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Inhibition of HIV-1 Tat-Mediated Transactivation by Quinacrine and Chloroquine

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The replication of human immunodeficiency virus type 1 (HIV-1) requires cellular components to interact with regulatory elements located in the long terminal repeat (LTR) as well as viral proteins Tat and Rev. Several well known signaling transduction inhibitors were tested to determine their effects on the Tat-mediated transactivation using a transfection assay with the bacterial chloramphenicol acetyltransferase gene under the control of the HIV-1 LTR. The protein kinase C inhibitors curcumin and staurosporine, but not a tyrosine kinase inhibitor herbimycine A, inhibited Tat-mediated LTR-driven transactivation. Two antimalarial drugs quinacrine and chloroquine, that are also arachidonic acid metabolism inhibitors, were found to inhibit the Tat-mediated LTR-driven gene expression. Another inhibitor of arachidonic acid metabolism 4-bromophenacyl bromide was also found to inhibit Tat-mediated gene expression driven by HIV-1 LTR. However, the antimalarial drug quinine elicited no effects on Tat-mediated transactivation. These results suggest that the anti-arachidonic acid metabolism properties of quinacrine and chloroquine may be responsible for their ability to inhibit Tat-mediated LTR-regulated gene expression.

Human immunodeficiency virus type 1 (HIV-1) gene expression is governed by viral early proteins including Tat (1, 2) and Rev (3, 4), which are essential for viral replication, and by a complex arrays of cellular DNA- and RNA-binding proteins. Rev acts post-transcriptionally to induce the expression of HIV-1 structural proteins and thereby shifts the early phase of HIV-1 replication to the late cytopathic phase of the replication cycle (5). Tat enhances transcription initiation of the integrated proviral genome by interacting with the Tat-responsive element (TAR) located in the LTR (6-10).

Complete activation of HIV-1 LTR by Tat is also regulated by interactions of cellular transcriptional factors and regulatory proteins with specific *cis*-acting DNA target sequences within the LTR. In addition to TAR, these modulating elements include core promoter elements such as SP1 (11, 12) and TATA (9, 13) that are required for activation of HIV-1 gene expression in most cell lines. On the other hand, the state of T cell activation and proliferation also controls HIV-1 replication and gene expression. In particular, cellular signal transduction via protein kinase C is essential to the mitogenic stimulation of T cells. Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator, activates HIV-1 gene expression (14), in part, via activation of NF-κB, a family of mitogen and phorbol ester-inducible DNA binding transcription factors which bind to NF-κB binding sites, resulting in the activation of HIV-1 enhancer (15, 16). LTR-directed gene expression is perturbed by inhibition with protein kinase C activities (17, 18).

Intracellular signal transduction pathways are complex and these signaling pathways may cross-interact with each other. A component in one pathway may ultimately be affected by components in initially unrelated signaling pathways. Arachidonic acid, serving as a biologi-

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cally active signaling molecule as well as an important component of membrane lipid, exerts many biological functions including modulation of the activities of protein kinases and ion channels (review see 19). Although arachidonic acid has been proposed to activate protein kinase C in many biological systems (19-22), whether it is involved in HIV-1 Tat-mediated gene expression under the control of HIV-1 LTR remains unknown.

In the present study we employed a quantitative bioassay based on the transactvation of a chimeric gene containing the HIV-1 LTR fused to the bacterial chloramphenicol acetyltransferase (*cat*) gene. This construct was tranfected into cells together with Tat expression plasmid and cells were then incubated with inhibitors that are known to block protein kinase C or arachidonic acid pathways. We found many clinical antimalarial drugs currently in use can inhibit Tat-mediated gene expression regulated by LTR. The anti-arachidonic acid properties of these drugs may be responsible for their HIV-Tat inhibitory capability. This finding may have an important implication for the development of drugs that can intervene HIV-1 replication cycle by targeting the viral LTR.

MATERIALS AND METHODS

Chemicals. Curcumin, staurosporine, quinacrine, quinine, chloroquine hydrochloride, 4-bromophenacylbromide and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT) were all purchased from Sigma. [^{14}C]-Chloramphenicol and [^{35}S]methionine were purchased from Amershan. Acetyl CoA was purchased from Bohringer Mannheim.

Cells. A monocytic cell line, U937, and two CD4$^+$ lymphoma cell lines, Jurket and SupT1, were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Life Science, Gaithersburg, MD).

Plasmids. pU3R-III CAT is a reporter gene construct that contains *cat* gene fused to HIV-1 LTR (2, 23). pIIIextat is a vector that expresses Tat protein under control of HIV-1 LTR (23), and is required to *trans*-activate expression of *cat* gene from pU3R-III CAT.

DNA transfection and drug treatment. 5×10^6 cells were co-transfected with 2 μg of pIIIEXtat and 5 μg of pU3R-III CAT by the DEAE-dextran method as described elsewhere (24). Drugs were added 24 hr post-transfection and cultures were further incubated at 37°C for additional 12 hr. For each set of experiments, we multiplied the number of cells and amount of DNA used in each trasnfection by the number of total transfections. After transfection the cells were divided into aliquots which contained equal number of cells for each drug treatment. Total cell lysates were prepared 36 hr after transfection and CAT assay were performed as described elsewhere (24). All experiments were performed in triplicate unless otherwise indicated and all showed similar results.

MTT assay. Spectrophotometric quantitation of cell viability was performed using MTT as an indicator as previously described (25). Cells at a density of 10^4 cells/well were grown in medium containing indicated concentrations of drugs in 96-well plates for 12 hr. MTT was added and cultures were incubated for another 2 hr at 37°C. The formed brown crystal was dissolved by DMSO and the relative optical density at 540 nm and 650 nm was read by a microplate reader (Molecular Devices).

[^{35}*S*]*Methionine labeling.* 5×10^6 cells were grown in RPMI (without methionine): RPMI (with methionine) = 9:1 that was supplemented with 10% dialysed FBS in the presence of drugs and 0.1 mCi/ml of [^{35}S]methionine at 37°C for 12 hr. Cells were then washed with phosphate-buffered saline (PBS) and lysed in ice cold RIPA buffer [25 mM Tris-HCl (pH 7.2), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl and 1 mM EDTA] containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, 1 mM sodium orthovanadate and 5 μg/ml of leupeptin. Protein concentration was determined by the BCA assay and lysates containing equal amounts of proteins were subjected to polyacrylamide gel electrophoresis (PAGE) or TCA precipitation for determination of ^{35}S incorporation into macromolecules. After PAGE the gels were dried and exposed to X-ray film.

RESULTS

Inhibition of Tat-mediated, LTR-driven gene expression by curcumin, staurosporine and quinacrine, but not by herbimycine A. Curcumin is a dietary pigment responsible for the yellow color of curry and has been shown to have an inhibitory effect on the PMA-induced protein kinase C activation (26). Nonetheless, whether curcumin inhibits PMA-stimulated gene expression driven by HIV-1 LTR has not been resolved. To address this question, U937 cells were transfected with 5 μg of pU3R-III CAT, a *cat* reporter gene linked to HIV-1 LTR, and 2 μg of pIIIextat, which synthesizes Tat protein and thus, trans-activate CAT expression via HIV-

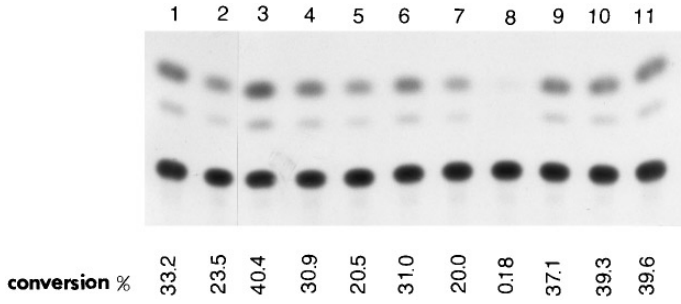

FIG. 1. Inhibition of Tat-mediated transactivation by curcumin, staurosporine and quinacrine. Cells were co-transfected with 2 μg of pIIIextat and 5 μg of pU3R-III CAT by the DEAE dextran method. Twenty four hr posttransfection cell cultures were incubated with drugs for an additional 12 hr. Total cellular proteins were harvested and CAT assays were performed. The relative signals of acetylated and native chloramphenicol were determined using a phosphorimager. The relative conversion activity (RCA) were defined as the ratio of signals representing the acetylated forms to the sum of signals of the acetylated and unreacted chloramphenicol. Lane 1, control; lane 2, 15 μM curcumin; lanes 3-5, 10 nM, 45 nM, and 120 nM staurosporine, respectively; lanes 6-8, 1 μM, 4 μM, and 10 μM quinacrine, respectively; lanes 9-11, 0.2 μM, 0.7 μM, and 1.5 μM herbimycine A, respectively.

1 LTR. Twenty four hr posttransfection, different concentrations of curcumin were added to the cell cultures. Cells were harvested 36 hr posttransfection and cell lysates were prepared. Equal aliquots of samples were assayed for *cat* activity and quantitated by a phosphorimager. Cat expression was decreased in the presence of curcumin and the decrease in the percentage of [^{14}C]chloramphenicol conversion to acetylated forms was curcumin concentration-dependent; the Cat expression dropped from 43.5% of the control to 13.2% in the presence of 30 μM curcumin. This result indicates that inhibition of protein kinase C by curcumin results in inhibition in Tat-mediated HIV-1 LTR-regulated gene expression.

Next, inhibitors of some signal transduction pathways were tested to examine their effects on Tat-responsive gene expression under control of HIV-1 LTR. Staurosporine, a protein kinase C inhibitor (27), was found to inhibit CAT expression in a dose-dependent manner (Fig. 1, compare lanes 1 and 3-5). Quinacrine, an inhibitor of arachidonic acid metabolism (28) was also capable of inhibiting Tat-LTR gene expression (Fig. 1, compare lanes 1 and 6-8). Essentially, no CAT expression could be detected in the presence of 10 μM quinacrine. In a separate experiment, 2 μM quinacrine was found to be enough to inhibit the Tat-induced transactivation. On the other hand, herbimycine A, a tyrosine kinase inhibitor (29), did not inhibit CAT expression even at 1.5 μM (Fig. 1, compare lanes 1 and 9-11).

Effects of anti-malarial drugs on the inhibition of Tat-mediated transactivation. Because quinacrine is a clinical drug currently being used in malarial treatment (30), we further investigate whether other anti-malarial drugs, such as chloroquine (Feldmann et al., 1994) and quinine (31), had the ability to inhibit Tat-LTR gene expression. As shown in Fig. 2, chloroquine inhibited Tat-mediated gene expression under control of LTR in a dose-dependent manner (Fig. 2A). In contrast, quinine did not show any inhibitory effect on CAT expression even at 64 μM (Fig. 2B, lanes 1-4). As a positive control, quinacrine at 8 μM significantly blocked Tat-mediated transactivation (Fig. 2A, lane 5).

Inhibition of Tat-mediated LTR-directed gene expression by 4-bromophenacyl bromide. To further examine whether the anti-arachidonic acid metabolism activity of quinacrine and chloroquine may be responsible for their inhibitory effects on Tat-mediated gene expression, transfected cells were treated with 4-bromophenacyl bromide, which is a known arachidonic acid metabolism inhibitor (32). The 4-bromophenacyl bromide at 10 μM was strikingly abolished Tat-mediated gene expression (Fig. 3), suggesting that the inhibitory effects in Tat-

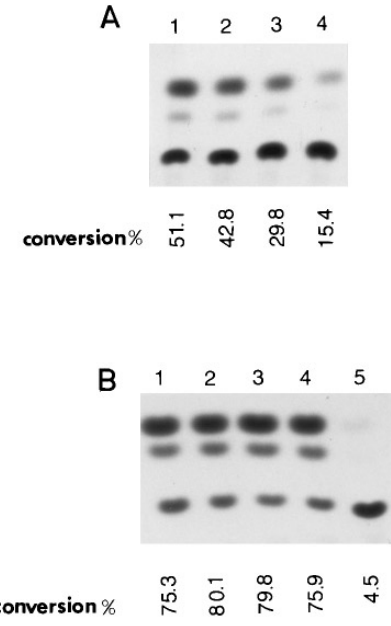

FIG. 2. Inhibition of Tat-LTR transcriptional activity by chloroquine but not by quinine. Cells were cotransfected with pU3R-III CAT and pIIIextat as described in the legend to Fig. 1 in the presence of various drugs. (A) Lane 1, H_2O; lanes 2-4, 15 μg/ml, 30 μg/ml, and 50 μg/ml chloroquine, respectively. (B) Lane 1, H_2O; lanes 2-4, 8 μM, 32 μM, and 64 μM quinine, respectively; lane 5, 8 μM quinacrine.

mediated gene expression of quinacrine and chloroquine may target the mechanism underlying arachidonic acid metabolism.

Cytotoxicity of these drug. To determine whether the concentrations of drugs used in the study may be cytotoxic, [^{35}S]methionine incorporation and MTT assays were performed. Chloroquine at 15 μg/ml had only a slight cytotoxic effect oo cells (Fig. 4, lane 5 and Fig. 5, treatment B) but inhibited Tat-mediated gene expression. At 50 μg/ml of chloroquine cell viability, as determined by MTT assay, was decreased by 37% as compared to the control (Fig. 5). Nevertheless, CAT expression was decreased by 70% as compared to the control (Fig. 2A, compare lanes 1 and 4). Treatment with 1.5 μM herbimycin A decreased the O.D. value to 0.340 (Fig. 5, herbimycin A treatment, D). Although this value was lower than the O.D. value 0.353 of 15 μg/ml chloroquine treatment (Fig. 5, chloroquine treatment, B),

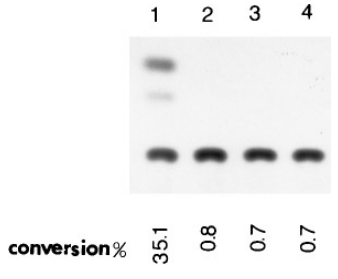

FIG. 3. Inhibition of Tat-LTR transcriptional activity by 4-bromophenacyl bromide. Cells were cotransfected with pU3R-III CAT and pIIIextat as described in the legend to Fig. 1. Lane 1, DMSO; lane 2, 10 μM; lane 3, 20 μM; lane 4, 30 μM 4-bromophenacyl bromide, respectively.

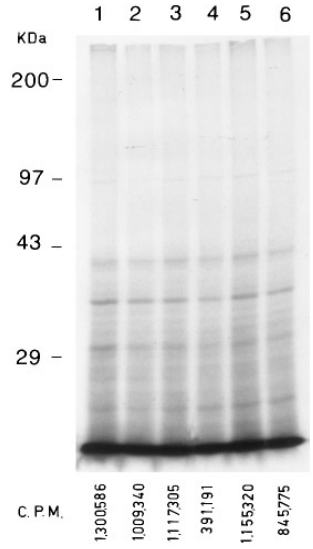

FIG. 4. Effects of drugs on [^{35}S]methionine incorporation. Transfected cells were treated with various drugs for 12 hr and [^{35}]methionine metabolic labeling was performed as described in the ''Materials and Methods.'' Equal amounts of proteins (50 μg) from each sample were resolved by SDS-PAGE and also determined for TCA-precipitable counts. Lane 1, DMSO; lane 2, 15 μM curcumin; lane 3, 2 μM quinacrine; lane 4, 4 μM quinacrine; lane 5, 15 μg/ml chloroquine; lane 6, 30 μg/ml chloroquine.

herbimycine A did not have any effect on Tat-mediated transactivation (Fig. 1, lane 11). Nevertheless, chloroquine did inhibit Tat-LTR transcriptional activity at this dose (Fig. 2A, lane 2). These results show that the decrease in cell viability is not necessarily concomitant with the decrease in Cat expression regulated by LTR.

DISCUSSION

Protein kinase C has been well documented to play an important role in Tat-mediated gene expression directed by HIV-1 LTR. The activation of Tat-mediated, LTR-driven gene expression by phorbal ester-activated protein kinase C (34) may, in part, be attributed to the phosphorylation of an inhibitor of NF-κB such as IκB. This leads to dissociation of NF-κB from IκB and the enhanced binding of the active NF-κB moiety to its recognition sites, resulting in activation of HIV-1 enhancer (15, 16, 33-35). On the other hand, the transactivation effect of Tat on LTR has also been shown to be regulated by protein kinase C in a mechanism that apparently is distinct from the effect of mitogens on LTR-driven basal transcription (36).

In this study we observed that two arachidonic acid metabolism inhibitors quinacrine and chloroquine have inhibitory effects in Tat-mediated gene expression in CD4$^+$ T-cell lines such as SupT1 and Jurkat as well as a monocytic U937 cell line. Although the molecular events underlying the involvement of arachidonic acid metabolism pathway in LTR-controlled gene expression are poorly understood, it is likely that the arachidonic acid metabolic products may activate NF-κB (37). Also, the arachidonic acid metabolism pathway has been shown to activate protein kinase C (19-22), thus, resulting in the enhanced Tat-mediated LTR-regulated gene expression.

Our finding is intriguing because these arachidonic acid metabolism inhibitors are currently being used as antimalarial drugs. Although these compounds may have anti-proliferative effects on cells, the decrease in Tat-mediated transactivation cannot be simply attributed to their adverse effects on cell viability. Nevertheless, our finding suggests a possible link of arachi-

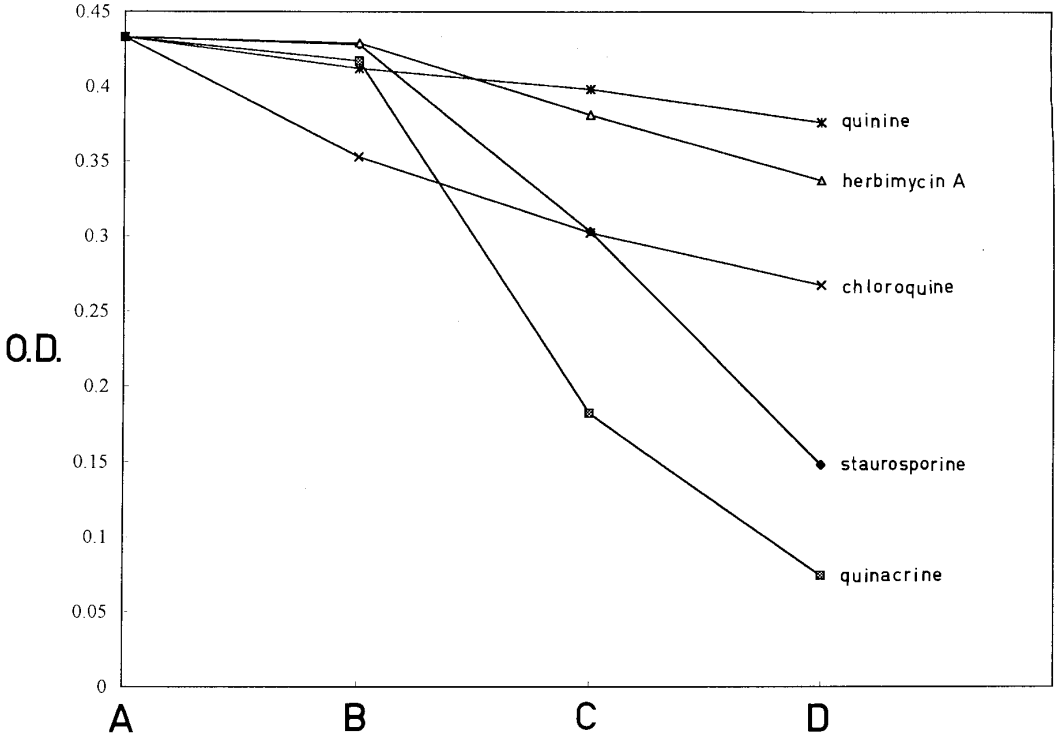

FIG. 5. MTT assays of drugs treatment. Transfected cells were treatment with various drugs as indicated for 12 hrs. Point at A represents DMSO treatment. Points at set B represent treatments by 8 μM quinine, 0.2 μM herbimycine A, 15 μg/ml chloroquine, 10 nM staurosporine, and 2 μM quinacrine. Points at set C represent treatments by 32 μM quinine, 0.7 μM herbimycine A, 30 μg/ml chloroquine, 40 nM staurosporine, and 4 μM quinacrine. Points at set D represent treatments by 64 μM quinine, 1.5 μM herbimycine A, 50 μg/ml chloroquine, 120 nM staurosporine, and 10 μM quinacrine treatment. The assays were performed twice each time with six replicates.

donic acid pathway to HIV-1 gene expression and raises an interesting question of whether these drugs inhibit HIV-1 replication and/or infectivity.

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