The protein kinase C (PKC) activator phorbol myristate acetate (PMA) was used to upregulate viral replication in a clone of promonocytic cells chronically infected with human immunodeficiency virus (HIV-1). Induction of virus could be inhibited by the triphenylethylene anti-estrogen tamoxifen at concentrations that had minimal effects on cellular DNA synthetic responses and cell cycle kinetics. This effect correlated with tamoxifen's ability to block PMA-mediated enhancement of HIV-promoter-driven transactivation in cells of monocyte and CD4 T-lymphocyte lineages. No interference with a primary infection was noted. Tamoxifen's mechanism of action may relate both to its capacity to inhibit PKC and to consensus sequences for gonadal steroid responsive elements in the HIV long terminal repeat, as it was able to partially inhibit another HIV activator, 5-azacytidine, which does not modulate PKC function. The finding that regulation of HIV in a model for low-level chronic or latent infection is amenable to a nonimmunosuppressive steroid antagonist may suggest approaches to pharmacologic intervention early in HIV infection.

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Effect of Tamoxifen on Regulation of Viral Replication and Human Immunodeficiency Virus (HIV) Long Terminal Repeat-Directed Transcription in Cells Chronically Infected With HIV-1

By Jeffrey Laurence, Henrietta Cooke, and Santosh K. Sikder

HUMAN IMMUNODEFICIENCY virus (HIV) is classified as a lentivirus. However, the slow progress of clinical symptomatology characteristic of HIV infection is not due to an intrinsically slow replication rate, but rather to the complex nature of viral gene regulation and virus-cell interactions. A variety of nuclear proteins acting as positive or negative transcriptional regulators binds to specific sequences within the long terminal repeat (LTR) of the two subtypes of HIV virus, HIV-1 and HIV-2. Proceeding upstream from the start site, there are consensus sequences for constitutively expressed host transcription factors, such as TFIID (TATA binding factor) and SP1, and two inducible cellular transcriptional activators, NF-kB and NFAT-1. Downstream of the start site, overlapping TAR, the target of the tat trans-activator, there are binding regions for at least two other molecules, CTFl (CCAAT transcription factor) and leader binding protein. A change in the quantity or activity of these proteins may underlie the sensitivity of HIV messenger RNA (mRNA) accumulation to a myriad of stimuli. These include signals as disparate as cytomegalovirus and herpes simplex virus coinfection, monocyte and lymphokine treatment, antigenic stimulation, and phorbol ester exposure. All are capable of converting a latent or chronic HIV infection to a productive one, and many appear to involve protein kinase (PK) activation.

A common motif among these pathways is the promiscuous cellular transcriptional regulator NFkB. NFkB is a 50-Kd protein often complexed to a 65-Kd molecule, IkB, which inhibits its activity. Phosphorylation of IkB by PKC may release the active species from its inert form, and permit translocation of NFkB to the nucleus.

DNA sequences responsive to steroid hormone regulation have also been located near mammalian retroviral promoters. For example, the rate of transcription of mouse mammary tumor virus (MMTV) DNA is augmented by glucocorticoids via a mechanism that increases, rapidly and selectively, the utilization of a transcription initiation site within the MMTV-LTR. Progesterone also elevates MMTV RNA expression in vivo and in vitro, an effect that is synergistically enhanced by estradiol. Consensus sequences for hormone-responsive elements have similarly been identified in HIV and membrane receptors for both adrenal and gonadal steroids are present on peripheral blood lymphocytes and monocytes, as well as their transformed counterparts.

This series of studies was designed to explore ways of prolonging the latent phase of HIV infection by preventing induction of the NFkB protein, or by activating negative regulatory sequences in the viral promoter. In our initial reports, we used a model system for phorbol ester-mediated induction of HIV-1 from a chronically infected promonocyte clone. We established the PKC dependence of this system, and defined its susceptibility to relatively toxic PKC inhibitors such as the isoquinolone H7 and 2-aminopurine. We also demonstrated that an immunosuppressive glucocorticoid, dexamethasone, could alter HIV promoter activity without inducing viral replication in a chronically infected monocytic cell line. We now report the ability of tamoxifen citrate, a nonsteroidal estrogen antagonist with anti-PKC, as well as hormone receptor binding properties, to block both upregulation of HIV replication and HIV-associated transactivation in cells of monocytic and T-cell lineages at clinically achievable concentrations.

MATERIALS AND METHODS

Cells
U1.A1 cells were subcloned from U1.1, a clone of U937.3 cells infected with the lymphadenopathy-associated virus (LAV) strain of HIV-1 and obtained from Dr T.M. Folks of the National Institutes of Health. They contain an average of two proviral copies of HIV-1...
as determined by Southern blotting. 8E5 is a CD4- subclone derived from LAV-infected A3.01 CD4+ T lymphoblasts and obtained from Dr Folks.13 Defective virions, with a termination codon affecting pol gene expression,14 are inducible on exposure of 8E5 cells to 5-iodo-2'-deoxyuridine15 or other agents. U937.3/HIV is a clone of U937.3 cells, which constitutively produce high levels of the LAV strain of HIV-1. All cells were cultured in RPMI-1640 plus 10% fetal bovine serum at a concentration of 2 x 10^5/mL.

Reagents

4B-phorbol-12-myristate-13-acetate (PMA), tamoxifen citrate (Nolvadex, 1-[p-dimethylaminoethoxyphenyl]-1, 2 diphenyl-1-butene) (TMX), and 5-azacytidine (5-AZA) were purchased from Sigma Chemical Co (St Louis, MO). PMA was prepared as a stock solution of 100 µg/mL in DMSO (dimethylsulfoxide), diluted in RPMI-1640, and used at final concentrations of 5 and 50 µg/mL. TMX was prepared as a stock solution of 10 µg/mL in DMSO, and 5-AZA as a stock solution of 1 mg/mL in 0.2N NaOH.

Cell Kinetic Studies

U1.1A cells were plated in macrowells at 0.5 x 10^6 cells/mL in the presence of varying concentrations of TMX. At various times (6 hours to 4 days), cells were washed twice with phosphate buffered saline (PBS), resuspended in 1 mL PBS, fixed by addition of 5 mL of cold 70% ethanol while vortexing, and stored at 4°C. Fixed cells were pelleted, washed with PBS, resuspended in 1 mL of PBS containing 300 U/mL RNase A (Sigma), and maintained for 30 minutes at 37°C. Propidium iodide (Sigma) was added to a final concentration of 50 µg/mL, and the cells kept on ice for 10 to 30 minutes. DNA histograms were obtained by scanning 1 x 10^6 cells on an EPICS-V flow cytometer, and analyzed by a computer-assisted method.15 DNA synthetic responses were concurrently evaluated as previously described.16

Viral Studies

U1.1A cells, 1 x 10^4, were cultured in 0.2 mL of medium in flat-bottom microwells, exposed to varying concentrations of PMA, 5-AZA and TMX, and supernatants assayed for HIV p24 core antigen at 48 hours. Preliminary experiments had shown that maximal HIV Ag was produced within 48 to 60 hours after PMA19 or 5-AZA treatment. HIV antigens were quantitated in supernatants by an enzyme-linked immunosorbent-based assay (ELISA) primarily detecting p24 core components using a human immunoglobulin (lg) directed against HIV-1 epitopes and contained in polystyrene beads (Abbott Labs, Chicago, IL).

HIV-Associated Trans-activation

The ability of the tat transcription unit of HIV to enhance the expression of the chloramphenical acetyl transferase (CAT) gene when CAT is linked to the LTR of HIV was measured as previously reported.20,21 Two plasmids were used either singly (HIV-LTR-CAT) or together (cotransfection of CAT- and tat-containing vectors). The tat plasmid pCV-1 contains a 1.8-kilobase (kb) fragment of HIV-1 complementary DNA (cDNA) encompassing the tat gene. The CAT plasmid pC15CAT contains the entire HIV-1 LTR and a portion of nef.

In Situ Hybridization

Cells. Twenty microliters of 1 x 10^6 U1.1A cells/mL in PBS were placed in each well of 4-well poly-L-lysine coated microscopic slides. Cells were allowed to settle, then immersed in 4% paraformaldehyde for 15 minutes at 22°C followed by rinsing in 0.15 mol/L Tris, pH 7.2. Twenty microliters of prehybridization buffer (6X SSC buffer [NaCl, trisodium citrate], 40% formamide, 5X Denhardt's solution, 100 µg/mL denatured salmon sperm DNA [Sigma]) was added per well and left at 22°C for 15 minutes. The solution was then shaken off and 5 µL of hybridization buffer (6X SSC, 45% formamide, 10% dextran sulfate, 5X Denhardt's solution, 4 ng digoxigenin-labeled probe) was added.

DNA labeling. A linearized plasmid, pIIIextat, containing the HIV tat, env, and LTR regions, was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN) by the random primer method.22 The reaction was stopped with 0.2 mol/L ethylene-diamine tetraacetic acid (EDTA) (pH 8.0). DNA precipitated with 0.3 mol/L sodium acetate and ethanol, and dissolved in 10 mmol/L Tris, 1 mol/L EDTA, 0.1% SDS, pH 8.0.

Hybridization and detection. Procedures are detailed elsewhere.23 Briefly, wells containing hybridization buffer were protected with cover slips, sealed with rubber cement, and denatured at 90 to 95°C for 5 minutes. After cooling on ice they were placed in a humidified atmosphere for 16 hours at 42°C. The coverslips were then detached and the slides washed twice for 15 minutes each in 6X SSC, 45% formamide at 42°C, twice for 5 minutes each with 2X SSC at 22°C, twice for 15 minutes each with 0.2X SSC at 50°C, and once with 0.1 mol/L Tris, 0.15 mol/L NaCl, pH 7.5, at 22°C. Slides were then incubated in 0.5% blocking reagent (Boehringer Mannheim), rinsed in Tris-NaCl, and 150 µmol/L polyclonal sheep anti-digoxigenin Fab of IgG conjugated with alkaline phosphatase added for 15 minutes at 22°C. They were then washed in Tris-NaCl followed by 0.1 mol/L Tris, 0.1 mol/L NaCl, 0.05 mol/L MgCl2, pH 9.5, NBT/BCIP (nitroblue tetrazolium/5-bromo-3-indolyl phosphate; Boehringer Mannheim) color solution containing 0.24 mg/mL levamisole (Sigma) was added to cover the slides, which were kept in the dark at 22°C. The reaction was stopped 16 hours later with 10 mmol/L Tris and 1 mmol/L EDTA, pH 8. Confirmation that the nucleic acid signal detected in stimulated cells was primarily RNA included control slides incubated with RNase-A (Sigma; 20 µL of 100 µg/mL stock for 60 minutes at 37°C) before hybridization.

RESULTS

U1.1A cells were exposed to PMA and supernatants assayed for HIV p24 core antigen at 48 hours. As previously reported,20 a four- to ninefold increase in p24 antigen occurred in the presence of PMA (Table 1). U1.1A cells were also exposed to TMX (1 to 20µmol/L) for 24 to 96 hours.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Compound</th>
<th>Concentration</th>
<th>HIV-1 p24 Core Antigen Concentration (ng/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>—</td>
<td>2,790 ± 110</td>
</tr>
<tr>
<td>PMA</td>
<td>5 ng/mL</td>
<td></td>
<td>9,980 ± 480</td>
</tr>
<tr>
<td>PMA</td>
<td>50 ng/mL</td>
<td></td>
<td>26,040 ± 1,100</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>—</td>
<td>3,110</td>
</tr>
<tr>
<td>PMA</td>
<td>1 µmol/L</td>
<td></td>
<td>2,600</td>
</tr>
<tr>
<td>TMX</td>
<td>5 µmol/L</td>
<td></td>
<td>3,290</td>
</tr>
<tr>
<td>TMX</td>
<td>10 µmol/L</td>
<td></td>
<td>3,140</td>
</tr>
<tr>
<td>TMX</td>
<td>20 µmol/L</td>
<td></td>
<td>2,520</td>
</tr>
</tbody>
</table>

Table 1. Effect of Various Agents on HIV-1 Replication in Chronically Infected U1.1A Cells

Cells were plated at 1 x 10^4/microwell in 0.2 mL culture medium with the appropriate concentration of inducing agent. Supernatants were harvested 48 hours later and tested for p24 antigen by an ELISA-based antigen capture system. PMA data represent the mean ± SD of three separate experiments, and for TMX, two separate experiments.
and culture supernatant was analyzed at 24 hour intervals for p24. Little effect was noted on the low basal level of antigen production by these chronically infected cells at 48 hours (Table 1), or at any other time point assessed.

Similar cultures were established, preincubated with TMX (1 to 10 pmol/L) for 2 hours, and exposed to 5 ng/mL PMA. As shown in Table 2, TMX blocked the ability of PMA to upregulate HIV replication by approximately 20% at 1 pmol/L, and by more than 90% at 5 pmol/L. These concentrations inhibited DNA synthetic responses by less than 20% (Table 3). In contrast, viral replication in parental U937.3 cells, constitutively producing HIV, was unaffected by TMX (Table 2).

TMX is thought to exert its effect by binding to estrogen receptors and translocating them to the nucleus, where processes dependent on estrogen-regulated events, including receptors and translocating them to the nucleus, where cytometric studies of propidium iodide-stained U1.1A cells we evaluated cell cycle kinetics of TMX-treated cells. Flow cytometric studies of propidium iodide-stained U1.1A cells showed no change in cell cycle distribution at 6 hours after TMX exposure (Fig 1). A 48 hour incubation with ≥5 pmol/L showed a reduction in S phase fraction and an accumulation in early G1 (Fig 1), in agreement with reports of the action of TMX on other endocrine-responsive cells. The magnitude of this alteration was consistent at the other time points evaluated (72 and 96 hours; data not shown).

Cellular responses to phorbol esters mimic responses to growth factors, hormones, and immunologic activation. As gonadal steroids are able to modulate MMTV expression through its LTR, we used a transient cotransfection assay to study possible repression of HIV-LTR-mediated gene expression in the presence of TMX. Conversion of radiolabeled chloramphenicol to its acetylated forms was markedly enhanced by PMA when the HIV-LTR-CAT and tat plas-

### Table 2. Effect of Tamoxifen on PMA-Mediated Induction of HIV-1 Versus Constitutive Viral Production

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PMA</th>
<th>TMX (pmol/L)</th>
<th>p24 Ag (pg/10^6 cells)</th>
<th>Inhibition of PMA Induction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1.1A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5,267 ± 124</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>16,723 ± 3,036</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14,643 ± 62</td>
<td>18.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5,640 ± 67</td>
<td>96.7</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>3,307 ± 431</td>
<td>100.0</td>
<td>—</td>
</tr>
<tr>
<td>U937.3/HIV</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14,690</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13,540</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13,672</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13,650</td>
<td>0</td>
<td>—</td>
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<td></td>
<td>+</td>
<td>16,670</td>
<td>0</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15,930</td>
<td>0</td>
<td>—</td>
</tr>
</tbody>
</table>

Cells were plated at 1 x 10^5/microwell in 0.2 mL culture medium with the appropriate concentration of TMX for 1 hour, followed by addition of 5 ng/mL PMA. Supernatants were harvested 48 hours later and p24 Ag concentration measured. Values for p24 Ag represent the mean ± SD of three separate experiments for the U1.1A cells (inducible cells), and the mean of pooled triplicate wells for the U937.3 experiment (constitutive viral producer).

### Table 3. Effect of Inducing Agents and TMX on Proliferation of U1.1A Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Agent</th>
<th>Concentration (h)</th>
<th>[3H]Thymidine Incorporation</th>
<th>cpm ± SD</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>—</td>
<td>—</td>
<td>5,085 ± 288</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>5 ng/mL</td>
<td>24</td>
<td>4,906 ± 348</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>50 ng/mL</td>
<td>24</td>
<td>3,343 ± 490</td>
<td>34.3</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>—</td>
<td>24</td>
<td>15,787 ± 2,715</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>TMX</td>
<td>1 µmol/L</td>
<td>24</td>
<td>15,644 ± 1,479</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td>TMX</td>
<td>5 µmol/L</td>
<td>24</td>
<td>15,992 ± 1,080</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>TMX</td>
<td>10 µmol/L</td>
<td>24</td>
<td>15,245 ± 41</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>TMX</td>
<td>15 µmol/L</td>
<td>24</td>
<td>5,359 ± 1,105</td>
<td>68.1</td>
</tr>
<tr>
<td>C</td>
<td>None</td>
<td>—</td>
<td>48</td>
<td>1,584 ± 173</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>TMX</td>
<td>1 µmol/L</td>
<td>24</td>
<td>1,427 ± 64</td>
<td>17.9</td>
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<tr>
<td></td>
<td>TMX</td>
<td>5 µmol/L</td>
<td>24</td>
<td>1,301 ± 209</td>
<td>22.2</td>
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<tr>
<td></td>
<td>TMX</td>
<td>10 µmol/L</td>
<td>24</td>
<td>1,232 ± 36</td>
<td>24.8</td>
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<tr>
<td></td>
<td>TMX</td>
<td>15 µmol/L</td>
<td>24</td>
<td>1,191 ± 66</td>
<td>—</td>
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<tr>
<td></td>
<td>5-AZA</td>
<td>1 µmol/L</td>
<td>24</td>
<td>8,356 ± 777</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-AZA</td>
<td>5 µmol/L</td>
<td>24</td>
<td>8,955 ± 773</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5-AZA</td>
<td>10 µmol/L</td>
<td>24</td>
<td>8,066 ± 701</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>5-AZA</td>
<td>20 µmol/L</td>
<td>24</td>
<td>5,215 ± 2,333</td>
<td>37.8</td>
</tr>
</tbody>
</table>

U1.1A cells were plated as described in Table 1, and DNA synthetic responses measured at 24 or 48 hours by determination of [3H]thymidine incorporation. Cpm represent the mean ± SD of triplicate wells.

mids were cotransfected into U1.1A cells (Fig 2). In contrast, TMX had no effect on baseline HIV-LTR directed CAT activity, yet completely abrogated PMA-upregulation of tat activity in a dose-dependent manner (Fig 2). An equivalent effect was demonstrated in 8E5 T cells. As shown in Fig 3, PMA caused a fourfold enhancement of HIV-LTR-CAT, an effect that was reduced by more than 40% with 10 pmol/L TMX. (The much higher number of proviral copies in these cells, as compared with U1.1A cells that harbor only two proviral copies per cell, is responsible for the high degree of CAT protein activity in 8E5 cells transfected with HIV-LTR-CAT alone.)

To ensure that TMX was not simply blocking virion release, its ability to inhibit upregulation of HIV mRNA in U1.1A cells was also investigated. In a previous report we demonstrated, by in situ hybridization, that less than 0.5% of U1.1 cells express HIV-specific structural or regulatory transcripts, an incidence that rises to more than 65% 24 hours after PMA treatment. We exposed cultures of U1.1 A cells to 5 ng/mL PMA or buffer in the presence or absence of varying concentrations of TMX. As shown in Fig 4, HIV tat or env transcripts were not expressed in the absence of PMA, while more than 80% of cells showed such messages at 24 hours after PMA exposure. This expression was reduced to less than 10% of cells with ≥5 pmol/L TMX.

To investigate whether TMX was having a dual effect and not simply blocking upregulation of HIV through inhibition of PKC- or alteration of cell cycle kinetics, a PKC-independent viral activator was evaluated. Stimulation by 5-AZA of reporter genes linked to the HIV-LTR occurs at the transcriptional level, related to the demethylation of cytosine residues throughout the LTR enhancer.
INHIBITION OF HIV ACTIVATION BY TAMOXIFEN

Fig 1. Effects of tamoxifen on HIV-1 infected promonocytic cell cycle kinetics. Cytofluorograph tracings of propidium iodide-labeled U1.1A cells were prepared. Each individual graph represents percent fluorescent cells (ordinate) versus fluorescence intensity (abscissa). The numbers at the right of each tracing represent computer estimates of the fraction (%) of cells in G1, S, or G2M phases of the cell cycle. (Upper row) Six hours after exposure of cells to buffer (O) or varying concentrations (1 to 10 µmol/L) of TMX. (Lower row) Forty-eight hours after a similar exposure of cells to buffer or TMX.

Discussion
HIV, as other lentiviruses, induces a latent infection characterized by relatively high frequencies of chronically infected T lymphocytes and monocyte/macrophages. The ability of HIV to escape host immune surveillance may relate in part to regulatory mechanisms that facilitate a rapid transition from latent infection to virion release in a manner that minimizes the opportunity for an effective host response. Compounds that interfere with transcriptional activation or posttranscriptional regulation of HIV might have clinical utility in asymptomatic HIV-seropositive individuals through blockade of viral induction, thereby prolonging the latent state and perhaps decreasing viral shedding.

We concentrated on chronic HIV-1 infection in immortalized cells and its relationship to potential inducers. We showed that, in contrast to upregulation of viral replication by PMA, the anti-estrogen tamoxifen has no stimulatory effect. In addition, while transcriptional enhancement could be demonstrated for two different classes of molecule, the PKC activator PMA and the demethylating agent 5-aza, TMX completely blocked only PMA-mediated, HIV-LTR-driven trans-activation. The mechanisms of these phenomena may relate both to tamoxifen's effects on PKC and hormone-responsive elements in the HIV-LTR.

TMX inhibits PKC in vitro, blocking PMA-dependent leukocyte functions with an IC50 of 6.1 ± 1.6 µmol/L. It does not interfere with the high-affinity phorbol ester receptor site, and appears to inhibit PKC by competing with phospholipids. In terms of HIV induction via PMA or immunologic stimuli (antigen, alloantigen, lymphokine), PKC activation presumably initiates an intracellular phosphorylation cascade, the end result of which is to stimulate pre-existing nuclear trans-acting factors. NF-kB is the enhancer protein first described as capable of upregulating HIV replication by interacting with the 5'-LTR at a site different from that of the tat-responsive TAR region. As PMA induces NF-kB, our data are consistent with TMX functioning through suppression of this enzymatic pathway.

However, another mechanism may also be operative. The IC50 for inhibition of HIV-LTR-directed tat activity by TMX was only 2 µmol/L (Fig 2). 5-aza acts apart from PKC activation, and MMTV-LTR hormone-receptor complexes have also been shown to regulate the transcription of DNA sequences proximal or distal to the locus whose expression is modulated. Thus we sought evidence for similar consensus sequences in the HIV-LTR.

For glucocorticoids, progestins, and androgens, nucleotide sequences required for receptor binding also act as hormone-responsive elements (HRE) in vivo. These nucleotides are not conserved absolutely, but a consensus, AGGTCANNNTGACCT, has been determined. It comprises an imperfect palindrome of 15 base pairs with the hexanucleotides AGAPuACA and TGTPyCT being the essential elements. Degeneracy in this sequence appears to lead to alterations in DNA-steroid receptor affinity, which in turn could lead to differences in the kinetics or magnitude of the hormone response. In the HIV-LTR, the core sequence AGAAC is absolutely conserved in two different HIV-1 isolates and mammalian retroviruses, and murine and human endogenous retroviral elements. The estrogen-responsive element has a different consensus sequence, AGGTCA, with the half-palindromic motif 5'-GGTCA the critical region. Tamoxifen-estrogen complexes bind to these nuclear acceptor sites in human lymphocytes for extended periods of time.

Defining potential negative regulatory phenomenon related to TMX is difficult. Almost all of the steroidal
transcriptional activators also function as repressors, either through their HREs or via negative elements (nHRE) which do not have clear consensus sequences. Receptor-TMX complexes also have some agonist activities, including induction of the progesterone receptor. Even the various adrenal and gonadal steroids that share a common cis-acting sequence may interact with different transcriptional activators already bound to a promoter. For example, the binding of two common regulatory proteins, nuclear factor-I and factor-1, to the MMTV promoter occurs only when cells are treated with steroids, and sequences responsive to these same two molecules are present in the HIV-LTR. In addition, steroids may inhibit the transcription of genes through negative regulation mediated by 5' flanking sequences, or affect posttranscriptional processes. Cell-specific factors have been shown to interact with the negative regulatory element in the HIV-LTR to retard HIV replication. Transfections with LTR deletion mutants should aid in defining whether any of these sequences serve as physiologic steroid-responsive elements susceptible to TMX.

The potential clinical utility of these in vitro hormonal manipulations remains to be established. Compounds need to be evaluated as potential therapeutics in chronic HIV infection as classic anti-viral agents, such as 3'azido-3'deoxynucleoside (AZT), have no effect on preventing induction of HIV expression in chronically infected cell lines, although alpha-interferon may have a salutory effect. The IC₅₀ for blockade of the PMA-inductive effect on HIV replication (Table 2) and HIV-LTR-directed trans-activation (Figs 2 and 3) in our cell lines is on the order of 1 to 2 μmol/L. This level had little effect on cell cycle kinetics (Fig 1) or cell proliferation (Table 3). Complete inhibition of viral induction, at least in the promonocyte line, occurred at 5 to 10 μmol/L, similar to the IC₅₀ for PKC inhibition by TMX, 6.1 μmol/L. TMX has been used as primary therapy or an adjuvant to surgery in carcinoma of the breast in females and males with few untoward effects, even when continuously taken over several years. Therapeutic plasma levels show considerable variability, but are in the range of 1 to 2 μmol/L with a twice daily dose of 20 mg taken orally. Much higher affinity analogs, including hydroxytamoxifen,

Fig 2. Effect of tamoxifen on PMA-mediated enhancement of HIV-LTR-CAT in a promonocytic cell line. The CAT plasmid (1 μg DNA) was cotransfected into U1.1A cells (2 x 10⁴) with a plasmid containing tat (1 μg DNA). PMA (50 ng/mL) and/or TMX (1 to 10 μmol/L) was present throughout the 48-hour culture period. The percent conversion of [³⁵S] chloramphenicol (Cm) to its acetylated forms (ACm) was determined. (A) Dose-response curve for inhibition of PMA-driven upregulation of HIV-LTR-CAT activity. This activity was measured in the presence (●) and absence (○) of PMA. (B) Chromatographic data for conversion of Cm to ACm.

![Graph showing relative activity of HIV-LTR directed CAT activity against Tamoxifen concentration.](image)

![Table showing conversion of Cm to ACm](image)

![Chromatogram showing ACm and Cm](image)

![Conversion data table](image)
have also been identified. Estradiol elevates total PKC levels in certain tissues for prolonged periods in animals, an effect blocked by TMX.

Hormonal manipulations may also have other, nonviral effects in HIV infection. A variety of adrenal and gonadal deficits occur in acquired immunodeficiency syndrome (AIDS). Plasma levels of the androgen dehydroepiandrosterone are significantly depressed in asymptomatic HIV carriers, with further lowering in individuals with AIDS, and testicular atrophy is a common finding. Also of interest is the fact that a model for one major manifestation of AIDS, Kaposi’s sarcoma, produced when tat under the control of the HIV-LTR is introduced into the germline of mice, was elicited in one study only in male transgenic animals, despite equivalent levels of tat mRNA expression in their female counterparts. The possibility that this sexual difference in phenotype is hormonally based is under investigation. Preliminary reports that 5-AZA could activate the virus in transgenic mice containing the entire HIV provirus suggests that experimental evaluation of TMX and related agents in vivo may soon be feasible. A further indication that hormonal therapy might have a therapeutic role in HIV infection was the preliminary observation that the synthetic progestosterone megestrol acetate (Megace) led to significant improvements in appetite, lethargy, and body weight in some, although not all, AIDS patients.

Finally, TMX has a variety of immunologic effects. In vitro modulation of human lymphocyte reactivity includes increases in pokeweed mitogen-driven Ig production, inhibition of CD8+ T-suppressor cell phenomena, and augmentation of natural killer cell activity.

Our data lend further support to the concept that molecules capable of blocking upregulation of HIV, including agents such as tamoxifen, should be evaluated as possible adjuncts in the therapy of chronic, asymptomatic HIV infection.

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