583 are found in the CPT-resistant topo I gene (26). The cytotoxicity measurements in our study showed that the TD_{50} of TPT in CPT-K5 cells was 13.7 μM, whereas it was 0.027 μM in the RPMI 8402 cells. TPT decreased expression of p24 antigen in both CPT-K5 and RPMI 8402 cells chronically infected with HIV-1 in a dose-dependent manner, with an ID_{50} of 0.009 μM (Fig. 1a). RNA hybridization studies showed that TPT treatment of CPT-K5 cells decreased the production of HIV-1 RNA and inhibited expression of HIV-1 p24 to similar extents (Fig. 1b). The ID_{50} of TPT for HIV-1 RNA in these cells was 0.007 μM. These results indicate that the anti-HIV activity of TPT is not a direct result of inhibiting topo I activity, the usual target of TPT.

TPT inhibited production of P24 by HIV-1 clinical isolates. Two HIV-1 clinical isolates were assayed with PBMC for the anti-HIV effect of TPT. p24 levels were decreased on day 3 and not detected by day 6 when 0.125 μM TPT was present in the culture (Fig. 2a). The ID_{50}s of TPT for clinical isolates 010792 and 010893 were 0.006 μM ($r^2 = 0.998$) and 0.002 μM ($r^2 = 0.981$), respectively. The corresponding PBMC viabilities were 89 and 100% compared with that of non-TPT-treated cells and thus were much more resistant than the RPMI 8402 cells. The TD_{50} of TPT for mock-infected PBMC is 0.037 to 0.072 μM, which is more sensitive than that for HIV-1-infected PBMC.

An AZT-resistant HIV-1 strain (A012D) was employed to show that TPT inhibited the expression of the p24 antigen of this isolate with the same efficiency as it did that of $HIV\text{-}1_{IIIB}$ (Fig. 2b). A012D is highly resistant to AZT, with an ID_{50} of 2 μM compared to an ID_{50} of 0.01 μM for the wild type (15). Inhibition of HIV p24 was tested at a range of 0.002 to 0.125 μM TPT, and 74% of PBMC cells were viable at 0.125 μM TPT as measured by trypan blue exclusion (Fig. 2b). The ID_{50}s of TPT for the AZT-resistant HIV-1 strain and $HIV\text{-}1_{IIIB}$ were 0.007 μM ($r^2 = 0.979$) and 0.005 μM ($r^2 = 0.936$), respectively. These results suggest that the mutations in the HIV RT gene which are associated with a high level of resistance to AZT do not interfere with the antiviral activity of TPT.

TPT inhibited HIV-1 activation by TNF-α in OM10.1 cells. The OM10.1 cell line is a promyelocytic cell line latently infected with HIV-1 in which each cell contains one genomic copy of HIV-1. Less than 10% of cells constitutively express a low level of HIV-1 p24, and cells remain $CD4^+$ when in culture. When treated with TNF-α, however, more than 95% of cells become $HIV\text{-}1^+$, CD4 is down-regulated, and p24 in the supernatant is increased by >30-fold (4). This TNF-α-induced viral replication was blocked by TPT in a dose-dependent manner with minimal cell toxicity. OM10.1 cells were stimulated by TNF-α for 36 h in the presence of 0.004 to 0.031 μM TPT. TPT decreased expression of p24 antigen to 7% of that of the nontreated control. A cell proliferation assay showed that cell growth continued in the presence of TPT. The cell number was 131% of that of the non-TPT-treated cells at 0.031 μM TPT, which was the highest TPT concentration used in this experiment (Table 1). The TD_{50} of OM10.1 cells with TNF-α is 0.078 to 0.1 μM. In the presence of PTX, an inhibitor of TNF-α mRNA expression, the activation of HIV p24 expression with exogenous TNF-α was not prevented at a concentration of up to 500 μM (Table 1).

TPT also suppressed expression of HIV-1 RNA and p24 in nonactivated OM10.1 cells (data not shown). Both HIV-1 RNA and p24 were sharply decreased 3 days after TPT was added to the culture and continually decreased until the end of the assay (day 6). In these cells, HIV-1 RNA was decreased by

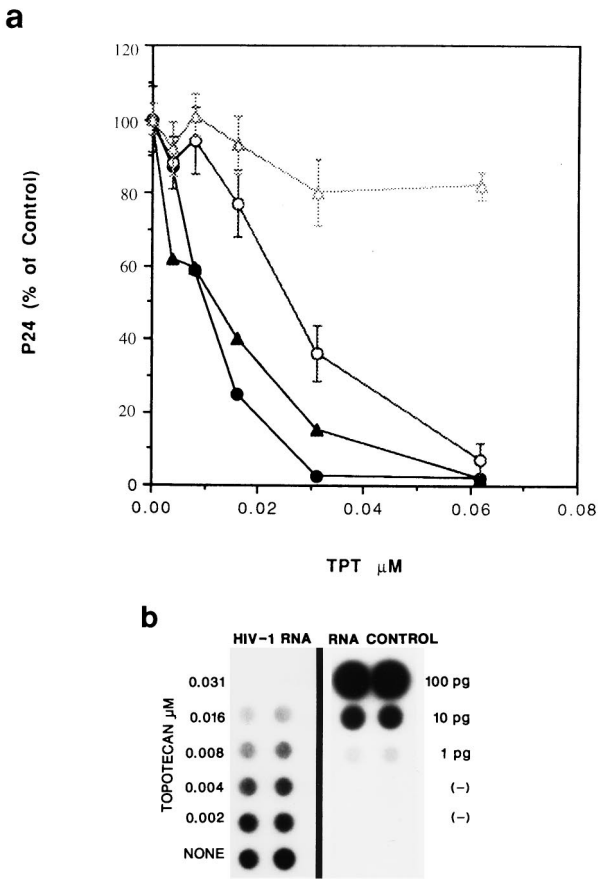

FIG. 1. (a) TPT inhibits production of HIV-1 p24 in chronically infected CPT-K5 cells. The supernatants were collected on day 6 after the drug treatment and tested as described in Materials and Methods. The cell viability was examined by MTT assay, and samples were run in duplicate. ——▲——, p24 in CPT-K5 cells; ——●——, p24 in RPMI 8402 cells; - - - -△- - - -, CPT-K5 cell viability measured by MTT assay; ——○——, RPMI 8402 cell viability measured by MTT assay. (b) TPT inhibits production of HIV-1 RNA in CPT-K5 cells. The culture condition and TPT treatment were the same as for panel a. Cells were harvested on day 6 for viability tests, and 4×10^4 cells from each well were put into 5 M guanidium thiocyanate and stored at −20°C. Each spot represents HIV-1 RNA extracted from 2×10^4 viable cells after TPT treatment. The samples were tested in duplicate. The HIV-1 RNA was detected by an antisense RT RNA probe. Sense RT RNA, used as an RNA concentration control, is shown in the right side of the panel.

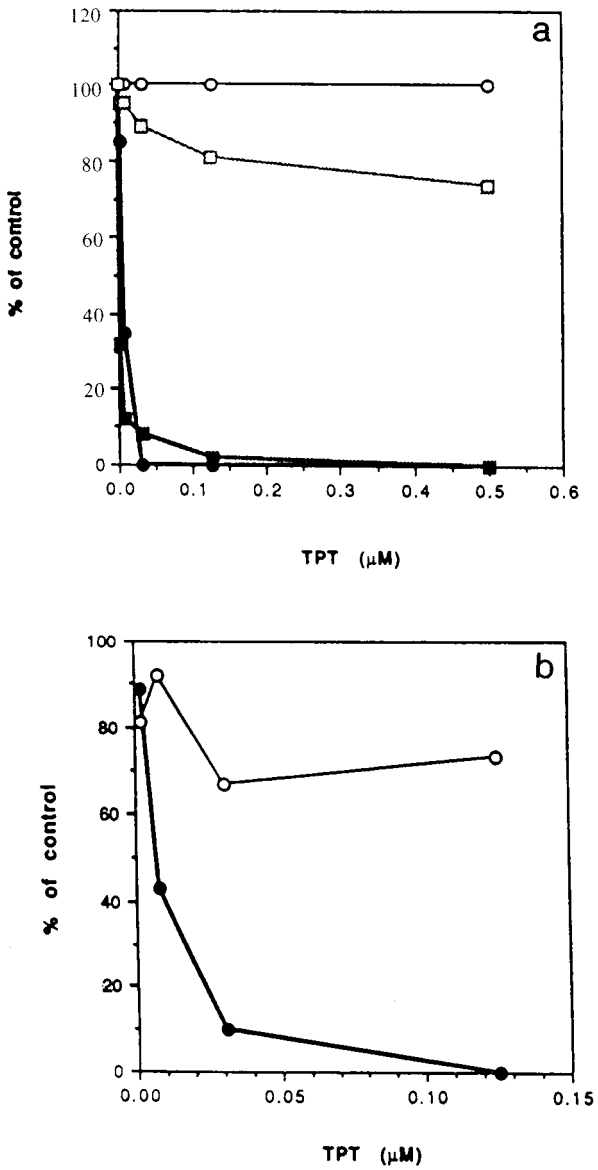

FIG. 2. (a) TPT inhibited expression of p24 in PBMC acutely infected with HIV-1 clinical isolates. p24 level and cell viability were tested on day 6 after TPT treatment and expressed as percentages of control levels. The viability was measured by trypan blue exclusion. ——●——, p24 level of clinical isolate 010792; ——○——, viability of cells infected with clinical isolate 010792; ——■——, p24 level of clinical isolate 010893; ——□——, viability of cells infected with clinical isolate 010893. (b) TPT inhibited expression of p24 in an AZT-resistant HIV-1 strain. ——●——, p24 level; ——○——, cell viability.

62% at 0.008 μM TPT. The HIV-1 p24 level was decreased by 70% at 0.016 μM TPT, whereas cell viability was maintained at 90% with this concentration. The ID_{50}s of TPT for inhibition of RNA and production of p24 in unstimulated OM10.1 cells were 0.006 and 0.007 μM, respectively ($r^2 = 0.988$).

TPT inhibited the replication of HIV-1 SP1 and NF-κB mutants. Two HIV-1 LTR mutants were grown in PBMC and tested for sensitivity to TPT, and the results were compared with those for their wild types. The NF-κB mutant pNFA has two NF-κB binding sites deleted. The SP1 mutant pSPC has two GG to TT point mutations in each upstream SP1 binding site. Both of the mutants were shown to be sensitive to TPT, as were the wild types (Fig. 3). The p24 titer of TPT-treated pSPC cultures was also measured on day 14, and no difference was observed between mutant and wild type (16).

TABLE 1. TPT inhibited expression of p24 in TNF-α-activated and HIV-infected OM10.1 cells[a]

Treatment	p24 (pg/ml)	% Inhibition	Cell no. (%)
None	28		100
TNF-α	624	0	100
TNF-α plus 0.004 μM TPT	386	40	89
TNF-α plus 0.008 μM TPT	326	50	98
TNF-α plus 0.016 μM TPT	243	64	154
TNF-α plus 0.031 μM TPT	70	93	131
TNF-α plus 50 μM PTX	608	3	96
TNF-α plus 100 μM PTX	642	0	105
TNF-α plus 250 μM PTX	600	4	92
TNF-α plus 500 μM PTX	602	4	93

[a] TPT was added to cultures of OM10.1 cells (10^5 cells/ml) at the same time as TNF-α (20 U/ml). p24 levels were measured and cell numbers were determined 36 h later, as described in Materials and Methods.

Decrease in production of p24 antigen in TPT-treated cells is not due to inhibition of cell proliferation. The anti-HIV effect of TPT was achieved at a concentration that showed little cytotoxicity to HIV-1-infected cells as measured by cell viability and cell proliferation assays. These assays were performed in RPMI 8402 cells, in PBMC acutely infected with HIV-1, and in OM10.1 cells latently infected with HIV-1. RPMI 8402 cells grew from an initial concentration of 2×10^3 to 10.6×10^3 cells/ml at day 3 in the presence of 0.01 μM TPT, representing a 5.3-fold increase in the number of cells plated. HIV-1-infected PBMC grew from an initial concentration of 3×10^5 to 1.7×10^6 cells/ml at day 6 in the presence of 0.031 μM TPT, representing a 5.7-fold increase. OM10.1 cells without TNF-α stimulation grew from an initial concentration of 3×10^5 to 2.0×10^6 cells/ml at day 6 in the presence of 0.031 μM TPT, representing a 6.3-fold increase. At this TPT concentration, the level of HIV-1 p24 antigen decreased to 4% of that of the control in these infected PBMC and OM10.1 cells. The anti-HIV effect of TPT was dose dependent and occurred at concentrations much lower than those which suppressed cell growth (Fig. 4). The ID_{50}s of TPT in these cells ranged from 0.002 to 0.009 μM, whereas the TD_{50}s were greater than 0.027 μM. The therapeutic indices (TD_{50}/ID_{50}) are thus calculated to be 3 to 14. These results suggest that the anti-HIV effect of TPT is achieved by a mechanism other than that responsible for killing HIV-1 host cells.

DISCUSSION

We report that TPT decreased replication of HIV-1 in the CPT-K5 cell line. The topo I in these cells has single amino acid changes from aspartic acid to glycine at residues 533 and 583, which cause a more than 125-fold decrease in the sensitivity of these cells to CPT. TPT inhibited production of HIV-1 RNA and p24 in CPT-K5 and wild-type cells equally effectively. The antiviral effects of TPT were not likely to be the result of nonspecific cytotoxicity, since TPT inhibited production of HIV-1 p24 antigen and viral RNA at concentrations that did not affect cell viability and growth. These observations suggest that TPT targets factors in virus replication other than cellular topo I.

These antiviral effects of TPT were observed not only in the

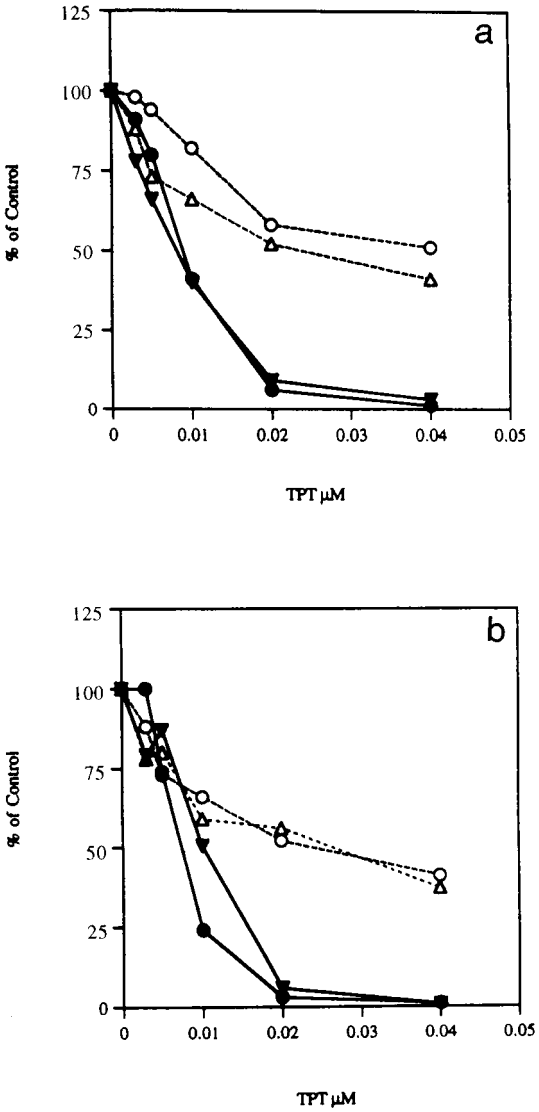

FIG. 3. (a) Effect of TPT on HIV-1 LTR mutant pNFA versus that on wild-type pILIC. The viability was measured on day 6 by MTT assay. ——▼——, p24 level of PBMC infected with pILIC; - - - -△- - - -, viability of PBMC infected with pILIC; ——●——, p24 level of PBMC infected with pNFA; - - - -○- - - -, viability of PBMC infected with pNFA. (b) Effect of TPT on HIV-1 LTR mutant pSPC versus that on wild-type pILIC. The viability was measured on day 6 by MTT assay. ——▼——, p24 level of PBMC infected with pILIC; - - - -△- - - -, viability of PBMC infected with pILIC; ——●——, p24 level of PBMC infected with pSPC; - - - -○- - - -, viability of PBMC infected with pSPC.

topo I-mutated CPT-K5 line but also in PBMC acutely infected with clinical isolates and in OM10.1 cells latently infected with HIV-1 and activated by TNF-α. It is noteworthy that TPT inhibited production of HIV RNA and p24 in latently infected cells as efficiently as in acutely infected PBMC. The finding that TPT treatment of OM10.1 cells prevented activation of HIV-1 by TNF-α provides an example that shows that HIV-1 activation in latently infected cells can be altered by drug treatment.

In this study, we also examined the anti-HIV activity of TPT on two pairs of HIV-1 LTR mutant viruses. pNFA has two NF-κB binding sites deleted, and pSPC has point mutations in each upstream SP1 binding site. TPT inhibited replication of mutant virus as efficiently as it inhibited that of wild-type virus

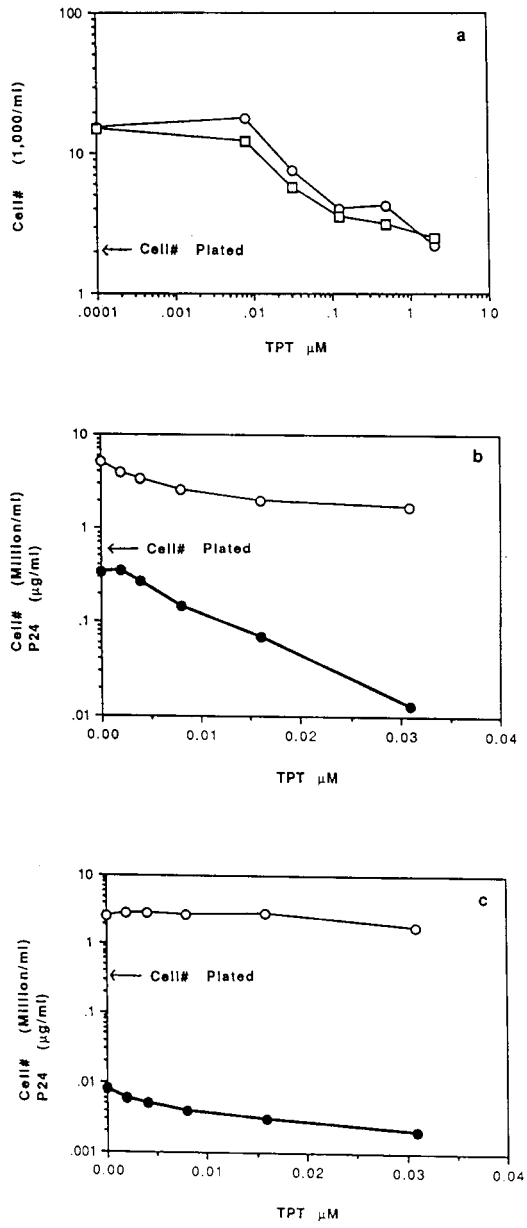

FIG. 4. (a) Effect of TPT on cell proliferation in RPMI 8402 cells. Cells were plated at 2×10^3 cells/ml in the presence of various concentrations of TPT. Proliferation was determined after 72 h by counting of cell numbers with a Coulter counter. Data represent the averages of triplicates in two separate experiments. ——□——, cell number from experiment I; ——○——, cell number from experiment II. (b) Effect of TPT on cell proliferation and p24 decrease in PBMC acutely infected with HIV-1. HIV-1 infection and cell culture condition were as described in Materials and Methods. The cell proliferation number was determined with a Coulter counter (ZM) in duplicate before and after cells were treated with TPT for 6 days. The level of HIV-1 p24 was assayed as described in Materials and Methods. ——○——, cell number after TPT treatment for 6 days (in millions per milliliter); ——●——, p24 level in the supernatant (in micrograms per milliliter). (c) Effect of TPT on cell proliferation and p24 decrease in unstimulated OM10.1 cells. The cell proliferation number was determined as for panel b. The OM10.1 cell culture and p24 assay were performed as described for Table 1 except without TNF-α stimulation. ——○——, cell number after TPT treatment for 6 days (in millions per milliliter); ——●——, p24 level in the supernatant (in micrograms per milliliter).

in PBMC. These results indicate that TPT may target regions other than the NF-κB and SP1 binding sites (17).

In the experiments with OM10.1 cells, the percentage of viable cells increased in the presence of 0.016 and 0.031 μM TPT. This increase may have resulted from the ability of TPT to inhibit replication of HIV-1 and thereby to decrease the cytotoxicity of HIV-1 to host cells. In addition, we compared the TD_{50} and ID_{50} in each tested cell type and found that the anti-HIV activity of TPT is distinguishable from its cytotoxicity. The therapeutic index is in the range of 3 to 14, which is reasonable for an anticancer drug having an anti-HIV activity and inhibiting both acute and chronic HIV-1 infections.

The pharmacology of TPT in advanced cancer patients has been established. At the approved dose and schedule of 1.5 mg/m^2/day for five consecutive days, the mean peak concentration of the active lactone species of TPT is 0.078 μM (22). This concentration compares favorably to the ID_{50}s for inhibition of HIV-1 ranging from 0.002 to 0.009 μM for acutely infected PBMC, chronically infected RPMI 8402 cells, and latently infected OM10.1 cells. Clinical trials are necessary to determine whether the pharmacokinetics of TPT in cancer patients are similar to those in HIV-infected patients and what role TPT will play in the management of these latter patients.

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