p53 Transactivation of the HIV-1 Long Terminal Repeat Is Blocked by PD 144795, a Calcineurin-Inhibitor with Anti-HIV Properties*

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Previous reports have indicated that benzothiophenes exhibit broad anti-inflammatory properties and inhibit human immunodeficiency virus-type 1 (HIV-1) replication. We show that the immunosuppressant cyclosporin A (CsA) and benzothiophene-2-carboxamide, 5-methoxy-3-(1-methyl ethoxy)-1-oxide (PD 144795) block the induction of p53 and NF-κB binding to the HIV-1 long terminal repeat (LTR) by the T cell receptor activator phytohemagglutinin. CsA and PD 144795 also inhibit the induction by phytohemagglutinin of the transcription mediated by an HIV-1 LTR fragment containing the p53 and NF-κB sites. These effects of PD 144795 on HIV-1 transcription correlate with its ability to inhibit the phosphatase activity of calcineurin and are similar to those previously described for CsA. Moreover, a constitutive active form of calcineurin is able to induce expression from the HIV-1 LTR in a p53- and NF-κB-dependent manner and PD 144795 is able to block this induction. These results demonstrate that the DNA binding of p53 to the HIV-1 LTR can be modulated by calcineurin and provide a framework to understand the anti-HIV properties of benzothiophene derivatives.

Benzothiophene derivatives have been shown to have both anti-inflammatory and anti-HIV1 effects. Originally, these compounds were shown to block expression of cellular adhesion molecules and to exhibit anti-inflammatory properties (1). Recently, benzothiophene derivatives were shown to block HIV-1 transcription in response to tumor necrosis factor α stimulation of promyelocytes (2). Additionally, these compounds blocked constitutive HIV-1 transcription in chronically infected cells and induced a latency state in cytokine-activated cells. The benzothiophene derivatives did not block the activation of NF-κB in response to tumor necrosis factor α treatment and did not block Tat function (2). In this report, we have studied the effects of PD 144795 (Parke-Davis Pharmaceuticals) (Fig. 1) on HIV-1 LTR-directed transcription in Jurkat T cells.

The expression of HIV-1 genes is controlled in part by the interaction of sequence-specific transcription factors with the LTR region of the provirus. NF-κB, Sp1, and other sequence-specific transcription factors have been shown to control transcription initiation directed by HIV LTR (3, 4). We have recently identified an inducible form of p53 in Jurkat T cells that directly interacts with a specific DNA-binding site positioned immediately downstream to the most 5′ Sp1-binding site in the HIV-1 LTR (5, 6). We have also shown that this DNA element mediates the induction of the HIV-1 LTR transcriptional activity by tumor promoting mutant forms of p53 (5). These results provide a mechanism to explain the dramatic increase in HIV-1 replication observed after the overexpression of mutant p53 in cells that completely lack expression of this protein (7).

It is well known that the replication of the HIV-1 virus in lymphocytes correlates with the activation/proliferation status of the infected cell (8, 9). Treatment of HIV-1-infected Jurkat cells with T cell receptor (TCR) or protein kinase C activators induces HIV-1 replication (10, 11). Previous reports have indicated that ligands of the TCR activate LTR-dependent transcription through a CsA-sensitive mechanism (12–14). CsA, through its interaction with cyclophilin (reviewed in Ref. 15), acts as an strong inhibitor of the serine/threonine phosphatase CN (16, 17). This enzyme is a heterotrimeric complex consisting of a 59-kDa catalytic subunit, calcineurin A subunit, a calcium-binding regulatory subunit of 19 kDa, calcineurin B subunit, and a 17-kDa calcium-binding protein, calmodulin.

CN plays a critical role in the regulation of calcium-dependent signaling pathways that are necessary for T cell activation (16, 18, 19). This enzyme regulates the nuclear translocation of NF-ATp, a transcription factor implicated in the expression of several cytokines (20). Also, CN inhibitors are known to decrease HIV-1 viral replication and to inhibit HIV-1 LTR-mediated transcription (12, 21). Inhibition of NF-κB binding to the LTR region of the HIV-1 provirus has been suggested as the mechanism by which CN inhibitors would repress the transcriptional activity mediated by this promoter (14, 22). Intriguingly, the relatively large number of genes whose expression is modulated by CN inhibitors in T cells (23, 24) suggests that, in addition to NF-AT and NF-κB, other transcription factors could be affected by these drugs.

In this study we have investigated the effect of CsA and PD 144795 on the transcriptional activity mediated by p53 and NF-κB. Using several approaches we demonstrate that calcineurin is implicated in the regulation of p53 transcriptional activity by tumor promoting mutant forms of p53.
activity, that PD 144795 is an inhibitor of calcineurin, and that the DNA binding and transactivation of p53 to the HIV-1 LTR can be modulated by calcineurin inhibitors. These results offer insight into the understanding of the molecular basis for the inflammatory and anti-HIV properties of benzothiophene derivatives.

**EXPERIMENTAL PROCEDURES**

**PD 144795**—Compound PD 144795 (Fig. 1) was synthesized by Parke-Davis Pharmaceutical Research as described previously (1).

**Electrophoretic Mobility Shift Assays**—The LTR B sequences were as described (5). DNA oligonucleotides were prepared with an Applied Biosystems 391EP DNA synthesizer using the phosphoramidite method, and purified by Sep-Pak C_{18} cartridges (Waters Associates). Plasmids were prepared with Qiagen columns according to the manufacturer's instructions. The HIV-1 LTR—BCAT have been previously described (5, 26). Plasmids were prepared and assayed for nucleoprotein complex formation as in Hubbard and Klee (28). CN activity was determined in the presence of two complexes, which we called PIC 1 and 2 (Fig. 2), identification of p53 and Sp1 in the PICs. Jurkat cells were incubated with PHA as in A and nuclear extracts prepared. 2 μg of nuclear extracts were incubated for 4 min at 4 °C with 0.2 μg of serum (NA), monoclonal anti-p53 antibodies PAb 240 (240, conformational), 421 (421, C-terminal), 1801 (1801, N-terminal); or polyclonal anti-Sp1 (Sp1), and then assayed by EMSA as in A. C, CsA and PD 144795 inhibit the formation of PICs. Jurkat cells cultured as in part A were preincubated for 30 min with 0.03% MeSO (NA), 0.5 μg/ml CsA, 100 ng/ml rapamycin (Rap), 3 μM PD 144795 (144795), or 100 ng/ml pentoxifylline, and then incubated for 4 h in the presence of 5 μM LFA. Equal volumes of MeSO were added in each condition. Nuclear extracts were prepared and assayed for binding to the LTR B probe. D, binding of the nuclear extracts from the MeSO (NA), CsA-, and PD 144795-treated cells to an Oct-1 probe. Conditions as in C. E, effect of PD 144795 (144795) on the induction of p53/Sp1 binding to the HIV-1 LTR by PHA and PMA. Jurkat cells cultivated as in A were preincubated for 30 min in the presence or absence of 3 μM PD 144795, and then incubated with 5 μg/ml PHA or 50 ng/ml PMA. Nuclear extracts were prepared and assayed for nucleoprotein complex formation as in A.

**RESULTS AND DISCUSSION**

CsA and PD 144795 Block p53 and NF-κB Binding to the HIV-1 LTR—The HIV-1 LTR contains a binding site for a proliferative-associated form of p53 (5). The p53 protein bound to this site is in a proliferative or mutant-like unfolded state based on recognition by specific monoclonal antibodies. This DNA region mediates the induction of the HIV-1 LTR by tumor necrosis factor α and by transforming mutant forms of p53. Importantly, tumor necrosis factor α induces a physical interaction between p53 and Sp1 and this interaction is required for the induction of HIV-1 LTR-mediated transcription by this cytokine (6). We investigated whether or not p53/Sp1 binding to the HIV-1 LTR is also induced by activation of the T cell receptor. For that purpose, we performed EMSA using an oligonucleotide probe (LTR B), CAGGGAGGCCTGGGCTGGGACTGGGG, that contains the composite p53 (underlined) and Sp1 (italics) site in the HIV-1 LTR, and nuclear extracts prepared from Jurkat T cells either unstimulated or stimulated with PHA (Fig. 2A). Incubation with PHA induced the appearance of two complexes, which we called PIC 1 and 2 (Fig. 2A). Subsequent experiments indicated that maximal complex formation by PHA was obtained at a 5-h incubation time (not...
shown). To confirm that these complexes contain p53 and Sp1, extracts were incubated with antibodies against these proteins prior to the mobility shift assay. Monoclonal anti-p53 PAb 421 and polyclonal anti-Sp1 demonstrated that p53 was present in PIC 1 and 2 while Sp1 was detected only in PIC 1 (Fig. 2B). A supershift was obtained with the anti-Sp1 antibody, whereas disruption of Sp1/p53 binding to the LTR was observed with the anti-p53 antibody Pab 421. We have previously shown that PAb 421 specifically blocks p53 interaction with LTR B p53/Sp1 composite site (6).

Subsequent experiments were directed to determine if the PHA-inducible binding of p53/Sp1 to the HIV-1 LTR is sensitive to pharmacological modification. We performed EMSA with nuclear extracts of Jurkat T cells incubated with PHA and exposed to a series of pharmacological agents. Incubation of Jurkat cells for 30 min with CsA or PD 144795 blocked the induction of PICs by PHA, however, incubation with pentoxifylline (data not shown), an NF-κB inhibitor (30), or the immunosuppressant rapamycin (15) had no effect (Fig. 2C). Other 5-methoxybenzothiophene derivatives had minor or no effect on PIC formation (not shown). Also, titration experiments indicated that inhibition of the induction of the p53/Sp1 complexes was reached at 3 μM PD 144795 and 0.5 μg/ml CsA (not shown).

As a control, the nuclear extracts used in Fig. 2C were tested for their ability to bind the HLA DRα octamer site using an oligonucleotide probe from this gene (46). No effect of CsA or PD 144795 was detected on Oct-1 binding (Fig. 2D). The identity of the Oct-1 complex was determined by supershift with an anti-Oct-1 antibody (not shown). CsA and PD 144795 also inhibited the induction of p53/Sp1 complexes in another T cell line, CEM, when these cells were incubated in the presence of the calcium ionophore A23187 (47) (not shown). Finally, the effect of CsA and PD 144795 was specific for the TCR-dependent signal and no effect of these compounds was detected when Sp1/p53 complexes were induced by the incubation of the Jurkat cells with phorbol myristate acetate (PMA) (Fig. 2E).

Previous reports have shown that CsA inhibits the induction by calcium-dependent signals of the DNA binding activity of the transcription factors NF-AT and NF-κB (14, 22, 31–33). An inhibition of NF-κB by PD 144795 could be critical for HIV-1 replication, as NF-κB is a major regulator of HIV-1 LTR-mediated transcription (34, 35). We tested whether or not PD 144795 was able to block the induction by PHA of NF-κB binding to its recognition sequence in the HIV-1 LTR. Nuclear extracts from Jurkat cells, incubated as described above, were assayed by EMSA using an oligonucleotide probe containing the HIV-1 LTR NF-κB II site. A typical result of these experiments is shown in Fig. 3A. Two specific complexes were identified using this probe. The faster mobility complex contained NF-κB/p50/p50 homodimers whereas the slower mobility complex was comprised of NF-κB p50/p65 heterodimers as indicated by supershift with specific anti-NF-κB antibodies (Fig. 3B). Incubation of Jurkat cells with PHA increased p50/p65 binding while a minor effect was observed on p50/p50 homodimers. PD 144795, as well as CsA, inhibited the PHA-induced increase in the levels of p50/p65 heterodimers. No effect of these drugs was observed on the binding of p50/p50 homodimers. The decrease in p50/p65 binding to a basal or lower than basal level was observed in several experiments (not shown). A certain variability in the level of basal p50/p65 binding was observed, probably resulting from activation of NF-κB by growth factors present in the serum. CsA and PD 144795 also inhibited NF-κB p50/p65 binding in CEM lymphocytes when these cells were incubated in the presence of the calcium ionophore A23187 (47) but not in the presence of PMA (not shown).

In summary, these results indicate that CsA and PD 144795 specifically inhibit the activation by PHA of p53 and NF-κB binding to the HIV-1 LTR. Intriguingly, these results suggested that both compounds interfere with a common signal transduction pathway that regulates transcription factors that are critical for the expression of the HIV-1 promoter.

CsA and PD 144795 Inhibit the PHA-induced Transcrip-
tional Activation of the HIV-1 LTR—To test the sensitivity of the p53 and NF-κB-mediated transcriptional activities to CsA and PD 144795 in vitro, transfection experiments were performed in Jurkat T cells using the reporter plasmid −121,+232 HIV-1 LTR CAT that contains the HIV-1 LTR κB II and p53 sites (5). After a 24-h incubation period, transfected cells were aliquoted and incubated with PHA alone or in the presence of CsA or PD 144795 for an additional 48 h. The results of these experiments are shown in Fig. 4A. Incubation of the cells with PHA and CsA or PD 144795 reduced CAT activity to approximately 50% of PHA alone. However, the PMA induction of the transcriptional activity mediated by this promoter fragment was not altered by the incubation with CsA or PD 144795. Consistent with the idea of a common role for PD 144795 and CsA, we observed that the activation of an interleukin-2 reporter plasmid by calcium-dependent signals was inhibited by PD 144795 and CsA (not shown). These results parallel the signal-specific inhibition by CsA and PD 144795 of p53 and NF-κB binding in vitro.

To define the HIV-1 LTR DNA elements that are targeted by PD 144795 and CsA, we tested the effect of these compounds on the activity of NF-κB/p53-E1BTATA CAT, a minimal promoter-
Thus, our results demonstrate that PD 144795 targets the same enzymatic activity as CsA, suggesting that inhibition of calcineurin is a common mechanism of action of both drugs that explains their similar effect on the transcriptional activity of p53 and NF-κB. Current experiments in our laboratory indicate that PD 144795 has additional effects on early events associated with the activation of the TCR. For example, low concentrations of PD 144795 induced a slow and steady increase in the intracellular calcium levels in T lymphocytes and inhibited the PHA-induced rise of intracellular calcium in these cells.3

The effect of PD 144795 on the activity of calcineurin was further investigated by experiments of overexpression of this enzyme in vivo. It has been shown that overexpression of calcineurin in Jurkat cells renders them more resistant to the effects of CsA and FK 506 (48), another calcineurin-inhibitor agent, and augments both NF-AT- and NF-interleukin-2A-dependent transcription (18, 19). We co-transfected −121,+232 HIV-1 LTR CAT with pSRα-ΔCAM, a pSRα expression vector containing a constitutively active form of calcineurin, in Jurkat cells and normal human fibroblasts. Calcineurin induced the transcriptional activity of this HIV-1 LTR fragment in both cell types (Fig. 5C). A higher induction was obtained in fibroblasts (15-fold) than in Jurkat cells (6-fold). Incubation of the cells with PD 144795 inhibited the effect of calcineurin in a dose-response manner. Fibroblasts were more sensitive than Jurkat cells to the inhibition by PD 144795. Half-maximal inhibition of CAT activity by PD 144795 was reached at approximately 0.2 μM in normal human fibroblasts and 1 μM in Jurkat cells. A possible explanation for the cell-type difference in the response to PD 144795 could be the existence of a mutant p53 allele in the Jurkat cells (36). This mutant p53 protein may already display in basal conditions the conformation necessary for the interaction with HIV-1 LTR. Alternatively, Jurkat T cells may carry other genetic alterations that result in a certain degree of constitutive activation of NF-κB or p53. Interestingly, low concentrations of PD 144795 (0.3 μM) induced a moderate activation of −121,+232 HIV-1 LTR CAT in both cell types. This paradoxical effect may result from an indirect activation of endogenous calcineurin by PD 144795 secondary to an increase in the intracellular calcium levels induced by low concentrations of the drug. This effect disappeared at higher concentrations of PD 144795 as it was expected from its inhibitory effect on calcineurin activity in vivo (Fig. 5A). The effect of the low concentration of PD 144795 was not observed in cells transfected with pSRα-ΔCAM. This calcineurin mutant is not affected by alterations in the intracellular calcium levels (18). Finally, since PD 144795 inhibits calcineurin and the transcriptional activity mediated by NF-κB and p53, we investigated whether or not calcineurin is involved in the regulation of the transcriptional activity of NF-κB and p53.

**PD 144795 Inhibits Calcinurin Activity**—Since engagement of the TCR activates HIV-1 LTR-mediated transcription through a calcineurin-dependent pathway (12–14), and since benzothiophene showed similar properties to CsA, we tested the effect of PD 144795 on the activity of calcineurin, the enzymatic activity that is targeted by CsA (16, 17). Incubation of Jurkat cells with PD 144795 resulted in a dose-dependent inhibition of the phosphatase activity of calcineurin (Fig. 5A). PD 144795 did not decrease the activity of the enzyme lactate dehydrogenase, indicating that this compound was not toxic to the cells (Fig. 5A). PD 144795 and CsA also inhibited the activity of calcineurin in Jurkat cell extracts (Fig. 5B). However, they did not affect the activity of the enzyme alkaline phosphatase (Fig. 5B, inset), indicating that the effect of PD 144795 on calcineurin was specific.

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2 A. Gualberto, unpublished data.

3 R. Montaño and F. Sobrino, unpublished data.
regulatory functions of this protein. It has been shown that proliferative forms of p53 may work as positive regulators of cell growth (Refs. 41–43 and references therein). In addition, recent data indicates that certain members of the NF-AT(Rel) family of transcription factors can synergize with NF-xB and Tat in the transcriptional activation of HIV-1 (44). Since NF-AT activity is regulated by calcineurin (31, 40, 45), it is possible that an NF-xB/NF-AT synergistic effect on HIV-1 LTR transcription could be affected by PD 144795. In our experimental conditions, NF-xB was solely accounted for by HIV-1 kb site nucleoprotein complex formation (Fig. 3 and data not shown). However, we cannot discard that a physical or functional interaction between NF-xB and certain NF-AT family members may take place in vivo and could be targeted by PD 144795.

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