Bis-Anthracycline Antibiotics Inhibit Human Immunodeficiency Virus Type 1 Transcription

Olaf Kutsch,1* David N. Levy,2 Paula J. Bates,2 Julie Decker,2 Barry R. Kosloff,1 George M. Shaw,2,4 W. Priebe,5 and Etty N. Benveniste1

Departments of Cell Biology1 and Medicine,2 The University of Alabama at Birmingham, and The Howard Hughes Medical Institute,3 Birmingham, Alabama; The Department of Medicine, University of Louisville, Louisville, Kentucky3; and The University of Texas M. D. Anderson Cancer Center, Houston, Texas5

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The increasing numbers of human immunodeficiency virus type 1 (HIV-1) strains that exhibit resistance to antiretroviral agents used at present require the development of new effective antiretroviral compounds. Tat transactivation was recognized early on as an attractive target for drug interference. To screen for and analyze the effects of compounds that interfere with Tat transactivation, we developed several cell-based reporter systems in which enhanced green fluorescence protein is a direct and quantitative marker of HIV-1 expression or Tat-dependent long terminal repeat activity. Using these reporter cell lines, we found that the bis-anthracycline WP631, a recently developed DNA intercalator, efficiently inhibits HIV-1 expression at subcytotoxic concentrations. WP631 also abrogated acute HIV-1 replication in peripheral blood mononuclear cells infected with various primary virus isolates. We demonstrate that WP631-mediated HIV-1 inhibition is caused by the inhibition of Tat transactivation. The data presented suggest that WP631 could serve as a lead compound for a new type of HIV-1 inhibitor.

The advent of highly active antiretroviral therapy (HAART) in 1995 profoundly increased the life expectancy of human immunodeficiency virus (HIV) type 1 (HIV-1)-infected people in developed countries (31), but despite its tremendous benefits, HAART is not able to eradicate HIV from treated patients (for reviews, see references 2 and 34). As cessation of antiretroviral therapy results in an immediate rebound of viremia, lifelong treatment is required. In this scenario, the continuously increasing numbers of viral strains that are resistant to therapy pose a serious threat to the current treatment success and require the development of new anti-HIV-1 compounds (29).

A possible alternative therapeutic target is Tat transactivation. Tat transactivation is dependent on the interaction of HIV-1 Tat with the conserved RNA structure of the transactivation responsive (TAR) element, which consists of RNA with a complex three-dimensional structure which contains a stem-loop and a bulge (5, 11, 17). The TAR element is a part of the nascent viral RNA and is essential for efficient initiation of viral transcription and subsequent elongation (1, 9, 14, 23).

To screen for and analyze the effects of compounds that would target HIV-1 transcription, we established several reporter cell lines in which HIV-1 expression or Tat-mediated long terminal repeat (LTR) activity is directly and quantitatively linked to enhanced green fluorescence protein (EGFP) fluorescence. For this purpose we infected Jurkat (T-cell lymphoma) cells with an HIV-1 strain engineered to express EGFP (22). Following the initial infection, we established several cell clones that constitutively expressed EGFP and secreted infectious viral particles. Using these cell lines and two additional reporter cell lines in which EGFP expression is controlled by the HIV-1 LTR in the absence or presence of Tat, we discovered that the bis-anthracycline WP631 efficiently and specifically inhibits HIV-1 expression by interfering with Tat transactivation. WP631 was designed by using the established anticancer drug daunorubicin as a scaffold compound (4). Two daunorubicin molecules were linked by a p-xylene linker, while the individual DNA-binding capacity of each daunorubicin molecule was maintained. While daunorubicin preferentially binds to triplets of the type 5′-(A/T)CG or 5′-(A/T)GC (3), WP631 binds to a CG(A/T)(A/T)CG hexanucleotide sequence, thereby greatly increasing its DNA-binding specificity (16, 38) and DNA-binding affinity (24). Interestingly, although WP631 had been described to act as a DNA intercalator, we could not find evidence that WP631 would interact with any of the HIV-1 key promoter elements (e.g., NF-κB and Sp1) and suppress LTR activity at the DNA level. Although it is possible that WP631 binds to the double-stranded parts of the TAR elements, the inhibitory kinetics of WP631 rather suggest interaction with an unknown cellular factor, as has been described for the Tat inhibitor Ro24-7429 (15).

By using WP631 as a lead compound, it should be possible to design bis-anthracyclines by altering the major scaffold molecule, the side chains, or the linker type that specifically inhibits HIV-1 while exhibiting very low DNA-binding activities and, thus, cytotoxities. On the other hand, bimodal agents of the WP631 type with HIV-1-inhibitory and antineoplastic properties could be ideal compounds for the treatment of AIDS-related malignancies.

MATERIALS AND METHODS

Reagents. WP631 was purchased from ALEXIS (San Diego, Calif.) and was dissolved in dimethyl sulfoxide at a stock concentration of 5 mg/ml. Daunorubi-
cin and doxorubicin were obtained from Calbiochem (San Diego, Calif.) and were dissolved in methanol at stock concentrations of 5 mg/ml. All antibodies were purchased from BD Pharmingen (San Diego, Calif.). Ro24-7259 was kindly provided by Hoffmann-La Roche, Inc. (Nutley, N.J.).

Cell lines and cell culture. All cell lines were maintained in RPMI 1640 medium supplemented with 2 mM l-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% heat-inactivated fetal bovine serum.

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of HIV-1-seronegative volunteers after informed consent was obtained from each individual. The lymphocyte fraction was isolated by using Ficoll-Paque gradients (Pharmacia). Cells were stimulated with phytohemagglutinin (PHA-L; 5 μg/ml), streptomycin per ml, and 10% heat-inactivated fetal bovine serum, interleukin-2 (IL-2; 100 U/ml) for 4 days, and then the cells were infected with HIV-1 isolates at multiplicities of infection of 0.06 for HIV-1 WEAU and OVW1 and 0.03 for HIV-1 CUCY. After 2 h, the inocula were removed, and the cells were infected with HIV-1 Tat and EYFP vector that expresses HIV-1 Tat and EYFP under the control of the viral LTR, and EGFP fluorescence is a quantitative marker of HIV-1 LTR activity. The clone chronically infected cells was probably mediated by integration of the reporter plasmid in a transcriptionally active environment, as no mutation in the integrated LTR was detected (data not shown).

Flow cytometric analysis of HIV-1 expression and cytotoxicity. JNLG cells were seeded at a density of 10³ cells/ml in 24-well plates and were then treated with the respective drugs. EGFP expression was analyzed with a FACStar Plus flow cytometer (Becton Dickinson). Dead-cell exclusion was performed by addition of propidium iodide (PI) and staining with PI-negative cells. Data were analyzed with CellQuest software (Becton Dickinson). Quantification of cytotoxicity levels for each sample was based on the ratio of PI-negative cells in the live gate to the total cell counts by using forward scatter (FSC) and side scatter (SSC) analysis. In comparison to the level of cell death determined with a Neubauer chamber and by trypan blue staining, this method underestimated cell death by 5%.

Analysis of WP631-mediated HIV-1 inhibition in PBMC cultures. To analyze the effect of WP631 on HIV-1 infection in PBMCs, freshly isolated cells were stimulated and infected as described above. Supernatants for HIV-1 p24 envelope protein were collected on day 5 postinfection. In each cell line, regulation of HIV-1 expression or LTR activity was monitored by a reporter system. For this purpose, the viral LTR, JLTRG, was used as a direct and quantitative marker of HIV-1 expression. To study Tat-dependent and Tat-independent regulation of HIV-1 LTR activity, we stably transfected Jurkat cells with a Tat-epitope vector, which was designed to express HIV-1 Tat and EYFP under the control of the viral LTR, and EGFP fluorescence is a quantitative marker of HIV-1 LTR activity. The clone chronically infected cells was probably mediated by integration of the reporter plasmid in a transcriptionally active environment, as no mutation in the integrated LTR was detected (data not shown).

RESULTS

EGFP-based reporter systems for the study of compounds suppressing HIV-1 expression. To screen for and analyze the effects of potential pharmaceutical compounds that may inhibit HIV-1 expression at the transcriptional level, we established several reporter cell lines based on Jurkat (T-cell lymphoma) cells (Fig. 1A). In these cell lines EGFP fluorescence is a quantitative marker of HIV-1 LTR activity. The clone chronically infected cells was probably mediated by integration of the reporter plasmid in a transcriptionally active environment, as no mutation in the integrated LTR was detected (data not shown).

Analysis of WP631-mediated HIV-1 inhibition in PBMC cultures. To analyze the effect of WP631 on HIV-1 infection in PBMCs, freshly isolated cells were stimulate...
EGFP-Expression
full length virus

Transfection \(\rightarrow\) pLTRGFP

G418 Selection

cloning \(\rightarrow\) R1

LTR: Tat-independent

LTR: Tat-dependent

pLZRS-YFP-Tat Transduction

Clone
WP631. On day 4 following treatment with Ro24-7429, 75% inhibition of EGFP expression was achieved with both compounds, which, in the case of Ro24-7429, is consistent with the results presented in previous reports (15). In the case of WP631, WP631-mediated HIV-1 inhibition and WP631 uptake can be measured simultaneously, as the compound is a fluorochrome. The inability of Ro24-7429 and WP631 to completely inhibit EGFP fluorescence as an indicator of HIV-1 expression suggests that part of EGFP expression in these cells is Tat independent.

**HIV-1-inhibitory effect of the bis-anthracycline WP631.** Treatment of JNLG cells with the bis-anthracycline antibiotic WP631 resulted in a decrease in the level of EGFP expression. The observed inhibitory effect of WP631 was concentration dependent. WP631 at 0.05 μg/ml decreased the level of EGFP expression by 25%. WP631 at 0.2 μg/ml decreased the level of EGFP expression by 65% (Fig. 2B). Higher concentrations of WP631 did not further decrease the level of EGFP fluorescence but resulted in increasing levels of cell death.

Following treatment with a single dose of WP631, the level of EGFP fluorescence, which is used as a marker of HIV-1 expression, in JNLG cells continuously decreased for 4 to 6 days (Fig. 2B). The level of EGFP fluorescence then slowly increased again, until it finally regained full expression in the treated JNLG cells after 3 weeks, indicating that a single dose of WP631 can inhibit HIV-1 expression in cell culture over an extended period. With respect to the concentration-dependent WP631-mediated decrease in the level of EGFP expression and the kinetics of HIV-1 inhibition, similar results were obtained with J89G cells (data not shown). In both cell lines, WP631 concentrations of 0.3 μg/ml and higher started to cause cell death, as shown in the FSC-SSC dot plot shown in Fig. 2C. By using the ratio of the cells in the live gate and the total event counts, it is possible to determine the 50% cytotoxic concentration (CC50), which for WP631 was 0.4 μg/ml. The ability of EGFP to serve as a marker for HIV-1 expression is demonstrated by the tight correlation of EGFP fluorescence and the level of p24 Gag protein production (Fig. 2D). The slight difference in the slopes of the two curves can be explained by the antiproliferative properties and the cytotoxic effect of WP631, which decrease the levels of p24 Gag protein production in the supernatants of the cells treated with higher concentrations of WP631.

Interestingly, treatment of JNLG and J89G cells with daunorubicin, the parent compound of WP631, and the related compound doxorubicin, which are part of the standard treatment protocols for AIDS-related malignancies (10, 25), increased the levels of EGFP expression in the cell lines. The levels of EGFP fluorescence, which was used as an indicator of HIV-1 expression, were increased by daunorubicin and doxorubicin to 170 and 300%, respectively, of the levels observed in untreated control cells (Fig. 2E). As expected, daunorubicin and doxorubicin also exhibited very high levels of cytotoxicity in comparison to that exhibited by WP631 when JNLG cells and PBMCs were treated with the two compounds (CC50 of daunorubicin and doxorubicin for JNLG cells, 0.001 μg/ml).

**WP631 inhibits HIV-1 replication in acutely infected PBMCs.** PBMCs activated with PHA-L and IL-2 were infected with various primary HIV-1 isolates (HIV-1 CUCY, OVVWI, and WEAU). At 2 h following infection, PBMCs were treated with WP631 at various concentrations (0.001 to 0.3 μg/ml), and at 7 days following infection, the p24 Gag protein concentrations in the culture supernatants were determined. The concentration-dependent ability of WP631 to suppress the infectivities of the primary HIV-1 isolates is depicted in Fig. 3A to C. Although the results varied slightly for the different donors and the different HIV-1 isolates tested, addition of WP631 to the infected cultures at 0.3 μg/ml reduced the level of HIV-1 p24 Gag expression by 80 to 90%.

Similar results were obtained under more stringent experimental conditions when we tested the ability of WP631 to inhibit HIV-1 replication in a cell-to-cell viral transmission assay, which generally results in higher levels of virus replication. PBMCs were infected with HIV-1 CUCY, and at 4 days postinfection they were mixed with syngeneic uninfected PBMCs and were then treated with WP631. Supernatants for the p24 protein ELISA and cells were harvested on day 8, and the cells were subsequently stained for CD3 and CD4 to determine the influence of WP631 on the CD4+ T-cell count in the infected culture. The effect of WP631 on HIV-1 replication varied slightly for the three donors (Fig. 3D). Again, in contrast to the cell lines used in the initial experiments, WP631 concentrations of up to 0.3 μg/ml had no influence on cell viability but suppressed HIV-1 replication by 80 to 90% compared to the level of replication of HIV-1-infected but untreated cells, as measured by the p24 Gag protein ELISA. WP631 also stabilized the CD4+ T-cell count, another measure of HIV-1 expression, in the infected cultures in a concentration-dependent manner (Fig. 3E).

**Influence of WP631 on binding of transcription factors to the HIV-1 LTR Sp1 element.** We initially investigated whether WP631 would interfere with the binding of transcription factors that are vital for HIV-1 expression after time was allowed for intercalation into the integrated viral LTR. The HIV-1 LTR contains three Sp1 sites located in close proximity to each other, and these are essential for HIV-1 expression (12). As WP631 has been described to bind with an extremely high affinity to an Sp1 consensus sequence (27), we tested whether
FIG. 2. WP631-mediated inhibition of HIV-1 expression and EGFP fluorescence in JNLG cells. (A) JNLG cells were cultivated in medium only or were treated with Ro24-7429 (10 μM) or WP631 (0.2 μg/ml). The level of EGFP fluorescence, which was used as a measure of HIV-1 expression, was determined by flow cytometric analysis 4 days posttreatment. Changes in EGFP expression are presented over a logarithmic scale. Numbers represent the EGFP mean channel fluorescence (MCF). The results are representative of those of five independent experiments. (B) JNLG cells were treated with various concentrations of WP631, and the level of EGFP fluorescence was monitored over time by flow cytometric analysis. Gray circles, the theoretical decay rate of EGFP if WP631 treatment would have resulted in an immediate and complete block of HIV-1 LTR activity. The results represent the means ± standard deviations of six independent experiments. (C) JNLG cells were treated with WP631 (0.3 μg/ml) for 4 days. At that time, viability could be determined by comparing the ratios of cells in the live gate (R1) to the total cell counts in the FSC-SCC dot plots of untreated and WP631-treated cells. Simultaneously, the levels of EGFP expression as measures of HIV-1 expression and relative drug uptake were determined. (D) On day 4 following treatment of JNLG cells with WP631 at various concentrations (0 to 0.4 μg/ml), the level of EGFP fluorescence, as measured by flow cytometry, and p24 Gag expression, as measured by ELISA, in the supernatants of the respective cultures were determined. The results represent the means ± standard deviations of six independent experiments. (E) JNLG cells were treated with either daunorubicin (DA; 0.001 μg/ml) or WP631 (0.2 μg/ml) for 2 days (C, control). Then, cell viability and EGFP mean channel fluorescence were determined by flow cytometry. The results represent the means ± standard deviations of three independent experiments.
WP631 could compete away nuclear factors binding to a 38-bp fragment containing all three Sp1 sites of the HIV-1 HxB LTR (Fig. 4). Nuclear extracts derived from HeLa cells formed a complex with the labeled LTR-Sp1 probe (lane 1). Addition of WP631 (0.5 to 8 μM, equivalent to 0.6 to 9.2 μg/ml; lanes 2 to 6) or doxorubicin (0.5 to 8 μM, equivalent to 0.3 to 4.6 μg/ml; lanes 7 to 11) inhibited the formation of the complex in a concentration-dependent manner. In these experiments, abro-

FIG. 3. Influence of WP631 on acute HIV-1 infection in PBMCs. PBMCs from three different donors (donors MP, MS, and OL) were infected with free virus from three different primary HIV-1 patient isolates, isolates OVWI (A), WEAU (B), and CUCY (C). At 2 h following the initial infection, WP631 was added at various concentrations (0 to 0.3 μg/ml). At 5 days postinfection, virus infectivity in the absence or presence of WP631 was determined on the basis of the level of HIV-1 p24 Gag protein expression. (D) To determine the ability of WP631 to inhibit cell-to-cell viral transmission, PBMCs from three different donors (donors 46-5, 59-4, and 63-7) were infected with primary HIV-1 patient isolate CUCY. At 4 days postinfection, 5 × 10^5 infected cells were mixed with 1 × 10^6 syngeneic PBMCs, and WP631 was added at the indicated concentrations (0.03 to 0.3 μg/ml). On day 8 of the experiment, HIV-1 replication was determined by HIV-1 p24 ELISA. (E) Relative CD4 counts were determined by staining the cells for CD3 and CD4 and subjecting them to flow cytometric analysis.
gation of the complex required more than 4.6 μg of WP631 per ml, which is more than 20 times the dose required for inhibition of HIV activity in JNLG cells and PBMCs. Furthermore, doxorubicin, which we demonstrated increases the level of HIV-1 expression in JNLG cells (data not shown), exhibits a similar, concentration-dependent ability to compete with the binding of transcription factors to the LTR-Sp1 probe.

Using the Mercury TransFactor system, we further investigated whether WP631 would inhibit the binding of transcription factors to NF-κB p50, NF-κB p65, c-rel, c-fos, CREB1, and ATF2 consensus binding sequences, all of which have been described to be important for HIV-1 expression (6, 8, 20, 21, 30, 33, 36, 37, 39, 41). Binding of transcription factors present in the nuclear extracts of JNLG cells to the respective consensus binding sites was analyzed in the absence or the presence of 0.3 μg of WP631 per ml. We found that nuclear extracts of JNLG cells contain high levels of NF-κB p50 and ATF2 and low levels of NF-κB p65, c-rel, c-fos, and CREB1; but binding of these transcription factors to the respective consensus sequences was not influenced by the presence of WP631 (data not shown).

**Influence of WP631 on expression of immune-relevant genes.** To determine the mechanism underlying the observed WP631-mediated inhibition of HIV-1, we investigated the specificity of the HIV-1-inhibitory effect of WP631. We initially analyzed whether WP631 influences the expression of several immune-relevant genes, such as major histocompatibility complex (MHC) class I, MHC class II, CD3, CD4, CD8, CD28, CCR, or CXCR4, by PBMCs or immortalized cell lines (Jurkat and THP-1 cells) but found that none of them were influenced by WP631 in any of the cell types tested (Table 1). These results suggest that WP631 most likely does not modulate antigen presentation and recognition, which would result in additional immune suppression. The inability of WP631 to regulate expression of CD4, CCR5, and CXCR4 further suggests that WP631-mediated inhibition of HIV-1 replication in PBMCs does not occur at the level of receptor modulation.

**WP631 specifically targets Tat transactivation.** As we had observed that WP631 did not alter the expression of a variety of cellular genes, we wanted to investigate whether the compound would be generally active against retroviruses. For this purpose, we established Jurkat cell lines that constitutively express EGFP under the control of the murine leukemia virus (MuLV) promoter (JEGFP) and monitored the level of EGFP expression in the respective cells over a period of 7 days following treatment with WP631 at various concentrations (0.03 to 0.3 μg/ml). In contrast to EGFP expression in JNLG and J89G cells, EGFP fluorescence in JEGFP cells was not regulated by WP631 over the entire period of the experiment. Figure 5 depicts the relative levels of EGFP expression in JNLG, J89G, and JEGFP cells 4 days following treatment with WP631. WP631 also did not influence EGFP expression in Jurkat cells controlled by the CMV promoter (data not shown). As MuLV, unlike HIV-1, is a retrovirus that expresses its genes in a transactivation-independent manner, we continued to investigate whether WP631 would specifically target Tat transactivation.

For this purpose, we treated JLTRG/Tat-Y cells with WP631 at various concentrations (0.03 to 0.3 μg/ml). The level of Tat-dependent LTR activity in these cells is indicated by the expression of EGFP, whereas the levels of Tat expression are linked to the level of EYFP fluorescence. In the presence of WP631, EGFP expression in JLTRG/Tat-Y cells is reduced in a manner similar to that seen in JNLG cells containing full-length HIV-1, indicating that WP631-mediated HIV-1 inhibition is a result of specific interference with Tat transactivation (Fig. 5 and Fig. 6). Four days following treatment with 0.3 μg of WP631 per ml, the level of EGFP expression was reduced by 70% compared to that for untreated JLTRG/Tat-Y cells. At the same time, the level of EYFP expression under the control of the CMV promoter was not reduced (Fig. 6A and B), indi-

**TABLE 1. Regulation of expression of several cellular immune-relevant genes following treatment with WP631**

<table>
<thead>
<tr>
<th>Gene</th>
<th>PBMCs</th>
<th>Jurkat</th>
<th>THP-1</th>
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<tbody>
<tr>
<td>CD2</td>
<td>→</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CD3</td>
<td>→</td>
<td>NP</td>
<td>NP</td>
</tr>
<tr>
<td>CD4</td>
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<td>→</td>
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<tr>
<td>MHC class II</td>
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<td>→</td>
</tr>
<tr>
<td>CCR5</td>
<td>→</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>CXCR4</td>
<td>→</td>
<td>→</td>
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* PBMCs, Jurkat, or THP-1 cells were treated with WP631 (0.2 μg/ml) for 4 days. No differences in the expression of the various cell surface molecules were found for 4 days following WP631 treatment, as measured by flow cytometry.

* →, no regulation; ND, not done; NP, not present.

**FIG. 4.** Influence of WP631 on binding of transcription factors to the HIV-1 LTR Sp1 element. Electrophoretic mobility shift assay experiments were performed with a 38-bp oligonucleotide comprising the three Sp1 elements of the HIV-1 LTR and HeLa nuclear extracts. Samples were incubated without competitor (lane 1) or in the presence of various concentrations of WP631 (0.5 to 8 μM; lanes 2 to 6) and doxorubicin (Doxo; 0.5 to 8 μM; lanes 7 to 11).
cating that Tat expression in JLTRG/Tat-Y cells was not affected by WP631, which was also confirmed by Western blotting analysis (data not shown). The inhibitory effect determined from the levels of EGFP reduction in JLTRG/Tat-Y cells achieved with WP631 at this time was comparable to the inhibitory effect demonstrated by the Tat inhibitor Ro24-7429 (Fig. 2A). To determine the level of Tat-independent EGFP expression that can occur in these cells, we stimulated parental JLTRG cells (Fig. 6C) with tumor necrosis factor alpha (TNF-α; 10 ng/ml), which resulted in a 1-log shift in the level of the EGFP fluorescence intensity (Fig. 6D). The level of EGFP fluorescence observed in JLTRG cells following stimulation with TNF-α is equivalent to the level of Tat-independent EGFP expression seen in JLTRGon cells (Fig. 1). Hence, it is expected that a specific Tat inhibitor could not reduce the level of EGFP expression to levels lower than this, as long as the treated cells remain activated due to the previous presence of Tat. Indeed, EGFP expression in cells of the JLTRGon cell line, a Jurkat cell-based cell line in which the cells exhibit constitutive levels of EGFP expression under the control of the HIV-1 LTR in the absence of HIV-1 Tat, was not inhibited by WP631 (Fig. 5). This result indicates that WP631 inhibits only Tat-mediated LTR activity and does not interfere with HIV-1 expression by binding to elements on the integrated HIV-1 LTR.

The idea that WP631 does not interfere with HIV-1 expression at the DNA level is further strengthened by the absence of putative WP631 binding sites of the CG(A/T)(A/T)CG type in the 5′ LTR regions of integrated HIV-1 NL4-3 and 89.6 (Table 2). Only some related hexanucleotide sequences (GCT/TGC, GCG/GCG, GCT/AGC, GCG/CGC) that could serve as potential WP631 binding sites were identified, but none of these overlapped with LTR elements that have been identified to be important for HIV-1 expression (e.g., NF-κB, Sp1, and AP-1 elements).

**DISCUSSION**

The increasing number of HIV-1 strains that are resistant to commonly used retroviral inhibitors requires the development of new drugs, possibly drugs that target viral structures other than the viral protease or reverse transcriptase (29). A possible alternative strategy that can be used to suppress HIV-1 expression would be the inhibition of Tat transactivation that is essential for the initiation of viral transcription and elongation (9, 14, 23). In the present study, we demonstrate that at subcytotoxic concentrations the bis-anthracycline antibiotic WP631 (4, 16, 38) is capable of suppressing HIV-1 expression in infected cell lines and PBMCs by specifically inhibiting Tat transactivation.

To screen for and study the effects of compounds that interfere with HIV-1 transcription, we established several reporter cell lines that allow the direct monitoring of HIV-1 LTR activity. JNLG and J89G cells are EGFP-based reporter cell

**FIG. 5.** Effect of WP631 on LTR-controlled Tat-dependent and Tat-independent expression of EGFP. The influence of WP631 on Tat-dependent EGFP expression in the context of a full-length virus (JNLG and J89G) and in the context of a stable integrated LTR-EGFP reporter plasmid (JLTRG/Tat-Y) was compared to the influence of WP631 on EGFP expression controlled by the HIV-1 LTR in the absence of Tat (JLTRGon) and by the MuLV promoter (JEGFP). All cells were cultured in medium or were treated with WP631 at various concentrations (0.03 to 0.3 μg/ml) for 4 days and then subjected to flow cytometric analysis of EGFP fluorescence. The results represent the means ± standard deviations of three independent experiments.
lines that allow the study of the modulation of active HIV-1 expression in the context of a full-length virus (22). JLTRG/Tat and JLTRG/Tat-Y cells are stable T-cell lines that allow the study of modulation of Tat-dependent LTR activity by using EGFP as a direct and quantitative readout, whereas the use of JLTRGon cells allows us to study regulation of Tat-independent LTR activity.

In JNLG and J89G cells, WP631 down regulated HIV-1 expression by 70%, as measured by EGFP fluorescence levels and p24 Gag ELISA, without any apparent cytotoxicity. The level of HIV-1 inhibition achieved in these cells at this time point was comparable to that seen with the established Tat inhibitor Ro24-7249 (15). As EGFP expression in these cells is controlled by the integrated viral LTR, these results suggest that WP631 inhibits HIV-1 expression by suppressing LTR activity or interfering with viral RNA transcription. The observed long-lasting inhibition of HIV-1 expression indicates that WP631 binding to the LTR or to other structures involved in HIV-1 transcription is of extremely high affinity. Alternatively, WP631 treatment could result in alterations of the HIV-1 LTR, such as histone acetylation or DNA methylation (13, 19, 28, 35, 42, 45). WP631 not only inhibited chronic HIV-1 expression in JNLG and J89G cells, but it also efficiently abrogated acute HIV-1 infection in PBMC cultures

![Diagram](http://aac.asm.org)  

**FIG. 6.** WP631-mediated inhibition of Tat transactivation. JLTRG/Tat-Y cells were cultured in medium (A) or were treated for 4 days with 0.3 μg of WP631 per ml (B). EGFP expression was determined as a measure of Tat-mediated HIV-1 LTR activity, and changes are indicated as mean channel fluorescence intensity (MCF; arbitrary units). EGFP expression serves as an indicator of HIV-1 Tat expression and is presented as Y-MCF. To determine the level of possible Tat-independent LTR activity in these experiments, parental cell line JLTRG was cultured in medium (C) or was stimulated with TNF-α (10 ng/ml) (D). The results are representative of those of three independent experiments.

<table>
<thead>
<tr>
<th>Table 2: Frequency of putative WP631-binding motifs in the HIV-1 NL4-3 and 89.6 LTR and genes</th>
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<tbody>
<tr>
<td>Sequence</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>CGATCG</td>
</tr>
<tr>
<td>CGAACG</td>
</tr>
<tr>
<td>CGTTCG</td>
</tr>
<tr>
<td>CGTACG</td>
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<tr>
<td>CGATGC</td>
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<td>GAAACG</td>
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<td>GCATGC</td>
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*The HIV-1 NL4-3 and 89.6 LTRs were analyzed for the presence of the high-affinity-binding sequences described for WP631 (sequences in boldface) (16, 24, 38) and other related sequences. The HIV-1 NL4-3 and 89.6 LTRs were tested for the presence of total of 32 variations of this motif. Numbers indicate the frequency of binding sites in the LTR or the indicated HIV-1 gene.
infected with various primary HIV-1 isolates. When WP631 was applied at subcytotoxic concentrations, HIV-1 replication in infected PBMC cultures was inhibited by 80 to 90%, and the inhibition was independent of the viral strain and the donor. Notably, the cytotoxicity that was observed for the T-cell reporter lines with higher WP631 concentrations was reduced in the primary T-cell cultures, probably due to the slow proliferation rates of these cells.

As WP613 was published to be a major-groove DNA intercalator, we suspected that HIV-1 inhibition is caused by the specific binding of WP631 to a key regulatory site within the viral LTR. As previous work (27) suggested that WP631 interferes with Sp1 binding, we initially focused on whether WP631 could inhibit the binding of transcription factors to the three Sp1 sites that are located in the HIV-1 LTR and that have been reported to be essential for HIV-1 expression (12). Interference of WP631 with the binding of transcription factors could thus explain the observed downregulation of HIV-1 activity, but inhibition of transcription factor binding to the HIV-1 LTR Sp1 element required WP631 concentrations that greatly exceeded (20-fold higher) the concentration needed to inhibit HIV-1 infection in JNLG cells or PBMCs. These results indicate that the anti-HIV-1 effect of WP631 most likely is unrelated to the ability of WP631 to compete away transcription factor binding to the HIV-1 LTR Sp1 sequence in this system. The different abilities of WP631 to inhibit Sp1 binding in different systems might be explained by the high DNA sequence-binding specificity described for WP631 (4, 24), which would allow intercalation in the Sp1 consensus sequence used (5'-GAA TTC GGG GCG GGG CGA ATT-3') (27) but not in the HIV-1 Sp1 element (5'-AGG GAG GCG TGG CCT GGG CGG GAC TGG GGA GTG CGG AG-3').

We also found no evidence that WP631 interferes with the binding of several other cellular transcription factors, such as NF-kB p50, NF-kB p65, c-rel, c-fos, ATF2, and CREB1, that have been described to be essential for HIV-1 expression.

Sequence analysis further revealed that the HIV-1 LTR does not contain any binding sequences for WP631 [5'-GAA TTC GGG GCG GGG CGA ATT-3'] (27) but not in the HIV-1 Sp1 element (5'-AGG GAG GCG TGG CCT GGG CGG GAC TGG GGA GTG CGG AG-3').

As DNA usually forms B-form helices, whereas dsDNA forms A-form helices, it is unlikely that WP631 would intercalate into the dsDNA stretches of the TAR element, as it has been described to bind to DNA (4, 16, 24). Nevertheless, as the TAR element alters between different conformations with similar free energies, it is tempting to speculate that a complex molecule such as WP631 could also bind in alternative ways other than intercalation. Binding of WP631 to the TAR element would in turn prevent efficient binding of Tat, cyclin T1, or P-TEFb. Alternatively, WP631 may interfere with the binding of RNA polymerase II. In either case, direct binding of WP631 to the TAR element or WP631-mediated inhibition of RNA polymerase II, the inhibitory onset kinetics of WP631 reflected by the decrease in the level of EGFP expression should be much faster (Fig. 2B), as binding of WP631 should result in an immediate and complete block of viral transcription. In contrast, the inhibitory onset kinetic is comparable to the one seen for the established Tat inhibitor Ro24-7249 (data not shown). This inhibitor does not directly interfere with the Tat protein or binding of Tat to the TAR element but, rather, seems to inhibit an unknown cellular factor. We thus hypothesize that WP631 interferes with a cellular transcription factor important for Tat transactivation. This hypothesis is further strengthened by the finding that WP631 has a long-lasting
inhibitory effect that could not be explained by the binding of WP631 to the TAR element.

To our knowledge, this is the first description of a DNA bis-intercalator that specifically interferes with Tat transactivation. The bis-anthracycline WP631 at this time can only serve functionally interferes with Tat transactivation. The bis-anthracycline WP631 with respect to their cytotoxicities and their abilities to inhibit HIV-1 expression indicate that both effects can be modulated independently.

In this context the structural similarity to temacrazine, which has been described to inhibit HIV-1, is noteworthy (44). Temacrazine is a bis-triazolocarboxilic analog that has, on the basis of its DNA-intercalating abilities, shown promise in the treatment of colon cancer. As both agents, WP631 and temacrazine, have been described to exert their anti-HIV-1 properties at subcytotoxic levels, it is likely that the structural properties of the compounds are more important than their DNA-binding abilities for their anti-HIV-1 effects.

Although WP631, because of potential toxicities, is not suitable as a specific HIV-1 inhibitor at present, its ability to exert simultaneous antineoplastic and HIV-1-inhibitory effects at high concentrations predestines WP631 to be a compound that should be considered for the treatment of AIDS-related malignancies. Antineoplastic compounds that control HIV-1 infection would allow the interruption of HAART during cancer treatment without the fear of the evolvement of drug-resistant virus. Thus, compounds such as WP631 should reduce the added toxicities of two simultaneous chemotherapies, which should improve the overall outcomes of treatment for AIDS-related malignancies.

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