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A platelet-activating factor antagonist, RP 55778, inhibits cytokine-dependent induction of human immunodeficiency virus expression in chronically infected promonocytic cells

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ABSTRACT **A platelet-activating factor antagonist, RP 55778, potently suppressed the induction of human immunodeficiency virus (HIV) expression in chronically infected promonocytic U1 cells. RP 55778 inhibited the production of reverse transcriptase activity in U1 cells stimulated with the transcriptionally active inducers of virus production, tumor necrosis factor α and phorbol 12-myristate 13-acetate. This effect was correlated only in part with a reduction in the levels of HIV RNA, suggesting that this agent was also affecting posttranscriptional levels of virus production. In this regard, RP 55778 effectively blocked the induction of HIV expression in U1 cells stimulated with interleukin 6 and granulocyte–macrophage colony-stimulating factor, which act predominantly as posttranscriptional activators of HIV expression. Finally, RP 55778 inhibited the production of endogenous tumor necrosis factor α in phorbol 12-myristate 13-acetate-stimulated cells, thereby interfering with an autocrine pathway of virus expression. The suppressive effects of RP 55778 on HIV expression appeared to be independent of the platelet-activating factor cell surface receptor on U1 cells. RP 55778 inhibited acute HIV replication in primary T-cell blasts and the proliferative capacity of these cells. This study suggests that RP 55778 may represent potentially useful compounds in the treatment of HIV infection.**

After infection with the human immunodeficiency virus (HIV), most patients enter into a prolonged state of clinical latency that persists for years before the onset of the symptomatic phase of HIV infection (1). As HIV disease advances, increased levels of virus replication have been observed both in terms of plasma viremia (2, 3) and number of infected circulating cells (4). A mechanism of induction and/or modulation of HIV expression *in vivo* is the stimulation of infected T lymphocytes and mononuclear phagocytes by immunoregulatory cytokines as demonstrated *in vitro* (1, 5). In this regard, tumor necrosis factor α (TNF-α) and other cytokines have been shown to induce expression of HIV-1 in chronically infected T-lymphocytic and monocytic cells, as well as in primary T cells and macrophages acutely infected with HIV (for review, see ref. 5). Cytokines can be produced locally by uninfected cells (6), thereby influencing HIV-infected cells in a paracrine manner or secreted by the infected cells themselves generating an autocrine loop of viral induction (7, 8). In *ex vivo* studies, both mononuclear phagocytes (9) and B lymphocytes from HIV-infected patients have been found to constitutively secrete high levels of TNF-α and interleukin (IL) 6 (10). These findings, coupled with the observation that TNF-α, IL-6, and other potentially relevant cytokines have been found in plasma or cerebrospinal fluid of HIV-infected patients at higher levels than in uninfected individuals (11–13), suggest that pharmacologic modulation of cytokine-induced HIV replication could represent a potential form of antiviral therapy (for review, see ref. 5).

Platelet-activating factor (PAF) is a potent lipid autacoid formed from phosphoglycerides in the cell membrane. It is produced by many cell types including macrophages, platelets, basophils, neutrophils, eosinophils, and endothelial cells, and it has been found to play an important role in the pathogenesis of asthma, inflammation, and septic shock (for review, see ref. 14). In particular, a functional linkage between PAF- and TNF-mediated effects has emerged from multiple experimental systems (15–20). For example, lipopolysaccharide (LPS) injection into animals resulted in an increase in TNF and PAF plasma levels both of which contributed to the symptoms of sepsis (15, 16), whereas injection of TNF and LPS in normal mice caused hypotension, bowel necrosis, and complement activation, which were blocked by the addition of a PAF antagonist (19). Similarly, TNF-α injected intravitreally in mice induced PAF synthesis and uveitis, which were blocked by a PAF antagonist (20).

Despite the fact that the role of TNF in HIV expression has been extensively investigated in a number of studies (7, 8), little or no information is available on the potential involvement of PAF in these effects. However, PAF activated the HIV-1 long terminal repeat (LTR) in transiently transfected MOLT-4 T-lymphocytic cells, and this effect was completely blocked by a PAF antagonist (21), suggesting that PAF antagonists may represent a potential pharmacologic tool in the treatment of HIV disease. RP 55778 (Acopafant) is a synthetic competitive inhibitor of PAF (22) that has been shown to protect mice from LPS-induced septic shock and to block TNF secretion from murine peritoneal macrophages stimulated *in vitro* with LPS (23).

In the present study, we have investigated the effect of RP 55778 in the chronically HIV-1-infected promonocytic cell line U1 (24), in which virus expression is upregulated by several cytokines and phorbol 12-myristate 13-acetate (PMA) (5).

MATERIALS AND METHODS

Reagents. RP 55778 {Acopafant, (+)-3-(3-pyridyl)-1*H*,3*H*-pyrrolo[1,2-*c*]thiazole-7-carboxamide} and the L-enantiomer (RP 55779) were generously provided by Rhone-Poulenc Rorer. RP 55778 was prepared in 0.1 M HCl and diluted into RPMI 1640 medium (Whittaker Bioproducts). PAF (Sigma) was dissolved in 95% ethanol at 100 mM.

Abbreviations: PAF, platelet-activating factor; TNF-α, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; HIV, human immunodeficiency virus; RT, reverse transcriptase; IL, interleukin; GM-CSF, granulocyte–macrophage colony-stimulating factor; LPS, lipopolysaccharide; LTR, long terminal repeat; CAT, chloramphenicol acetyltransferase; PHA, phytohemagglutinin.

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HIV-1 Induction in Chronically Infected Cells. The chronically HIV-1-infected cell line U1 was maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (Whittaker Bioproducts) and glutamine (Biofluids, Rockville, MD). U1 cells (10^5 cells per ml) were pretreated with RP 55778 or medium in 96-well microtiter plates (Costar) at various times before stimulation with PMA (10^{-8} M; Sigma), recombinant TNF-α (100 units/ml; Genzyme), recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF; 100 units/ml; Amgen Biologicals), or recombinant IL-6 (100 units/ml; Amgen Biologicals). Aliquots of culture supernatants were harvested 3 days after stimulation and tested for the presence of reverse transcriptase (RT) activity.

Acute HIV Infection. Peripheral blood mononuclear cells of seronegative donors were isolated by Ficoll/Hypaque gradient centrifugation and stimulated for 3 days with phytohemagglutinin (PHA; 2 μg/ml; Sigma) to obtain T-cell blasts. Cells were resuspended in RPMI 1640 medium containing 10% fetal calf serum plus 10% (vol/vol) semi-purified IL-2 (Pharmacia Diagnostics, Silver Spring, MD) and a neutralizing anti-interferon α antibody (50 units/ml; Interferon Sciences, New Brunswick, NJ) before incubation with a 10^{-4} dilution of purified HIV (IIIB strain; Advanced Biotechnologies, Columbia, MD).

Cell Proliferation. U1 cells or PHA-blasts were pulse-labeled with [^{3}H]thymidine (New England Nuclear) at 0.5 μCi per well and harvested with a PhD cell harvester (Cambridge Technology, Cambridge, MA) 4 hr or 24 hr later, respectively. Radioactivity in dried filters was measured in a Beckman LS 5000 counter.

RT Activity Assay. An RT reaction mixture (25 μl) containing 60 mM Tris·HCl (pH 7.8), 63 mM KCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1.04 mM EDTA, poly(A) (Pharmacia) at 5 μg/ml, oligo(dT) (Pharmacia) at 0.16 μg/ml, 4 mM dithiothreitol, and ^{32}P-labeled dTTP (Amersham) at 1 μl/ml was added to 5 μl of culture supernatant. After a 2-hr incubation at 37°C, 6 μl was spotted onto DE81 paper and air-dried. The filters were then washed five times with 2× standard saline citrate (SSC) and twice with 95% ethanol and air-dried. Dried filters were cut and radioactivity was measured in a Beckman LS 5000 scintillation counter.

Northern Blot Analysis. Total RNA from 25 × 10^6 U1 cells was extracted by the guanidine thiocyanate/phenol method (Stratagene). RNA (10 μg per lane) was electrophoresed on a 0.8% agarose/formaldehyde gel and then transferred to nitrocellulose membranes. The nitrocellulose was baked and then hybridized with a ^{32}P-labeled HIV LTR homologous probe (*Sst* I-*Bss*HII) for 18 hr. The nitrocellulose filter was washed in 0.1× SSC at 65°C and exposed to x-ray film. Filters were stripped in 0.1× SSC at 80°C and rehybridized with TNF-α or β-actin probes as described (7).

HIV-Specific Immunofluorescence. U1 cells isolated 48 hr after stimulation were cytocentrifuged at 700 rpm for 5 min. The slides were air-dried and fixed with acetone. A 1:200 dilution of an anti-HIV plasma (4) was applied for 30 min at room temperature. The slides were then washed three times with phosphate-buffered saline and stained with fluorescein isothiocyanate-conjugated goat anti-human IgG (1:200 dilution in PBS; Jackson ImmunoResearch) as described (24). Slides were coded and examined blind by two investigators.

Chloramphenicol Acetyltransferase (CAT) Assay. U1 cells were transfected with an HIV-1 LTR-CAT construct (1 μg/ml) by the hypotonic DEAE-dextran method as described (25). Four hours after transfection the cells were treated with 10^{-4} M RP 55778 and 24 hr later cells were stimulated with PMA or cytokines. Proteins were extracted 42 hr after stimulation by multiple cycles of freezing and thawing in 0.25 M Tris·HCl (pH 7.8). Approximately 40 μg of protein was combined with 0.1 ml of chloramphenicol (3 mg/ml) containing [^{3}H]acetyl-CoA (New England Nuclear) at 2 μl/ml. The aqueous solution was overlaid with Econofluor scintillation fluid (Beckman) and radioactivity was measured in a Beckman LS 5000 scintillation counter.

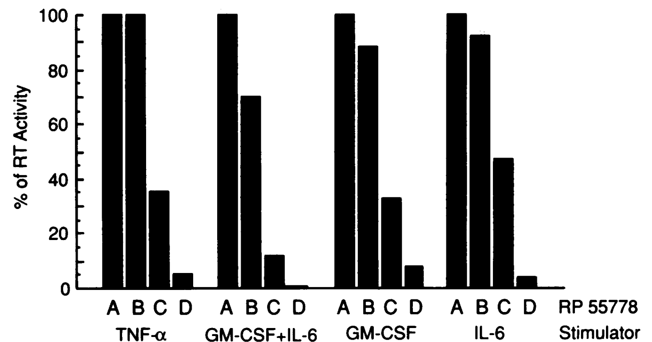

FIG. 1. RP 55778 inhibits HIV expression from U1 cells stimulated by PMA (10^{-8} M), TNF (100 units/ml), or GM-CSF (100 units/ml) plus IL-6 (100 units/ml) in a concentration-dependent manner. Bars: A, no RP 55778; B, 10^{-6} M RP 55778; C, 10^{-5} M RP 55778; D, 10^{-4} M RP 55778. Results are expressed as percentage of the RT activity of control cultures stimulated with cytokines.

RESULTS

RP 55778 Suppresses HIV Expression in U1 Cells. A 30-min pretreatment with RP 55778 inhibited cytokine-dependent expression of HIV in a concentration-dependent manner, exceeding 95% inhibition at 10^{-4} M (Fig. 1). Supernatants harvested 7 days after RP 55778 treatment gave similar results (data not shown). Cell proliferation measured 1–4 days after RP 55778 treatment by [^{3}H]thymidine incorporation or cell viability determined by trypan blue dye exclusion were not affected by any concentration of RP 55778 tested. Addition of RP 55778 at the same time as or after cytokine or PMA stimulation revealed no inhibition of RT

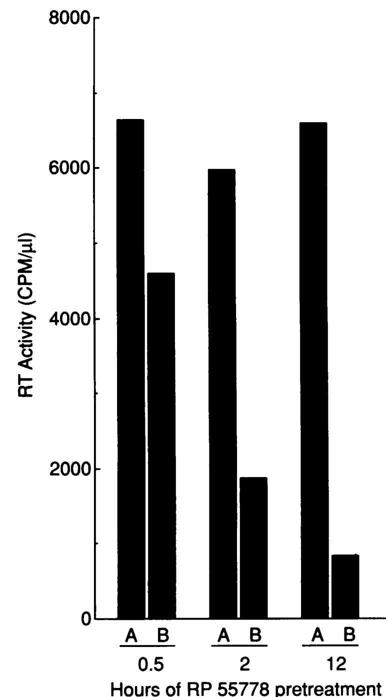

FIG. 2. Increasing the time of RP 55778 pretreatment augments the inhibition of PMA induction of HIV expression from U1 cells. Bars: A, 10^{-8} M PMA; B, 10^{-4} M RP 55778 pretreatment then PMA. Twenty-four-hour (not shown) and 12-hr pretreatments with RP 55778 before PMA stimulation resulted in similar levels of suppression.

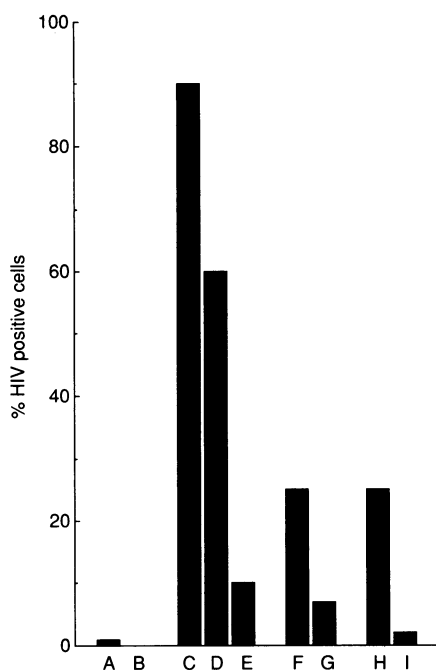

FIG. 3. RP 55778 suppresses HIV protein synthesis in U1 cells. Results are expressed as percentage of cells showing specific fluorescence for HIV proteins in duplicate cultures. Bars: A, unstimulated; B, 10^{-4} M RP 55778; C, 10^{-8} M PMA; D, 30-min pretreatment with RP 55778 then PMA stimulation; E, 24-hr pretreatment with RP 55778 then PMA stimulation; F, TNF-α (100 units/ml); G, RP 55778 (30-min pretreatment) then TNF-α; H, GM-CSF (100 units/ml) plus IL-6 (100 units/ml); I, RP 55778 (30-min pretreatment) then GM-CSF plus IL-6.

activity. U1 cells pretreated with RP 55778 for 30 min and then stimulated with PMA demonstrated minimal (up to 30%) reduction in RT activity. However, increasing the time of incubation with RP 55778 prior to the addition of PMA resulted in significantly reduced virus production (Fig. 2).

RP 55778 also decreased HIV protein expression as measured by indirect immunofluorescence in U1 cells stimulated with TNF-α or GM-CSF plus IL-6 (Fig. 3). In PMA-treated cells, the degree of inhibition of HIV protein expression after RP 55778 treatment was dependent on the duration of pretreatment with RP 55778 (Fig. 3), similar to the findings for RT activity (Fig. 2). Ultrastructural studies by transmission electron microscopy confirmed that RP 55778 suppressed HIV particle expression in PMA- and cytokine-stimulated U1 cells (J. M. Orenstein, personal communication). In contrast to U1 cells, RP 55778 did not inhibit PMA- or TNF-α-induced HIV expression in two chronically HIV-1-infected T-cell lines, J1 and ACH-2 (data not shown).

RP 55778 Partially Suppresses Transcription and Accumulation of HIV mRNA. Total cellular RNA was isolated from U1 cells 24 hr after stimulation with PMA or TNF-α and hybridized with an HIV-specific probe. Only a modest suppression of steady-state HIV RNA was observed in TNF-α-stimulated U1 cells pretreated for 30 min with RP 55778 (Fig. 4*A*, lanes 3 and 4) in contrast to the significant inhibition of RT activity (Fig. 1) and viral protein expression (Fig. 3). A 30-min pretreatment with RP 55778 prior to PMA stimulation demonstrated only a modest decrease in the amount of HIV-specific RNA (Fig. 4*B*, lanes 3 and 4) consistent with the RT and viral protein data (Figs. 2 and 3). However, U1 cells maintained in the presence of RP 55778 for 24 hr prior to stimulation with TNF-α or PMA showed a more profound, although incomplete, reduction in the amount of steady-state HIV RNA (Fig. 4 *A*, lanes 5 and 6, and *B*, lanes 3 and 4). Transient transfection with an HIV-1 LTR–CAT construct also showed only a modest decrease in activation of the virus promoter in U1 cells pretreated for 24 hr with RP 55778 (Fig. 4*C*). These data suggest that RP 55778 may be acting on later events of the virus life cycle that follow the synthesis and accumulation of HIV RNA.

RP 55778 Inhibits PMA-Induced TNF-α Production in U1 Cells. PMA stimulation of U1 cells leads to the synthesis and secretion of TNF-α, which then acts in an autocrine manner to upregulate HIV expression (8). U1 cells pretreated for 24

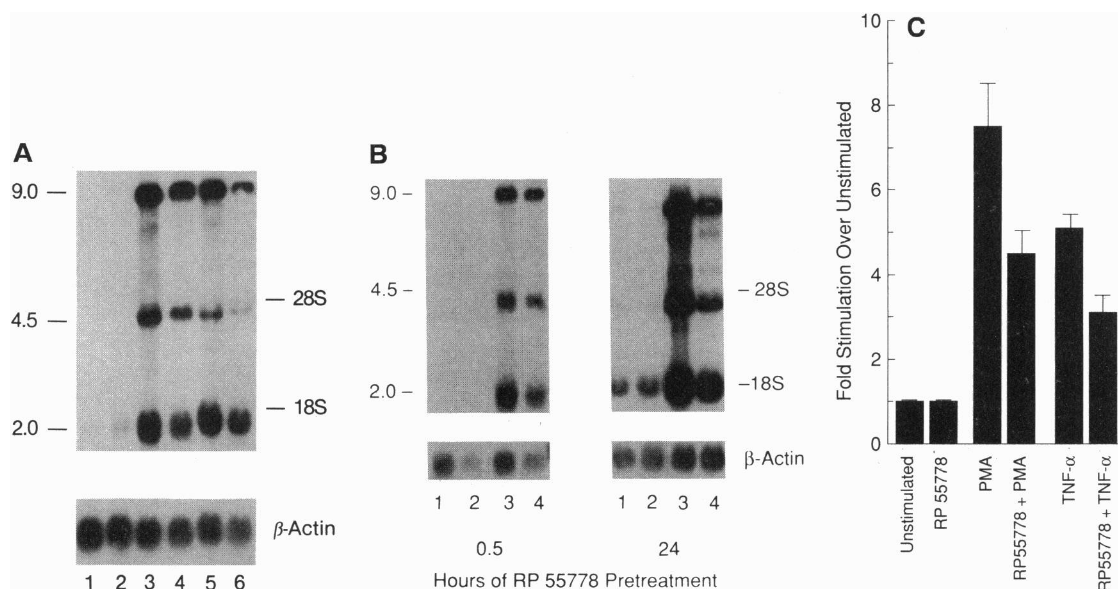

FIG. 4. Effect of RP 55778 treatment on HIV RNA accumulation and HIV LTR-CAT activation in TNF-α- or PMA-stimulated U1 cells. (*A*) Total RNA was isolated 24 hr after TNF-α stimulation and probed with HIV LTR and β-actin probes. Lanes: 1, unstimulated; 2, 10^{-4} M RP 55778; 3, TNF-α (100 units/ml); 4, 30-min pretreatment with RP 55778 followed by stimulation with TNF-α; 5, TNF-α; 6, 24-hr pretreatment with RP 55778 followed by stimulation with TNF-α. (*B*) Total RNA was isolated 24 hr after PMA (10^{-8} M) stimulation and probed with HIV LTR and β-actin probes. Lanes: 1, unstimulated; 2, 10^{-4} M RP 55778; 3, PMA; 4, RP 55778 pretreatment followed by PMA stimulation. (*C*) U1 cells transiently transfected with an HIV-1 LTR-CAT construct were pretreated for 24 hr with 10^{-4} M RP 55778 prior to stimulation with 10^{-8} M PMA or TNF-α (100 units/ml).

hr with RP 55778 and then stimulated with PMA secreted substantially less TNF-α than did cells stimulated with PMA in the absence of RP 55778 (Fig. 5). However, no significant change in the amount of TNF-α mRNA was seen in cells treated with RP 55778 (data not shown), suggesting that RP 55778 exerts its suppressive effect on TNF-α secretion at a posttranscriptional level. In contrast, RP 55778 did not inhibit either the constitutive or PMA-induced levels of cell surface expression of IL-1β (26) on U1 cells (data not shown).

PAF Neither Directly Induces HIV Expression Nor Synergizes with Other Inductive Agents in U1 Cells. Having established that RP 55778 exerted potent suppressive effects on the induction of HIV expression in U1 cells, we next investigated whether these effects were mediated via blocking of a PAF-dependent induction of HIV expression. No induction of HIV expression was observed in U1 cells incubated with PAF in a concentration range between 10^{-5} and 10^{-11} M (Fig. 6). Furthermore, no augmentation of the levels of HIV expression induced by any stimulator was observed in the presence of PAF (10^{-5} to 10^{-11} M).

PAF Does Not Inhibit the Suppressive Effect of RP 55778 on HIV Expression in U1 Cells. U1 cells were either preincubated with PAF (10^{-5} M) for 5–10 min and then treated with RP 55778 or were preincubated with PAF, treated with RP 55778, and retreated with PAF every 5 min until stimulation (data not shown). A maximal concentration of 10^{-5} M PAF was used due to significant cell toxicity observed at higher concentrations. However, the observation that PAF affinity for human neutrophil surface receptors (27) is $\approx$1000-fold higher than that of RP 55778 suggests that 10^{-5} M PAF would displace virtually any bound RP 55778 molecules. Thirty minutes later the cells were stimulated with TNF-α or GM-CSF plus IL-6. No inhibition of the suppressive effect of RP 55778 on HIV expression was observed when PAF was used as a competitive inhibitor (Fig. 6).

Effects of RP 55778 on Acute HIV Infection. Primary T-cell blasts acutely infected with HIV-1 were treated continuously throughout the infection with 10^{-4} M RP 55778. A 70% inhibition of peak RT activity and 40% inhibition of cell proliferation was observed (data not shown). Uninfected T-cell blasts were also treated with RP 55778 and proliferation was found to be similarly decreased. Flow cytometry analysis demonstrated a 50% reduction in IL-2 receptor and major histocompatibility class II antigen expression on the cell surface of both $CD4^+$ and $CD8^+$ T cells (data not shown), suggesting that RP 55778 may exert some immunosuppressive effect on PHA-activated T lymphocytes. Therefore, the decrease in proliferation induced by RP 55778 in T-cell blasts, an effect that was not observed in the chronically infected promonocytic and T-cell lines, was at least partially responsible for the reduced RT activity observed raising a question of whether a true antiviral effect was occurring in this acute infection model.

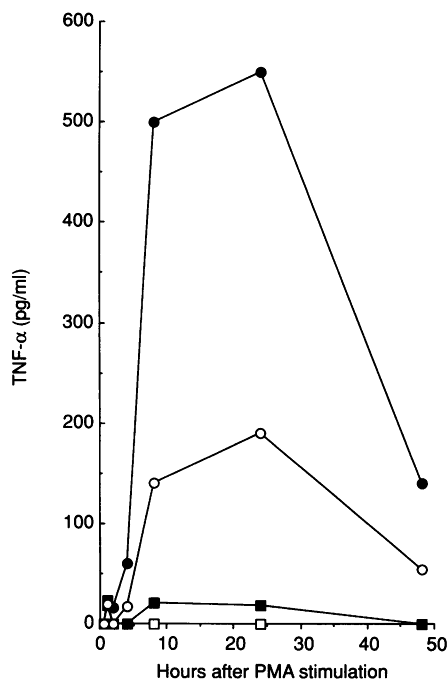

FIG. 5. RP 55778 inhibits TNF-α secretion in PMA-stimulated U1 cells. TNF-α was measured in U1 culture supernatants by use of an ELISA kit (R & D Systems, Minneapolis). ■, Unstimulated; □, 10^{-4} M RP 55778; ●, 10^{-8} M PMA; ○, RP 55778 24 hr pretreatment followed by PMA stimulation.

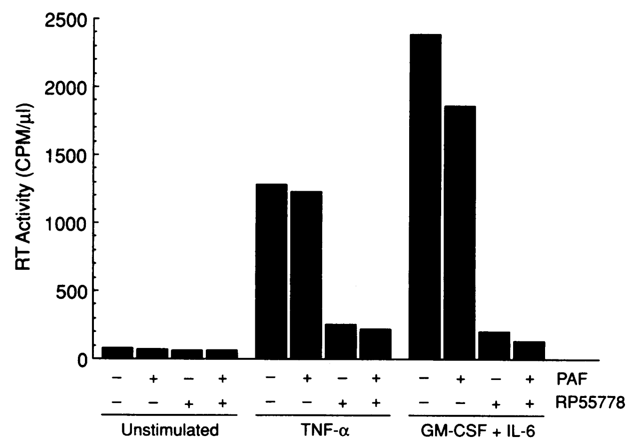

FIG. 6. PAF neither directly stimulates nor synergizes with TNF (100 units/ml) or GM-CSF (100 units/ml) plus IL-6 (100 units/ml) in inducing HIV expression in U1 cells or inhibits the suppressive effects of RP 55778 on HIV expression. U1 cells were treated with 10^{-5} M PAF or medium 5 min before treatment. RP 55778 (10^{-4} M)-treated cells were stimulated with cytokine 30 min later. Other concentrations of PAF (10^{-5} to 10^{-11} M) similarly did not affect cytokine stimulation or the inhibitory effect of RP 55778 in this model system. Similar results were observed in U1 cells stimulated with PMA, IL-6 alone, GM-CSF alone, or TNF, GM-CSF, plus IL-6 at suboptimal concentrations (each at 10 units/ml and 1 unit/ml).

DISCUSSION

In the present study we have investigated the effect of a compound with PAF antagonist activity on PMA- and cytokine-induced HIV expression in persistently infected promonocytic cells. RP 55778 inhibited HIV production induced by exogenously added cytokines such as TNF-α, IL-6, and GM-CSF. It was also effective in blocking TNF-α secretion from PMA-stimulated U1 cells and, thereby, reduced HIV expression by inhibiting a cytokine-dependent autocrine loop of virus induction. RP 55778 significantly reduced HIV protein synthesis and RT activity production in U1 cells but exerted only modest suppressive effects on the transactivation of the HIV LTR or the accumulation of steady-state HIV RNA, suggesting that the predominant effect of RP 55778 in U1 cells was a posttranscriptional inhibition of virus production.

We have reported (28) that certain cytokines, such as IL-6 and GM-CSF, induced HIV expression in U1 cells predominantly by posttranscriptional mechanisms in that RT activity and viral proteins increased proportionately to a much greater degree than did synthesis and accumulation of HIV RNA. In contrast, TNF-α, similar to PMA (29), has been found to transcriptionally induce HIV expression via activation of the cellular transcription factor NF-κB (30). The inhibition of HIV expression by RP 55778 in U1 cells induced by TNF-α, GM-CSF, or IL-6 was a rapid effect requiring only

30 min of pretreatment, and it was long lasting in that U1 cells were still unresponsive to cytokine stimulation 24 hr after a single RP 55778 treatment. This long-lasting effect of RP 55778 in cell suspensions suggested the possibility that this compound may be delivering a persistent negative signal ultimately resulting in the inhibition of HIV production. RP 55778 inhibition of TNF-α protein secretion but not mRNA is in partial contrast to a previous report demonstrating that RP 55778 reduced both TNF-α mRNA and protein secretion (26). The differences in these studies could be explained by interspecies differences and cell type examined. Membrane bound IL-1β expression and cellular proliferation were not inhibited by RP 55778 treatment of U1 cells (data not shown), suggesting a selective effect of RP 55778 on TNF-α and virus production. Finally, it has been reported that RP 55778 induces prostaglandin E_2, F_2, and $F_{1\alpha}$ synthesis,[§] but pretreatment with indomethacin did not alter RP 55778 suppressive effect on HIV induction in U1 cells (data not shown).

Exogenously added PAF neither directly induced nor synergized with cytokines or PMA in the stimulation of HIV expression in U1 cells. Furthermore, PAF failed to act as a competitive antagonist of RP 55778, suggesting that RP 55778 is probably mediating its suppressive effect on HIV expression via a mechanism independent of the PAF cell surface receptor. In support of this interpretation, RP 55779, the L-enantiomer of RP 55778, which has ≈50-fold less ability to inhibit PAF-mediated aggregation of human platelets via the surface PAF receptor (A.B., unpublished data), suppressed PMA- and cytokine-induced HIV expression in U1 cells at levels comparable to RP 55778 (data not shown). Among other potential sites of action of RP 55778 is that of an alternative PAF receptor. In support of this interpretation, three specific PAF receptors have been described in rat cerebral cortex, one at the cell surface and two associated with the microsomal membrane fraction (31). Furthermore, stimulated human neutrophils released extracellularly only a small proportion of their synthesized PAF, most of which remained cell-associated in the membranous and phagolysosome fractions (32), suggesting that PAF may mediate certain functions within the cell and not exclusively via the cell surface receptor. Finally, it is also possible that RP 55778 is acting on U1 by a yet unidentified PAF-independent mechanism.

RP 55778 has been used recently in dose-escalation trials in normal volunteers where it was well-tolerated (A.B., unpublished data). Its potential clinical usefulness in treating HIV disease needs further investigation. As an agent that can block expression of HIV from persistently infected cells, it may potentially be effective in combination with agents that block HIV spreading, such as zidovidine (AZT), dideoxyinosine (ddI), or dideoxycytidine (ddC) (for review, see ref. 33). RP 55778 has been reported to block TNF-α secretion in activated mouse peritoneal macrophages (26). We have found that RP 55778 blocks TNF-α secretion in PMA-, PHA-, or LPS-stimulated human peripheral blood mononuclear cells (D.W. and A.S.F., unpublished observations). This effect of RP 55778 may have additional benefits in treating HIV disease since elevated plasma levels of TNF-α have been reported in HIV-infected patients (14), and *ex vivo* studies have demonstrated increased TNF production from both mononuclear phagocytes (12) and B lymphocytes (13) of HIV-infected individuals. Although, a significant reduction of virus production in acutely infected T-cell blasts occurred after RP 55778 treatment, this effect was also associated with significantly reduced cell proliferation and IL-2 receptor expression in these cells, raising the question whether RP 55778 exerted a true antiviral effect in this system or merely suppressed T-cell activation.

In conclusion, RP 55778, a PAF receptor antagonist developed for the treatment of septic shock, exerts potent suppressive effects *in vitro* on PMA- and cytokine-induced HIV expression in persistently infected U1 cells. These studies may serve as a basis for further investigation of the role of RP 55778 and its analogues as potential therapeutic modalities in HIV infection.

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