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Inhibition of Human Immunodeficiency Virus Type 1 Replication In Vitro by a Novel Combination of Anti-Tat Single-Chain Intrabodies and NF-κB Antagonists

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Human immunodeficiency virus type 1 (HIV-1) Tat, an early regulatory protein that is critical for viral gene expression and replication, transactivates the HIV-1 long terminal repeat (LTR) via its binding to the transactivation response element (*TAR*) and, along with other cellular factors, increases viral transcription initiation and elongation. Tat also superactivates the HIV-1 promoter through a *TAR*-independent mechanism, including tumor necrosis factor alpha-induced and protein kinase C (PKC)-dependent activation of NF-κB, and inhibitors of Tat and NF-κB cooperatively down-regulate this Tat-mediated LTR superactivation. In this study, a combined pharmacologic and genetic strategy using two PKC (NF-κB) inhibitors, pentoxifylline (PTX) and Gö-6976, and a stably expressed anti-Tat single-chain intracellular antibody (sFv intrabody) was employed to obtain cooperative inhibition of both HIV-1 LTR-driven gene expression and HIV-1 replication. Treatment of cells with PTX and Gö-6976 resulted in cooperative inhibition of both HIV-1 LTR-driven gene expression and HIV-1 replication. In addition, the combined use of anti-Tat sFv intrabodies and the two NF-κB inhibitors retained the virus in the latent state for as long as 45 days. The combined treatment resulted in more durable inhibition of HIV-1 replication than was seen with the NF-κB inhibitors alone or the anti-Tat sFv intrabodies alone. Together, these results suggest that in future clinical gene therapy trials, a combined pharmacologic and genetic strategy like the one reported here may improve the survival of transduced cells and prolong clinical benefit.

Human immunodeficiency virus type 1 (HIV-1) is the primary etiologic agent of AIDS, a fatal disease that results from the gradual destruction of the helper T-cell population in infected individuals (21, 23, 28). Tat is a regulatory protein encoded by the HIV-1 genome that plays a unique role in the emergence of the virus from the latent state. Tat is known to be a potent transcriptional activator of the HIV-1 long terminal repeat (LTR) (14, 15, 33, 38, 42). The sequence in the 5′ untranslated region of all HIV RNAs called the transactivation response element (*TAR*) (nucleotides +1 to +44) is required for Tat-mediated transactivation (3, 19, 51, 58). Tat binds with high efficiency to a bulge region in a stable stem-loop structure of the *TAR* RNA and then interacts with a transcription initiation complex (26, 37) composed of Tat and DNA- and RNA-binding proteins (24, 25, 31, 32, 42, 48, 52, 56, 57) to primarily stimulate transcription initiation and to increase transcription elongation (33, 43).

Tat has other modes of action as well. Expression of Tat in human cells in culture leads to transactivation and overexpression of cellular genes encoding cytokines such as tumor necrosis factor alpha (TNF-α), TNF-β, and interleukin-2 (IL-2) (9, 10, 48a, 54, 59), some of which in turn can activate viral transcription through their activation of NF-κB (9, 10, 59). Activation of NF-κB by TNF-α occurs through a protein kinase C (PKC)-mediated pathway (6). NF-κB binding to enhancer elements in the HIV-1 LTR can lead to *TAR*-independent (NF-κB dependent) activation of viral transcription by Tat (7, 35). Superactivation of HIV-1 LTR-driven gene expression is thus induced by the concerted interaction of the cellular transactivator NF-κB and Tat (1, 4, 7, 31, 35). Therefore, due to its multifunctional roles in viral and cytokine gene transactivation, Tat represents an important target for HIV therapy (45).

Intracellular immunization is a form of gene therapy for the treatment of HIV-1 infection and AIDS that is aimed at the stable introduction into susceptible cells of genetic elements that inhibit viral replication, so called resistance genes (2, 26). Intracellular antibodies, or intrabodies, are a recent addition to the field of intracellular immunization-based strategies that are being used to treat HIV-1 infection (11–13, 20, 34, 36, 39, 40, 41, 44, 45, 49, 55). The effects of anti-Tat single-chain (sFv) intrabodies have been examined in stably transfected $CD4^+$ T lymphocytes (44). These studies demonstrated that anti-Tat sFv intrabodies effectively sequestered Tat in the cytoplasm, blocked its transport to the nucleus of the cell, and inhibited the Tat-mediated transactivation of the HIV-1 LTR (44). Moreover, in both stably transfected cells (44) and transduced peripheral blood mononuclear cells (PBMCs) from HIV-1-infected individuals (49), marked inhibition of HIV-1 replication was observed.

Previous studies have also demonstrated that superactivation of HIV-1 LTR-driven gene expression can be inhibited by cooperative inhibition of NF-κB and Tat (4). In these studies, cells were treated with pentoxifylline (PTX), an inhibitor of PKC, PKA, and NF-κB (4, 6, 16, 17), plus Ro24-7429, an inhibitor of Tat (30). However, the cooperative effect of these two inhibitors on HIV-1 replication was not examined. Therefore, the present study was undertaken to examine whether

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combined pharmacologic and genetic therapeutic strategies that are targeted to Tat and are aimed at inhibiting both the *TAR*-dependent and NF-κB-dependent Tat activation pathways simultaneously would result in a more potent inhibition of LTR-driven gene expression and HIV-1 replication than would any single agent or strategy alone. Because activation of NF-κB is known to be mediated through PKC, we tested two PKC inhibitors, PTX and Gö-6976, a specific inhibitor of PKC that has been previously shown to have anti-HIV-1 activity (48), and evaluated their effects alone and in combination on NF-κB activation and HIV-1 LTR-driven gene expression. The effects of PTX and Gö-6976 on HIV-1 replication were also examined in cells stably expressing anti-Tat sFv intrabodies. In this report, we demonstrate that treatment of cells with PTX and Gö-6976 results in cooperative inhibition of both HIV-1 LTR-driven gene expression and HIV-1 replication. In addition, the combined use of anti-Tat sFv intrabodies and the two NF-κB inhibitors resulted in more durable inhibition of HIV-1 replication than was seen with the NF-κB inhibitors alone or the anti-Tat sFv intrabodies alone.

MATERIALS AND METHODS

Cells. The human $CD4^+$ T-lymphocyte cell lines SupT1 (SupT) and Jurkat were cultured in RPMI 1640 medium (Gibco/BRL, Gaithersburg, Md.) with glutamine (2 mM) supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2 and 95% air. SupT cells stably transfected with pRc/CMV vector (Invitrogen) (SupT-vector cells) or constitutively expressing the anti-HIV-1 Tat sFv intrabody termed sFvtat1Ck (SupT-sFvtat1Ck cells) were previously described (44) and were propagated in RPMI 1640 medium with G418 (Sigma Chemical Company, St. Louis, Mo.) at 500 μg/ml.

PBMCs were isolated from leukopacks (obtained from the Dana-Farber Cancer Institute Blood Bank) by Ficoll-Hypaque density gradient centrifugation. The $CD4^+$ mononuclear cells were selected from the total PBMC population with anti-CD4-coated cell culture flasks (GenCell, Santa Clara, Calif.). A total of 20×10^6 PBMCs were resuspended in 4 ml of Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS/CMF)–1 mM EDTA–0.5% human gamma globulins (Sigma) and incubated on the flask for 1 h at room temperature. The flask was washed five times with PBS/CMF–1 mM EDTA. After the final wash, 10 ml of RPMI 1640 medium supplemented with 10% fetal calf serum and phytohemagglutinin (PHA) (1 μg/ml) was added to the flask, which was then incubated for circa 60 h at 37°C with 5% CO_2.

Reagents and plasmids. PTX [1-(5′-oxohexyl)-3,7-dimethyl xanthine] and Gö-6976 (a synthetic nonglycosidic indolocarbazole) were gifts from Hoechst-Roussel Pharmaceutical, Somerville, N.J., and Parke-Davis Company, Ann Arbor, Mich., respectively. DEAE-dextran, geneticin (G418), and PHA were obtained from Sigma. Acetyl coenzyme A was purchased from Boehringer Mannheim, and TNF-α was obtained from Endogen, Cambridge, Mass. pHIV-1 LTR-CAT was obtained from G. Nabel, Howard Hughes Medical Institute, University of Michigan Medical Center, Ann Arbor; expression plasmid pSVTat was obtained from J. Sodroski, Dana-Farber Cancer Institute, Boston, Mass.

Transfection experiments. Transient transfection of Jurkat cells was performed by the DEAE-dextran procedure as previously described (7, 44). Cells were transfected with either 5 μg of plasmid pHIV-1 LTR-CAT alone or, for cotransfection experiments, 2 μg each of plasmids pHIV-1 LTR-CAT and pSVTat. The cells were grown in media without or with different concentrations of PTX (10 to 300 μM) or Gö-6976 (10 to 300 nM) and were used either singly or in combination. Approximately 48 h after transfection with pHIV-1 LTR-CAT, the cells were stimulated with TNF-α or phorbol myristate acetate (PMA); 18 h later, chloramphenicol acetyltransferase (CAT) activity in cell extracts was determined as described previously (4, 5). In the pSVTat/pHIV-1 LTR-CAT cotransfection experiments, CAT assays were performed 60 h posttransfection.

In all of the transfection studies, control plasmid pSV-*lacZ* was routinely cotransfected with the specific HIV-1 LTR-CAT plasmid to normalize the transfection efficiency. The levels of β-galactosidase activity in control and drug-treated cells were the same, suggesting that the drugs did not influence another viral promoter that lacks the classic NF-κB motif and were not toxic to the cells. Furthermore, separate experiments demonstrated that Gö-6976, like PTX (5), did not inhibit the NF-κB-lacking Rous sarcoma virus promoter-driven reporter CAT gene expression (Fig. 1E).

EMSA. The level of active NF-κB in the nuclear extracts of control and treated cells was determined by electophoretic mobility shift assay (EMSA) (18). A ^{32}P-labeled double-stranded oligonucleotide probe encoding the NF-κB motifs was used for EMSA as described previously (4, 5).

HIV-1 challenge experiments. Stably transfected SupT-vector and SupT-sFvtat1Ck cells (10^6) were incubated overnight in the absence or presence of PTX (300 μM, final concentration) and/or Gö-6976 (300 nM, final concentration) and then infected with either the laboratory HIV-1 strain III-B or a syncytium-inducing (SI) European primary HIV-1 isolate (obtained from Jan Albert, Karolinska Institute, Stockholm, Sweden) at a multiplicity of infection (MOI) of 0.5. Six hours after challenge, the cells were thoroughly washed and fresh medium (without or with appropriate drugs) was added. Supernatants were then harvested on alternate days with resuspension of the cells in the corresponding RPMI 1640 medium (without or with appropriate drugs). Viral particles in collected supernatants were analyzed by using a radioimmunoassay kit for HIV-1 $p24^{gag}$ protein (DuPont) according to the manufacturer's instructions (44).

PBMCs were activated with either PHA (4 μg/ml; Sigma) or OKT3 (10 ng/ml), and the cells were cultured continuously in RPMI 1640 medium plus IL-2 (20 U/ml) without or with the NF-κB inhibitors. After 3 days of incubation at 37°C, the cells were challenged with either of two SI European primary HIV-1 isolates. Supernatants were collected every third day and assayed for p24 antigen.

Transduction of PBMCs and $CD4^+$ mononuclear cells and HIV-1 challenge. The sFvtat1Ck intrabody gene was cloned into the shuttle vector LNCX MuLV under the control of the cytomegalovirus immediate-early promoter (46). The empty LNCX (LN) or LN-sFvtat1Ck vector was transfected by the calcium phosphate method into the ecotropic cell line ϕCRE (46). After 48 h, the supernatants from the transfected cells were collected, cleared by low-speed centrifugation ($3,000 \times g$), and used to infect the amphotrophic packaging cell line PG13. Producer cell lines were established by using G418 and hypoxanthine-aminopterin-thymidine selection. Following establishment of stable PG13 subclones by limiting dilution, retrovirus-containing supernatants were harvested, filtered, and used to transduce PBMCs. Transduction was performed on 3 consecutive days with an initial 6 h of phosphate starvation, followed by a 1-h centrifugation of the cells and retroviral supernatants ($1,000 \times g$, 32°C) and overnight incubation at 32°C (8). The cells were then washed with fresh medium, resuspended in medium supplemented with IL-2 (20 U/ml) and G418 (800 μg/ml), and incubated at 37°C. The medium was changed on day 5. After 10 days in culture, the cell were washed and stimulated with irradiated human PBMCs (5,000 rads) at a ratio of 1:10 (transduced cells/feeders) and PHA (1 μg/ml). Four days later, the cells were fed with medium supplemented with IL-2 (20 U/ml) and G418 (800 μg/ml). When cells started to expand (circa days 10 to 14), they were washed, equilibrated in medium alone or with PTX (300 μM) plus Gö-6976 (300 nM), and then challenged with either of two SI European primary HIV-1 isolates of HIV-1. Viral replication was monitored by the p24 antigen capture assay as described earlier.

Transduction efficiency of the PBMCs was determined by PCR amplification with appropriate primers of the neomycin gene as described by Morgan et al. (47). Twenty-four hours after the last transduction, 20,000 cells were collected and PCR band intensity was compared to the intensity of a quantitation curve. The transduction efficiency was estimated to be around 10 to 15%.

RESULTS

Activation of the HIV-1 LTR by PMA and Tat. Basal reporter CAT activity, using our standard assay conditions and the wild-type, unmutated plasmid pHIV-1 LTR-CAT, was stimulated 23-fold by PMA (Fig. 1A). A markedly greater induction of CAT was observed with Tat (259-fold) compared to the unstimulated wild-type HIV promoter construct (1.5-fold). Tat induced an 18-fold up-regulation of the *TAR*-less HIV-1 promoter with functional NF-κB motifs compared to the basal level of activation seen in unstimulated cells (0.76-fold), whereas PMA induced a 25-fold upregulation of this *TAR*-less promoter. The NF-κB mutant HIV-1 promoter was not activated by PMA compared to the activity seen with unstimulated cells; however, a 25-fold level of activation was seen with Tat. These results demonstrate that in Jurkat cells, PMA-induced stimulation of CAT activity is mediated through activation of NF-κB in the HIV-1 promoter, whereas with Tat, stimulation of the HIV-1 promoter is through both *TAR*-dependent and *TAR*-independent pathways. Furthermore, the level of Tat-induced activation of the wild-type HIV-1 LTR is greater than the level of Tat-induced activation of the *TAR*-less and NF-κB mutant HIV-1 LTR promoters alone, suggesting that Tat induces a superactivation of the wild-type promoter through the combined effects of Tat on both the *TAR*-dependent and *TAR*-independent activation pathways.

Effect of Gö-6976 on Tat-, PMA-, and TNF-α-induced activation of the HIV-1 LTR. Gö-6976 is a synthetic nonglycosidic indolocarbazole and a selective inhibitor of PKC (50). Previous studies have demonstrated that Gö-6976 at noncytotoxic con-

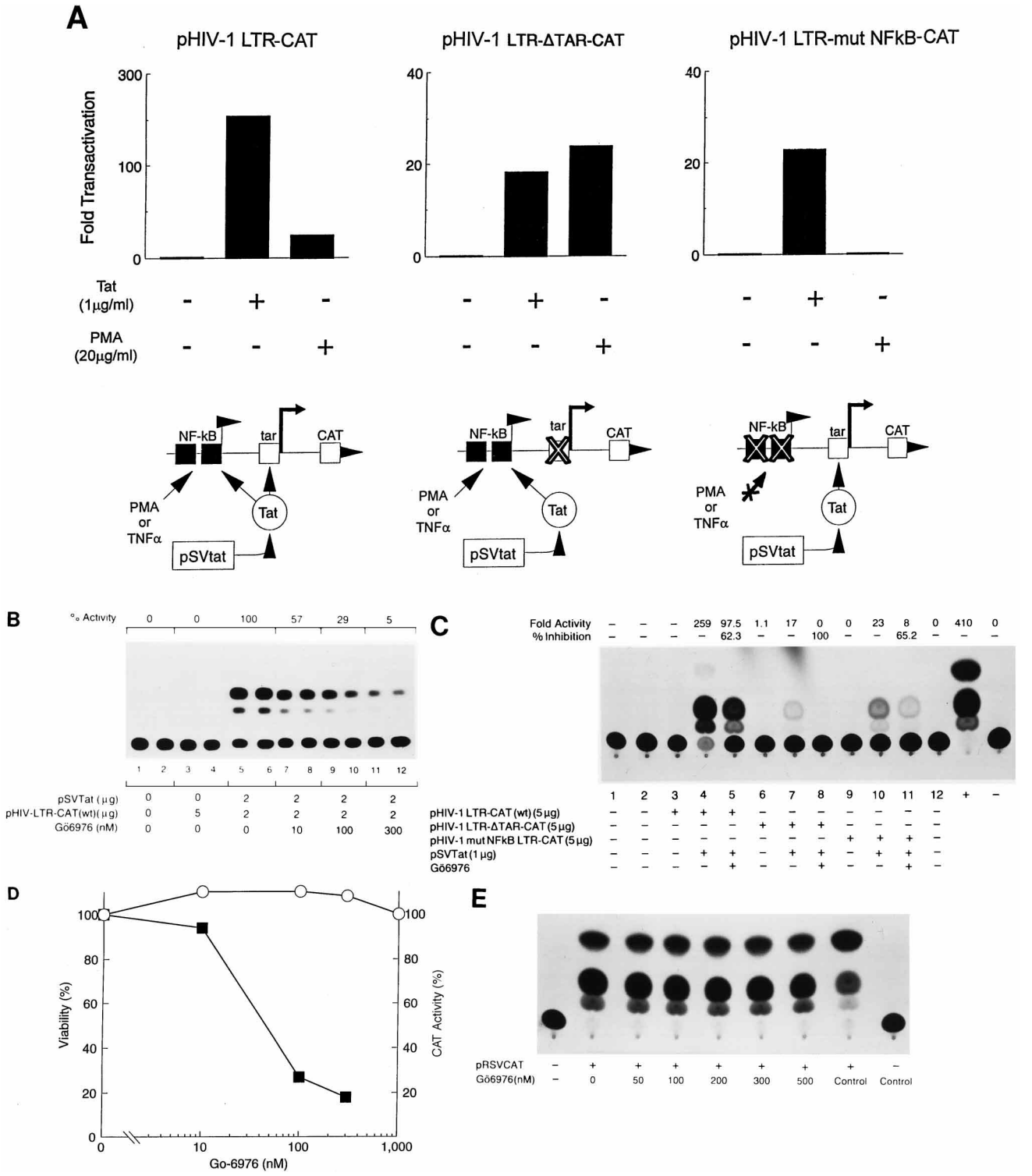

FIG. 1. (A) Tat or PMA-induced CAT expression in Jurkat cells transfected with plasmid pHIV-1 LTR-CAT, pHIV-1 LTRΔTAR-CAT, or pHIV-1 LTR-mut NFκB-CAT. (B) CAT expression in Jurkat cells untransfected (lanes 1 and 2), transfected with 5 μg of pHIV-1 LTR CAT (lanes 3 and 4), or cotransfected with 2 μg of pHIV-1 LTR-CAT and 2 μg of pSVTat in the absence (lanes 5 and 6) or presence (lanes 7 to 12) of increasing concentrations (0 to 300 nM) of Gö-6976. Transfections were done by the DEAE-dextran method (44). Cell lysates of untreated and Gö-6976-treated cells were made 48 h posttransfection and analyzed for CAT expression. wt, wild type. (C) Influence of Gö-6976 on activation of the HIV-1 promoter by Tat via the TAR- and NF-κB-dependent, TAR (alone)-dependent, or NF-κB (alone)-dependent activation pathway. Jurkat cells were transfected with the different LTR plasmids by electroporation (Bio-Rad Gene Pulser; 260 Volts; 960 μF). Jurkat cells were then resuspended in RPMI 1640 medium (with or without Gö-6976 [300 nM]). Cell extracts were prepared 60 to 72 h after transfection and analyzed for CAT activity. (D) Percent viability (○) and level of CAT expression (■) in TNF-α-treated Jurkat cells. Cells (10^6/10 ml) were grown in Gö-6976-equilibrated RPMI 1640 medium (Gö-6976 concentration ranging from 0 to 300 nM) and transfected with 10 μg of pHIV1 LTR-CAT plasmid by the DEAE-dextran method. The transfected cells were grown for an additional 48 h and then stimulated with TNF-α (10 ng/ml). The cells were incubated for another 18 h and then analyzed for percent viability and CAT expression. (E) CAT expression in Jurkat cells either untransfected or transfected with plasmid pRSV-CAT (5 μg) in the absence or presence of increasing final concentrations of Gö-6976 (0 to 500 nM).

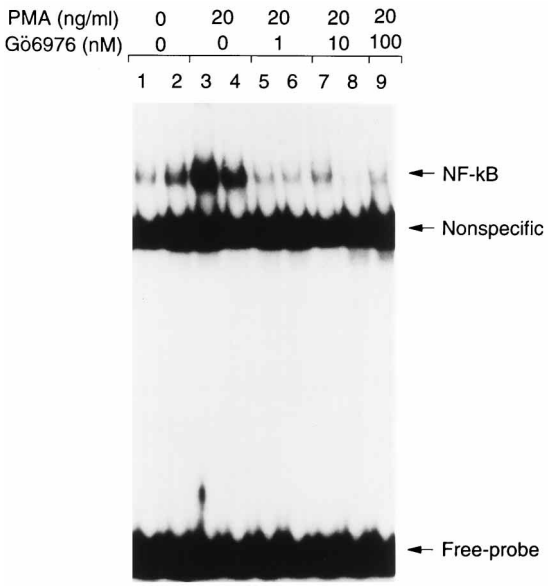

FIG. 2. PMA stimulation of NF-κB binding in the absence and presence of increasing concentrations of Gö-6976. Jurkat cells were stimulated for 6 h with PMA (20 ng/ml) in the absence (lanes 1 and 2) or presence of PMA (20 ng/ml) without (lanes 3 and 4) or with Gö-6976 (lanes 5 and 6, 1 nM; lanes 7 and 8, 10 nM; lane 9, 100 nM [final concentration]). Nuclear extracts were prepared as described elsewhere (18). Autoradiographic signals of active NF-κB binding to ^{32}P-labeled oligonucleotide in nuclear extracts of PMA-treated cells are shown.

centrations can inhibit HIV-1 replication from latent or low-level virus-producing cells by physiologic activators such as TNF-α and IL-6 (50). We examined the influence of Gö-6976 on HIV-1 LTR-driven reporter gene expression in CD4$^+$ Jurkat T cells in the presence or absence of Tat protein. As shown in Fig. 1B, little activation of the HIV-1 LTR is seen in the absence of Tat (lanes 3 and 4) compared to the activation seen in the presence of Tat (lanes 5 and 6). In the presence of increasing concentrations of Gö-6976 (lanes 7 through 12), a dose-dependent inhibition of Tat-mediated transactivation of the HIV-1 LTR is seen, with maximal inhibition occurring at 300 nM Gö-6976.

The effect of Gö-6976 on Tat and PMA-induced activation of NF-κB was studied by examining the level of HIV-1 LTR-driven reporter CAT gene activity in Jurkat cells transiently transfected with the different pHIV-1 LTR-CAT reporter plasmids. As shown in Fig. 1C, with the wild-type HIV-1 LTR promoter, Gö-6976 inhibited Tat-induced transactivation by 62.3%. With the *TAR*-less HIV-1 promoter, where the effect of Tat is mediated through activation of NF-κB alone, Gö-6976 inhibited Tat-induced transactivation completely. Using the pHIV-1 LTR-mut-NF-κB-CAT reporter in which the NF-κB motifs are mutated and in which only *TAR*-dependent LTR activation is measured, Gö-6976 inhibited 65.2% of Tat-induced transactivation of the mutant LTR. Thus, Gö-6976 inhibited the Tat-mediated *TAR*-independent LTR activation pathway. In parallel, transfected cells were stimulated with PMA for 12 h (20 ng/ml). Gö-6976 treatment inhibited PMA-induced transactivation of wild-type, TAR-less, and mutant NF-κB LTRs by 97, 98, and 0%, respectively (data not shown).

The effect of Gö-6976 on the TNF-α-induced activation of NF-κB was also studied by examining the level of wild-type HIV-1 LTR-driven reporter CAT gene activity in Jurkat cells treated with TNF-α and transiently transfected with plasmid pHIV-1 LTR-CAT. As shown in Fig. 1D, Gö-6976 also inhibited HIV-1 LTR activation by TNF-α with a dose-response curve similar to that seen with Tat protein (Fig. 1B). In addition, Gö-6976 was not cytotoxic at the indicated concentrations (Fig. 1D) and had no effect on Rous sarcoma virus promoter-driven CAT expression (Fig. 1E).

Effect of Gö-6976 on PMA-induced NF-κB activation of the HIV-1 LTR. To investigate the effects of Gö-6976 on NF-κB activation directly, the level of active NF-κB was determined by EMSA with nuclear extract from cells stimulated with PMA and treated with different concentrations of Gö-6976. As shown in Fig. 2, the level of active NF-κB was reduced to the unstimulated level following treatment of the stimulated cells with Gö-6976 at as low as 1 nM (final concentration). These results demonstrate that Gö-6976 is a potent NF-κB inhibitor and establish a site of antiviral action of the compound. In this respect, Gö-6976 is similar to PTX, which was previously established as an NF-κB inhibitor and which also inhibited HIV-1 LTR activation and virus replication (5, 22).

Effects of Gö-6976 and the Tat inhibitor Ro24-7429 in combination on HIV-1 LTR activation. Previous studies demonstrated that treatment of cells with PTX in combination with Ro24-7429, an inhibitor of Tat function, resulted in cooperative inhibition of HIV-1 LTR activation (4). The mechanism of inhibition by Ro24-7429 is not known (30). Therefore, the combined effect of Gö-6976 and Ro24-7429 on HIV-1 LTR-driven CAT gene expression was examined. Jurkat cells were transiently cotransfected with pHIV-1 LTR-CAT and pSVTat and in the presence of different combinations of Gö-6976

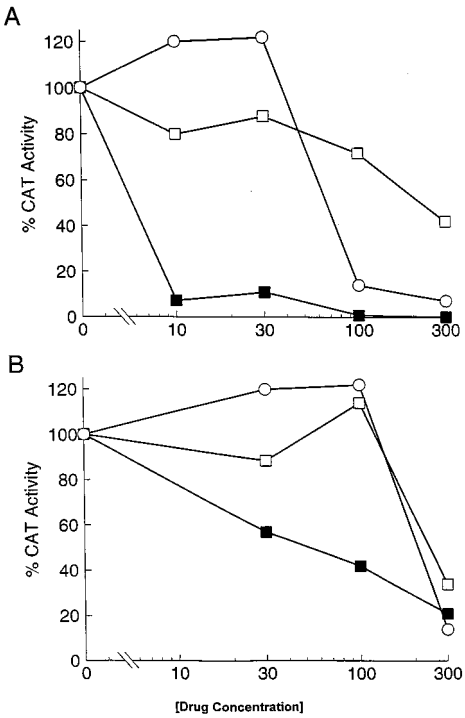

FIG. 3. Effects of Gö-6976 alone and in combination with Ro24-7429 (A) or PTX (B) on HIV-1 LTR-driven reporter gene expression. Jurkat cells were either untransfected or cotransfected with an equimolar concentrations of pHIV-1 LTR CAT and pSVTat (2 μg of each), using the DEAE-dextran method. Cells were grown in either the absence or presence of the test drug for 48 h prior to harvesting of cell lysates. (A) Percent CAT activity in the presence of increasing concentrations of either Ro24-7429 (nanomolar; ○), Gö-6976 (nanomolar; □), or Ro24-7429 plus Gö-6976 (■). (B) Percent CAT activity in the presence of increasing concentrations of either PTX (micromolar; ○), Gö-6976 (nanomolar; □), or PTX plus Gö-6976 (■).

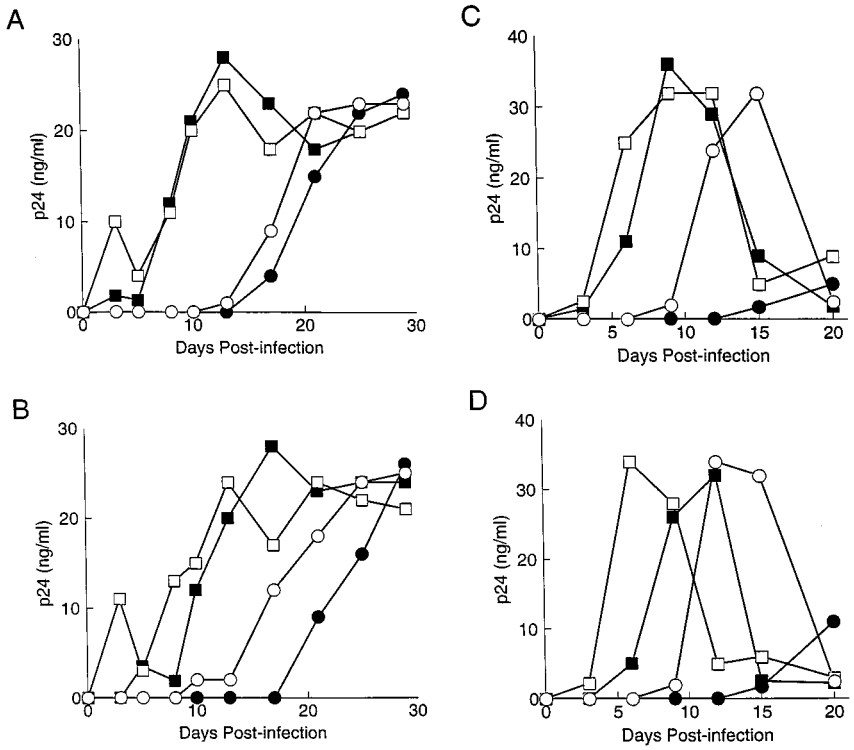

FIG. 4. Effects of NF-κB inhibitors on HIV-1 infection in human CD4$^+$ SupT cells (A and B) and PBMCs (C and D). (A and B) SupT cells (10^6) were either grown in untreated RPMI 1640 medium (□) or equilibrated in 300 μM PTX (■), 300 nM Gö-6976 (○), or 300 μM PTX plus 300 nM Gö-6976 (●). Cells were infected (MOI of 0.5) with either HIV-1_{III-B} (A) or primary isolate 1 (B), and cell-free p24 levels were recorded on alternate days. (C and D) PBMCs were stimulated with OKT3 (10 ng/ml) and were either grown in RPMI 1640 medium supplemented with IL-2 (□) or additionally equilibrated in 300 μM PTX (■), 300 nM Gö-6976 (○), or 300 M PTX plus 300 nM Gö-6976 (●). Cells were infected (MOI of 0.5) with primary isolate 1 (C) or 2 (D), and cell-free p24 levels were recorded on alternate days.

and/or Ro24-7429. As shown in Fig. 3A, significant inhibition of CAT gene activity is seen. Quantitative analysis of the CAT assay demonstrated that markedly lower concentrations of the compounds (10 nM Gö-6976 and Ro24-7429) which individually did not cause significant inhibition led to 93% reduction of CAT gene activity when used in combination. These results demonstrate that the NF-κB inhibitor Gö-6976 and the Tat inhibitor Ro24-7429 in combination cooperatively down-regulate Tat-mediated activation of the HIV-1 promoter.

Effect of two NF-κB inhibitors in combination on HIV-1 LTR activation. The combined effect of Gö-6976 and PTX on HIV-1 LTR-driven CAT gene expression was also examined. As seen in Fig. 3B, with Jurkat cells transiently cotransfected with pHIV-1 LTR-CAT and pSVTat and in the presence of different concentrations of PTX and/or Gö-6976, significant inhibition of CAT gene activity is observed. Quantitative analysis of the CAT assay demonstrated that lower concentrations of the compounds (10 to 50 μM PTX or 10 to 50 nM Gö-6976 which individually did not cause significant inhibition led to 50 to 70% reduction of CAT gene activity when used in combination. These results demonstrate that when used in combination, the two NF-κB inhibitors PTX and Gö-6976 also cooperatively down-regulate, in a *TAR*-independent pathway, Tat-mediated activation of the HIV-1 promoter.

Inhibition of HIV-1 infection in CD4$^+$ SupT cells and PBMCs in the presence of NF-κB inhibitors. To determine whether PTX and Gö-6976 alone and in combination were able to inhibit HIV-1 replication, we initially performed challenge experiments using human CD4$^+$ SupT cells and either a laboratory strain (III-B) or an SI European primary HIV-1 isolate. The cells were pretreated with PTX, Gö-6976, or both drugs overnight prior to challenge with the different viruses. Supernatants were isolated on alternate days, the cells were monitored for syncytium formation, and supernatants were quantitated for p24 levels. Cells treated with Gö-6976 alone gave marked protection against HIV-1 whether the cells were challenged with HIV-1_{III-B} (Fig. 4A) or with primary isolate 1 (Fig. 4B). This protection was not seen with PTX alone in either challenge experiment. The combination of PTX and Gö-6976 resulted in a small additional protective effect on cells challenged with primary isolate 1.

Next, we performed similar HIV-1 challenge experiments using PBMCs and two SI European primary isolates. Cells treated with Gö-6976 alone gave substantial protection against primary isolates 1 (Fig. 4C) and 2 (Fig. 4D). This level of protection was not seen with PTX alone. However, in contrast to the results of the challenge experiments with SupT cells (Fig. 4A and B), the combination of PTX and Gö-6976 resulted in a significant improvement in protection compared to cells treated with Gö-6976 alone. This pattern of inhibition of virus replication by the combination of NF-κB inhibitors correlated well with their effects on HIV-1 LTR-regulated gene expression (Fig. 3B).

Prolonged inhibition of HIV-1 infection in stably transfected CD4$^+$ SupT cells and in transduced PBMCs and CD4$^+$ mononuclear cells expressing Anti-Tat sFv intrabodies in the presence of NF-κB inhibitors. Since treatment of cells with the combination of NF-κB and Tat inhibitors results in cooperative down-regulation of Tat-mediated activation of the HIV-1 promoter (Fig. 3A and references 6 and 7), these observations suggested that a combined genetic and pharmacologic treatment strategy might result in a more durable inhibition of HIV-1 replication. In previous studies, we demonstrated that

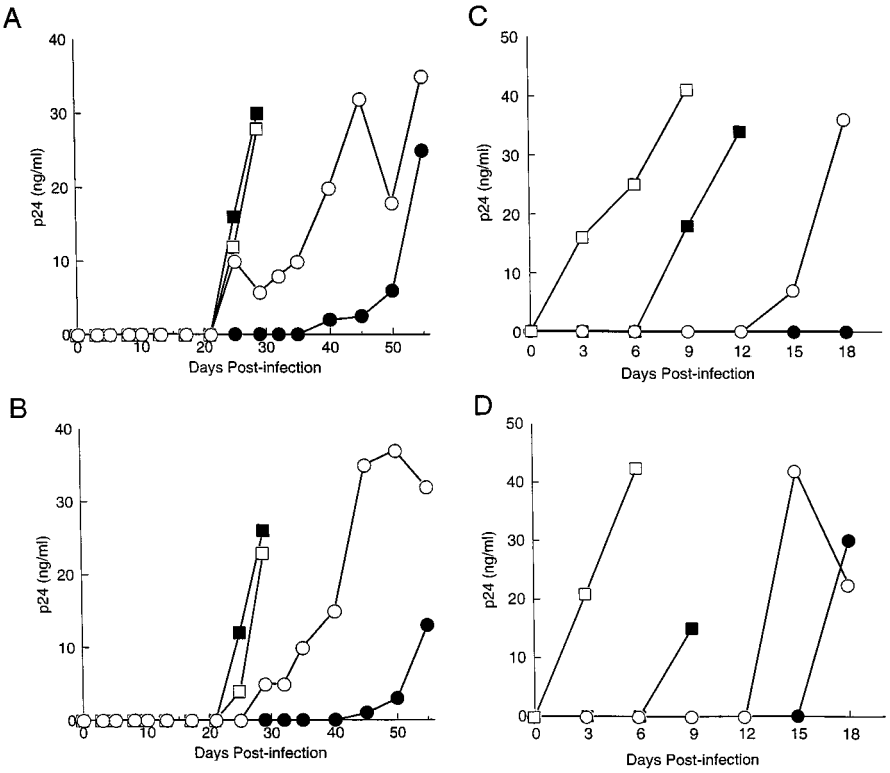

FIG. 5. Effects of NF-κB inhibitors on HIV-1 infection in stably transfected CD4$^+$ SupT cells (A and B) or transduced PBMCs (C and D) expressing anti-Tat sFv intrabodies. (A and B). SupT-sFvtat1Ck cells (10^6) were either grown in untreated RPMI 1640 medium (□) or equilibrated in 300 μM PTX (■), or 300 nM Gö-6976 (○), or 300 μM PTX plus 300 nM Gö-6976 (●). Cells were infected (MOI of 0.5) with either HIV-1_{III-B} (A) or primary isolate 1 (B), and cell-free p24 levels were recorded on alternate days. (C and D) Transduced PBMCs (C) or CD4$^+$ mononuclear cells (D) (squares, LN-vector supernatants; circles, LN-sFvtat1Ck supernatants) were either grown in RPMI 1640 medium supplemented with IL-2 (open symbols) or equilibrated in IL-2–RPMI 1640 medium with 300 μM PTX plus 300 nM Gö-6976 (closed symbols). Cells were infected (MOI of 0.3) with SI primary isolate 2 (C) or 1 (D), and cell-free p24 levels were recorded on alternate days.

an anti-Tat sFv intrabody, termed sFvtat1Ck, which is directed against the N-terminal activation domain of Tat protein could protect cells from HIV-1 challenge when stably expressed in human CD4$^+$ T cells (44). Accordingly, the influence of these two NF-κB inhibitors on HIV-1 infection in anti-Tat sFv intrabody-expressing cells was examined. Stable SupT-sFvtat1Ck and control SupT-vector cells were challenged with either HIV-1_{III-B} or SI primary isolate 1 (Fig. 5A or B, respectively). With the control SupT-vector-infected cells, large number of syncytia started appearing on days 4 to 5, and p24 release had peaked on day 12 (data not shown). Syncytium formation was delayed in the anti-Tat sFv intrabody-expressing cells in the absence of drugs until around day 20 postinfection, and subsequently a significant increase in p24 level was observed in both laboratory strain- and primary isolate-infected SupT-sFvtat1Ck cells at around day 25. In the presence of PTX alone (300 μM), p24 levels were similar to those in the untreated infected cells. In contrast, cells treated with Gö-6976 either alone (300 nM) or in combination with PTX (300 μM) showed significantly fewer syncytia or no syncytia until day 30 or 40, respectively (data not shown). After around 25 to 30 days, the SupT-sFvtat1Ck cells that were grown in Gö-6976-containing medium start to show increasing levels of p24, whereas cells grown in PTX-plus-Gö-6976 medium showed protection against viral infection for up to 45 days postinfection (Fig. 5B). Thus, addition of the two NF-κB inhibitors to the anti-Tat sFv intrabody-expressing cells substantially prolonged the protection against HIV-1 challenge seen with the anti-Tat sFv intrabody alone.

Two additional HIV-1 challenge experiments were performed on PBMCs and purified CD4$^+$ mononuclear cells that were transduced with an empty LN retroviral vector or with the same vector containing the sFvtat1Ck gene under the control of an internal cytomegalovirus immediate-early promoter. After G418 selection, the bulk-transduced PBMCs were challenged with SI primary isolate 2 in the absence or presence of PTX (300 μM) plus Gö-6976 (300 nM). As shown in Fig. 5C, vector-transduced PBMCs were significantly protected from HIV-1 in the presence of PTX plus Gö-6976 compared to the level of p24 seen in the absence of drugs, where cell-free p24 was first observed 3 days postinfection. Likewise, the sFvtat1Ck-transduced PBMCs were significantly protected from HIV-1 compared to the level of p24 seen in the vector transduced PBMCs. Moreover, for PBMCs that were transduced with the sFvtat1Ck gene and treated with the two NF-κB inhibitors, no cell-free p24 was detected for the entire 18-day experiment. As shown in Fig. 5D, a similar protective effect of the two NF-κB inhibitors on the vector- and sFvtat1Ck-transduced CD4$^+$ mononuclear cells that were challenged with SI primary isolate 1 was observed. Taken together, these studies demonstrate that the combination of anti-Tat sFv intrabodies and NF-κB inhibitors can prolong the antiviral action of either class of these agents alone.

DISCUSSION

The results of these experiments demonstrate that the PKC inhibitors PTX and Gö-6976 act cooperatively to inhibit Tat-

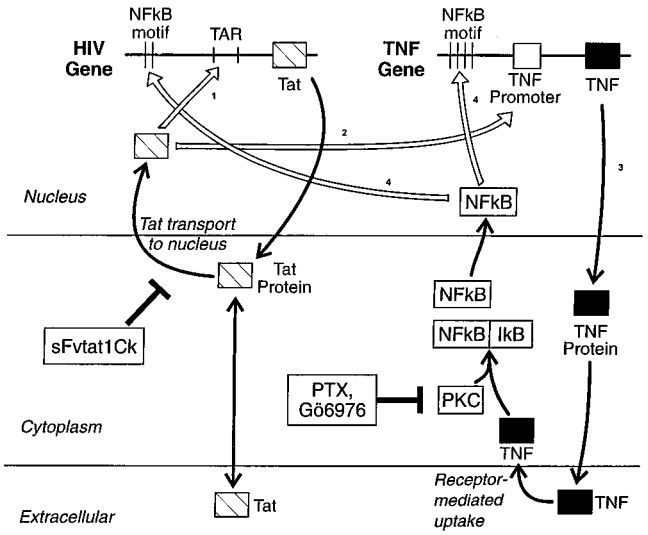

FIG. 6. Schematic description of Tat- and NF-κB-induced activation of the HIV-1 promoter. Steps of the *TAR*-dependent and *TAR*-independent pathways of Tat action in superactivating the HIV-1 promoter function are indicated by numerals. The HIV-1 LTR sequence is shown at the top left, and the TNF promoter is shown on the right. Steps: 1, production of Tat protein from the *tat* gene and its activation of the HIV-1 promoter via binding to *TAR*; 2, up-regulation of the TNF promoter by intracellular Tat protein or by uptake of Tat protein released from HIV-1-infected cells; 3, elevation of TNF mRNA levels, translation, cellular secretion, and receptor-mediated uptake of TNF, and PKC-mediated release and nuclear translocation of active NF-κB; 4, interaction of NF-κB with its motifs in the LTR and TNF promoter, leading to Tat/NF-κB-mediated superactivation of the HIV-1 promoter. The anti-Tat sFv intrabody sFvTat1Ck blocks nuclear translocation of Tat, and the PKC inhibitors PTX and Gö-6976 block release of active NF-κB.

mediated HIV-1 LTR transactivation by exerting their combined effects on the *TAR*-independent LTR activation pathway which is mediated through NF-κB. This inhibitory effect is also seen in HIV-1 challenge experiments, where the protection against HIV-1 was more durable in the presence of the combined PKC inhibitors than in the presence of either inhibitor alone. Moreover, when these agents were evaluated together with either anti-Tat sFv intrabody-expressing stably transfected $CD4^+$ T cells and transduced PBMCs and $CD4^+$ mononuclear cells, a even greater inhibition of HIV-1 replication was observed. Thus, these results support the idea that a combined pharmacologic and genetic strategy that blocks simultaneously both the *TAR*-dependent and NF-κB-dependent Tat activation pathways may be of value in the clinical setting.

The mechanisms by which Tat mediates LTR transactivation are complex. Previous studies and the data presented here demonstrate that Tat can activate the HIV-1 LTR promoter through both TAR-dependent and NF-κB-dependent pathways (7). Tat has been shown to transactivate several cytokine genes (9, 10, 48a). Tat-mediated transactivation of the TNF-α promoter occurs through activation of NF-κB, which in turn activates the HIV-1 LTR promoter in a *TAR*-independent manner. Activation of NF-κB also results in transactivation of TNF-α and other cytokine promoters and thereby establishes an auto-up-regulatory loop for superactivation of the HIV-1 LTR promoter (Fig. 6 and reference 7).

The cooperative effect of PTX and Gö-6976 on LTR-driven gene expression and HIV-1 replication was unexpected. Previous studies have demonstrated that treatment of Jurkat cells with PTX results in inhibition of both PKC- and PKA-induced activation of NF-κB and that both TNF-α- and PMA-induced activations of NF-κB are catalyzed predominantly by PKC (6). Likewise, Gö-6976 is a selective inhibitor of PKC and has been demonstrated to be a potent inhibitor of TNF-α-induced activation of viral transcription from latent or low-level virus-producing chronically infected cells (50). The present study demonstrates that like PTX, Gö-6976 is a potent inhibitor of NF-κB (Fig. 2) (5). Thus, although these drugs have overlapping sites of action on PKC and NF-κB, they appear to have an additive, if not cooperative, effect as inhibitors of both HIV-1 LTR-driven gene expression and virus replication. Further studies will be required to determine if other cellular kinases with nonoverlapping sites of action are inhibited by these two agents.

A previous study demonstrated that superactivation of HIV-1 LTR-driven gene expression can be inhibited by cooperative inhibition of NF-κB and Tat (4). In this study, treatment of cells with PTX and Ro24-7429 resulted in inhibition of HIV-1 LTR-regulated gene expression, and the inhibitory effect of these two drugs in combination, at concentrations that alone did not significantly influence viral promoter activity, was far more than additive. In the present study, a cooperative inhibition of Gö-6976 and Ro24-7429 on superactivation of LTR-driven gene expression was also observed (Fig. 3B). As with the PTX–Ro24-7429 study, the data presented here demonstrate that the inhibitory effect of Gö-6976 and Ro24-7429 in combination, at concentrations that alone did not significantly influence viral promoter activity, was far more than additive.

The results of the combined pharmacologic approaches to simultaneously target both NF-κB and Tat were extended to evaluate a combined pharmacologic and genetic strategy that targets both NF-κB and Tat. Indeed, when these two PKC inhibitors were evaluated together with anti-Tat sFv intrabody-expressing cells, either stably transfected $CD4^+$ T cells or transduced PBMCs, a greater inhibition of HIV-1 replication was observed than with either the combined PKC inhibitors alone or the anti-tat sFv intrabody-expressing cells alone. In addition, although HIV-1 infection eventually spread in the cell culture in these studies, these experiments were performed at high MOIs (0.5). Therefore, this novel combination of anti-Tat sFv intrabody and NF-κB inhibitors may have even greater therapeutic value against HIV-1 in vivo where exposure of $CD4^+$ cells to low MOIs may occur.

Interestingly, immune hyperactivation of HIV-1-infected primary T-cells via CD3 and CD28 receptors has been demonstrated, and expression of Tat protein recapitulates this phenotype and is associated with increased IL-2 secretion in response to costimulation with CD3 plus CD28 (48a). Since the anti-Tat sFv intrabodies are not secreted or released from the cells (data not shown), it is possible that the intracellular antibodies can inhibit both the induction of IL-2 secretion following T-cell activation via the CD3 and CD28 receptors and the release of Tat into the cultures that can result in Tat-mediated transcellular transactivation of cytokines such as TNF-α (29). Therefore, the inhibitory effects of the anti-Tat sFv intrabodies could result in the combined down-regulation of immune hyperactivation of HIV-1-infected T cells and in the auto-up-regulatory loop for Tat-mediated superactivation of the HIV-1 LTR promoter and the transcellular transactivation of cytokine genes in neighboring noninfected cells (Fig. 6).

In summary, these studies demonstrate that inhibitors of NF-κB and Tat in combination result in a more profound and durable inhibition of HIV-1 replication than inhibitors of either NF-κB or Tat alone. Such combination of pharmacologic and genetic approaches may enhance the efficacy and/or reduce the toxic effect of individual molecules; they may also minimize or retard the emergence of drug-resistant or escape

variants of HIV-1. In future clinical gene therapy trials that target the Tat protein, the addition of a pharmacologic arm, particularly when directed to inhibition of NF-κB, may improve the survival of the transduced cells and prolong the clinical benefit that is seen.

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