α-Lipoic acid blocks HIV-1 LTR-dependent expression of hygromycin resistance in THP-1 stable transformants

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Abstract Gene expression of human immunodeficiency virus (HIV) depends on a host cellular transcription factors including nuclear factor-κB (NF-κB). The involvement of reactive oxygen intermediates (ROI) has been implicated as intracellular messengers in the inducible activation of NF-κB. In this study, we compared the efficacy of two antioxidants, α-lipoic acid (LA) and *N*-acetylcysteine (NAC), which are widely recognized NF-κB inhibitors. Here, we demonstrate that LA has a more potent activity in inhibiting NF-κB-mediated gene expression in THP-1 cells that have been stably transfected with a plasmid bearing a hygromycin B resistance gene under the control of HIV-1 long terminal repeat (LTR) promoter. The spontaneous activation of NF-κB in this cell culture system leads to expression of the hygromycin phosphotransferase gene hence rendering the cells resistance to hygromycin B. In this study, the effect of the test compounds against transcriptional activity of HIV-1 LTR was evaluated based on the degree of cellular toxicity due to the inhibitory activity on the expression of hygromycin B resistance gene in the presence of hygromycin B. We also found that 0.2 mM LA could cause 40% reduction in the HIV-1 expression from the TNF-α-stimulated OM 10.1, a cell line latently infected with HIV-1. On the other hand, 10 mM NAC was required to elicit the same effect. Furthermore, the initiation of HIV-1 induction by TNF-α was completely abolished by 1 mM LA. These findings confirm the involvement of ROI in NF-κB-mediated HIV gene expression as well as the efficacy of LA as a therapeutic regimen for HIV infection and acquired immunodeficiency syndrome (AIDS). Moreover, this study validates the applicability of our present assay system which we primarily designed for the screening of candidate drugs against HIV-1 gene expression.

Key words: Gene expression; Reactive oxygen intermediate; Nuclear factor-κB; α-Lipoic acid; *N*-Acetylcysteine; HIV-1; AIDS

1. Introduction

α-Lipoic acid (LA), the oxidized form of 6,8-dimercaptooctanoic acid and its reduced form, dihydrolipoic acid (DHLA) have been gaining interest in the quest for an efficacious drug for the treatment of a wide variety of disorders that are mediated by reactive oxygen intermediates (ROI). LA is known to be reduced when it is incorporated into the cells in the form of DHLA. This LA/DHLA redox couple has been shown to have potent antioxidant abilities as investigated in cases of human immunodeficiency virus (HIV) infection [1], diabetes [2], diabetic polyneuropathy [3,4] and dermatitis [5]. LA and DHLA have also been observed to have neuroprotective capacity against direct and indirect excitotoxic insults [6], as well as protective effect toward oxidative hemolysis of human erythrocytes [7]. These documented effects of LA and DHLA have been mainly attributed to their intrinsic free radical-scavenging property [2,6–9]. It has been investigated by Matsugo et al. [8] that the hydroxyl radical quenching activity of LA is a function of the disulfide bond in the dithiolane ring structure of the compound. It has also been postulated that the anti-inflammatory action of LA is due to its ability to suppress nitric oxide production [2]. Another proposed mechanism of action of LA is the antioxidant's ability to chelate transition metals [3,8]. Other investigators further reported that LA can elevate the total level of the endogenous antioxidant, reduced glutathione (GSH), in some tissues and cell lines [4,6].

N-Acetylcysteine (NAC) is a precursor of GSH and is also used as a nontoxic drug widely prescribed as a mucolytic agent and antidote against drug-induced hepatotoxicity [10]. It has also been used clinically to treat acetaminophen toxicity [11]. Results of laboratory investigations have also implied the usefulness of NAC therapy in cases of HIV infection as well as inflammatory and oxidative stress-mediated pathologies including acute respiratory distress syndrome (ARDS), toxic and septic shock, porphyria and cerebral malaria [12] and melanoma [10]. It is believed that these therapeutic values of NAC are due to its direct scavenging actions on ROI [12–14]. On the other hand, others postulate that the effect of NAC could not be ascribed to its reducing activity but to the indirect effects on another cellular reducing system such as that of thioredoxin [15]. Moreover, it has been recognized that NAC, with its ability to penetrate the cell membrane, gets rapidly deacetylated to L-cysteine by *N*-acetylases present in various organs, thereby replenishing the intracellular cysteine required to produce GSH [4,10–13,16,17]. GSH is the major intracellular thiol, which comprises >90% of the cellular nonprotein thiols and an ROI scavenger present in all eukaryotic cells [10–12].

The therapeutic values of LA and NAC in acquired immunodeficiency syndrome (AIDS) and HIV infection have been looked into, mainly, in the light of redox regulatory mechanism that is involved in the activation of an inducible cellular

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Abbreviations: ROI, reactive oxygen intermediates; NF-κB, nuclear factor-κB; LA, α-lipoic acid; DHLA, dihydrolipoate; NAC, *N*-acetylcysteine; HIV-1, human immunodeficiency virus type 1; LTR, long terminal repeat; GSH, reduced glutathione; MTT, 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; TNF-α, tumor necrosis factor-α

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transcription factor, nuclear factor-κB (NF-κB) [4,11–13,15,16].

In the present study, we have further confirmed the antioxidant property of LA and NAC, including their role in inhibiting HIV-1 gene expression using recombinant THP-1, a $CD4^+$ monocyte-macrophage cell clone that has been stably transduced with a plasmid containing a phosphotransferase gene under the control of HIV-1 LTR. The findings were analyzed based on the results of a colorimetric cytotoxicity assay using 3(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) dye. The MTT assay utilized in this investigation was our modification of the original protocol of Mosmann [18] which we designed and established as a screening method for anti-Tat drugs [19]. To further support the findings with the current assay system, we have confirmed the effect of LA and NAC on viral p24 antigen expression in the culture fluid of TNF-α-stimulated OM 10.1 cells, a macrophage cell line that is latently infected with HIV-1 [20].

2. Materials and methods

2.1. Cell lines and plasmids

For this series of experiments, THP-1, a $CD4^+$ monocyte-macrophage cell line, was used. pKO plasmid was used to render the transfected cells resistant to hygromycin B. A detailed description of pKO plasmid construction has been presented previously [19]. However, for this particular experiment, the pKO plasmid was cut by *Xba*I as to render Tat nonfunctional. Thus, the cells were resistant to hygromycin B, solely dependent on host cellular transcription factors, in particular, NF-κB owing to the presence of its binding sites in the HIV-1 LTR promoter (Fig. 1A).

The HIV-1 p24 antigen detection was carried out with the culture supernatants of OM 10.1, a monocyte-macrophage cell line that is latently infected with HIV-1 [20] which was a gift from Dr. R.F. Schinazi of Emory University, VA Medical Center, GA, USA.

All cells were maintained in culture medium prepared from RPMI 1640 supplemented with 10% fetal calf serum (FCS).

2.2. Preparation of recombinant cells

THP-1 recombinant cells, referred to in this paper as T-Xba, were prepared by transfection of the *Xba*I-digested pKO plasmid [19]. Electroporation was carried out with Electro Cell Manipulator 600 (BTX, San Diego, CA, USA) under the following conditions: 2950 μF capacitance and 0.5 kV/cm field strength using 2-mm gap BTX cuvette containing 4×10^6 cells resuspended in 200 μl serum-free RPMI 1640 with 10 mM dextrose and 0.1 mM dithiothreitol. After 24 h incubation, cell viability was measured using trypan blue exclusion method. Stable transfectants were obtained by long-term cultivation in the presence of 300 μg/ml hygromycin B. Thereafter, cells were maintained in RPMI 1640 supplemented with 10% FCS and 300 μg/ml hygromycin B (Sigma Chemical Co., St. Louis, MO, USA). As dictated by the restriction site of the transfected plasmid, the expression of the hygromycin phosphotransferase gene is dependent on host transcription factors, particularly NF-κB [19].

2.3. Anti-NF-κB MTT assay

The test for the activity of LA and NAC on HIV-1 gene expression, referred to in this paper as anti-NF-κB MTT assay, was done according to the original cytotoxicity assay described in Kira et. al. [19], with some modifications. Carbenicillin was included to serve as negative control. Briefly, two-fold serial dilution of the drugs in 50-μl amounts was distributed onto triplicate wells of sterile flat-bottomed 96-well microtiter plates. In addition, 500 μg/ml final concentration of either hygromycin B (test) or culture medium (control), in 50-μl amounts, was dispensed to designated wells. The cells were then seeded at a density of 1×10^5/ml in 100-μl amounts. Incubation was carried out in an atmosphere of 5% CO_2 in air humidified at 37°C. On the sixth day of incubation, cell viability was quantified by adding 25 μl MTT dye to the cultures and allowing it to be reacted upon for 2 h by the mitochondrial dehydrogenases generated by viable cells. The resulting formazan crystals were dissolved in DMSO and Sorensen's glycine buffer. Absorbance was measured at 540/690 nm. Throughout the study, all the experiments were carried out in triplicate. Experimental variations of cytotoxicity values of the samples were within ±5%.

Interpretation of results was done by determining the effective (E) and control (C) doses of the drugs and evaluating the relative efficacy of the test compound using E:C ratios. This has been described in detail in our previous paper [19]. Briefly, 'E' refers to that which causes cytotoxicity due to the effect of compound against NF-κB, that is, cell death in the presence of both the test drug and hygromycin B. 'C' reflects the nonselective cytotoxicity by test compounds, that is, the cell-killing effect of the test drug in the absence of hygromycin B. An E:C ratio value less than 1.0 indicates that the test compound has a selective cytotoxicity due to the inhibition of the HIV-1 promoter activity. The concentration of the test compound showing an E:C ratio value of 0.5 represents its 50% effective concentration (EC_{50}). If the EC_{50} value of a test compound is close to the concentration of 50% nonselective cytotoxicity, the compound is not recommended as a candidate drug because of the narrow therapeutic window.

2.4. p24 antigen assay

The viral p24 antigen level in the culture supernatant of OM 10.1 was measured using a commercial kit, Abbott™ HIVAG-1 B1A010 (Abbott Park, IL, USA), a sandwich solid phase enzyme immunoassay (EIA) in bead format. The samples were harvested from OM 10.1 cultures which have been stimulated for 72 h with 2 ng/ml TNF-α (Boehringer Mannheim Biochemica, Mannheim, Germany). The p24 antigen concentration was determined based on a curve constructed from the standard p24 antigen.

3. Results

3.1. Establishment of hygromycin B-resistant cells

After transfection of the *Xba*I-digested pKO plasmid into $CD4^+$ THP-1, cultures were maintained in medium containing 300 μg/ml hygromycin B to select hygromycin B-resistant cells. To confirm the level of cellular resistance to hygromycin B, the cells were maintained for 6 days in culture containing varied concentrations (0–3000 μg/ml) of the antibiotic and cell viability was measured by MTT assay. As seen in Fig. 1B, THP-1 parental cells demonstrated a 26% fall in their survival, expressed as percent of the control, at 125 μg/ml hygromycin B. It then reached an almost undetectable level starting at 500 μg/ml at which concentration, recombinant T-Xba cells maintained 82% viability. Thus, it was decided that 500 μg/ml hygromycin B is suitable for the actual drug screening assay. Even at the highest concentration tested (3000 μg/ml), T-Xba showed some resistance to hygromycin B, with 20% of them remaining viable. These results confirmed the successful transfection and the constitutive expression of the hygromycin B phosphotransferase gene in T-Xba cells.

3.2. Evaluation of LA and NAC by anti-NF-κB MTT assay

Using the recombinant T-Xba cells which constitutively express the hygromycin B resistance gene, we investigated the effects of known NF-κB inhibitors, LA and NAC. Fig. 2 shows the representative results of anti-NF-κB MTT assay depicting the nonselective cytotoxicity by test compounds and their corresponding specific cytotoxicity due to their genuine effect on NF-κB-dependent gene expression. Carbenicillin, a β-lactam antibiotic agent was used as a negative control. T-Xba cells, at a density of 10^4 per well were treated with serial two-fold dilutions (1920 to 7.5 μg/ml) of the test drugs in the presence or absence of 500 μg/ml hygromycin B. Cell viability was quantitated on day 6 of treatment. As shown in Fig. 2A, the 50% effective dose of LA was found to be 267 μg/ml (1.29 mM), while the 50% nonselective cytotoxic dose was

750 μg/ml (3.6 mM). In contrast, NAC did not induce selective cell killing. It was not able to bring the level of hygromycin cytotoxicity to 50% even at the highest concentration tested, 1920 μg/ml (12 mM). Although there was an observed 50% control dose for NAC at 960 μg/ml (5.9 mM), this seems to be an artifactual cell killing as the cell viability at 1920 μg/ml increased considerably.

To better evaluate the effect of the drugs, E:C ratio at different drug concentrations was calculated based on their selective and nonselective cytotoxicities. As demonstrated in Fig. 2B, LA is expected to be effective at concentrations above 190 μg/ml (0.9 mM), the calculated EC_{50} dose. LA has a relatively wide therapeutic window as its 50% nonselective cytotoxicity dose which was determined to be 750 μg/ml (3.6 mM) is 4-times as high as the calculated EC_{50} value. The therapeutic window for NAC could not be determined since the EC_{50} was not reached. However, as seen in the p24 antigen detection assay in the foregoing section, NAC can be an effective NF-κB inhibitor when used at much higher concentrations than what was used for the MTT assay.

3.3. Evaluation of LA and NAC using p24 antigen assay

To substantiate the above findings, we used varying concentrations of LA and NAC to examine their inhibitory effect on HIV-1 p24 antigen expression in OM 10.1 cells. In addition to pretreatment with LA and NAC, test cultures were also exposed to a uniform concentration of TNF-α (2 ng/ml) to stimulate NF-κB activation. Cell-free culture supernatants were used for the assay. As shown in Fig. 3, there was a detectable amount of p24 antigen (between the range of 0.5–2 ng/ml) in the supernatant of OM 10.1 cell cultures which were not stimulated with TNF-α, indicating low-level expression of structural proteins and production of virions in these chronically infected cells. However, there was no evident inhibitory effect of LA and NAC on the viral antigen expression from the unstimulated OM 10.1 cells when a set of controls, that is, incubated with varied concentrations of the two antioxidants, was run in parallel with the antioxidant-pre-treated and TNF-α-stimulated cells.

The observed low-level expression of p24 antigen appears to reflect the actual latency period occurring between HIV infection and the development of full-blown AIDS wherein the virus exists in a proviral DNA form which is integrated within the host genomic DNA though lacking full transcriptional activity [21,22]. The augmentation of virus expression in chronically infected cells can be achieved by treatment with cytokines such as TNF which is associated with the induction of a nuclear factor(s) binding to the NF-κB sites in the HIV-1 LTR [11,21,23]. In fact, TNF-α is considered to play a critical role in the pathogenesis of AIDS as its level in HIV-infected individuals has been found to be considerably increased [11].

Thus in this experiment, the p24 antigen expression was efficiently augmented upon treatment with 2 ng/ml TNF-α, which was dramatically inhibited by LA or NAC. Greater than 1 mM LA was found to completely block HIV-1 p24 antigen induction thereby bringing p24 antigen levels to the baseline. Approx. 40% reduction in p24 antigen production was observed in cultures which were TNF-α-stimulated and pretreated with 0.2 mM LA as compared to TNF-α-stimulated cells but were not pretreated with LA. In contrast, it required 50-times as much NAC to cause a similar effect (38% reduction). These findings support the results of the anti-NF-κB MTT assay (Fig. 2) in which LA was found to have a strong inhibitory effect while NAC was not found to be as effective. These results were reproducible and essentially the same observations were obtained over three times. Additionally, we have also examined the effects of other compounds known to block NF-κB including pentoxyphillin and sodium salicylate (data not shown). Among these compounds we examined, LA was found to be the most effective.

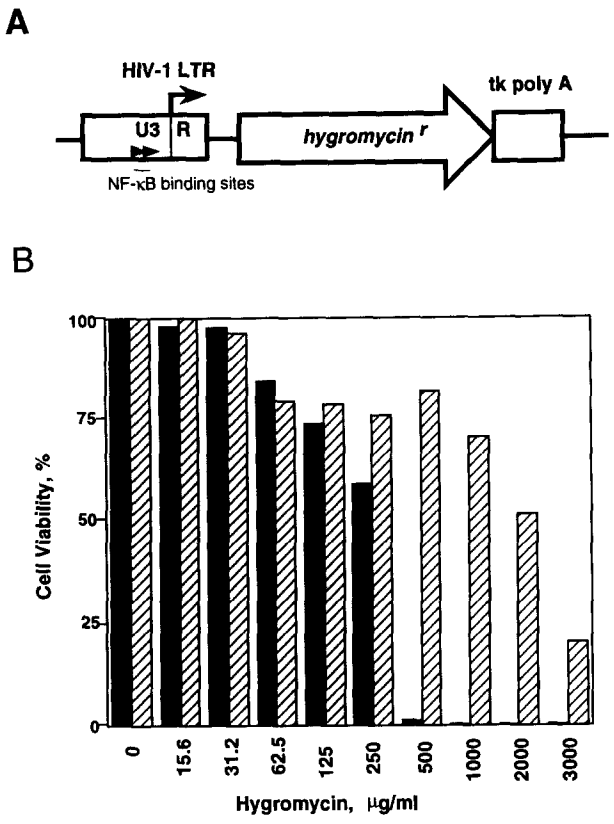

Fig. 1. Screening system for NF-κB inhibitors using recombinant THP-1 cells. (A) The DNA segment containing the transcription unit expressing the hygromycin phosphotransferase (hygromycin B resistance) gene under the control of HIV-1 LTR. The DNA fagment of *Xba*I-restricted pKO plasmid [19] was transfected into THP-1 human monocyte/macrophage cell line for the preparation of T-Xba recombinant clone. (B) Hygromycin resistance profile of THP-1 parental cells (closed bars) and T-Xba recombinant clone (cross-hatched bars). Cell viability, expressed as percent of negative control (no hygromycin B treatment), was measured by MTT assay after one week incubation of cells with varying concentrations of hygromycin.

4. Discussion

Data demonstrated in this study clearly indicate the feasibility of our test system in screening reagents that block HIV-1 gene expression. In the current assay, the promoter activity of HIV-1 LTR was examined as to the extent of expression of the hygromycin B phosphotransferase gene that endows cells with resistance to the toxicity of the antibiotic. The MTT assay used in this study is a modification of the test system described in our previous paper [19]. The pKO plasmid then used was cut by *Xba*I prior to transfection in this experiment to render Tat non-functional while leaving the NF-κB-dependent HIV-1 LTR promoter intact. Thus, in this study, the assay was redesigned to screen inhibitors of NF-κB and

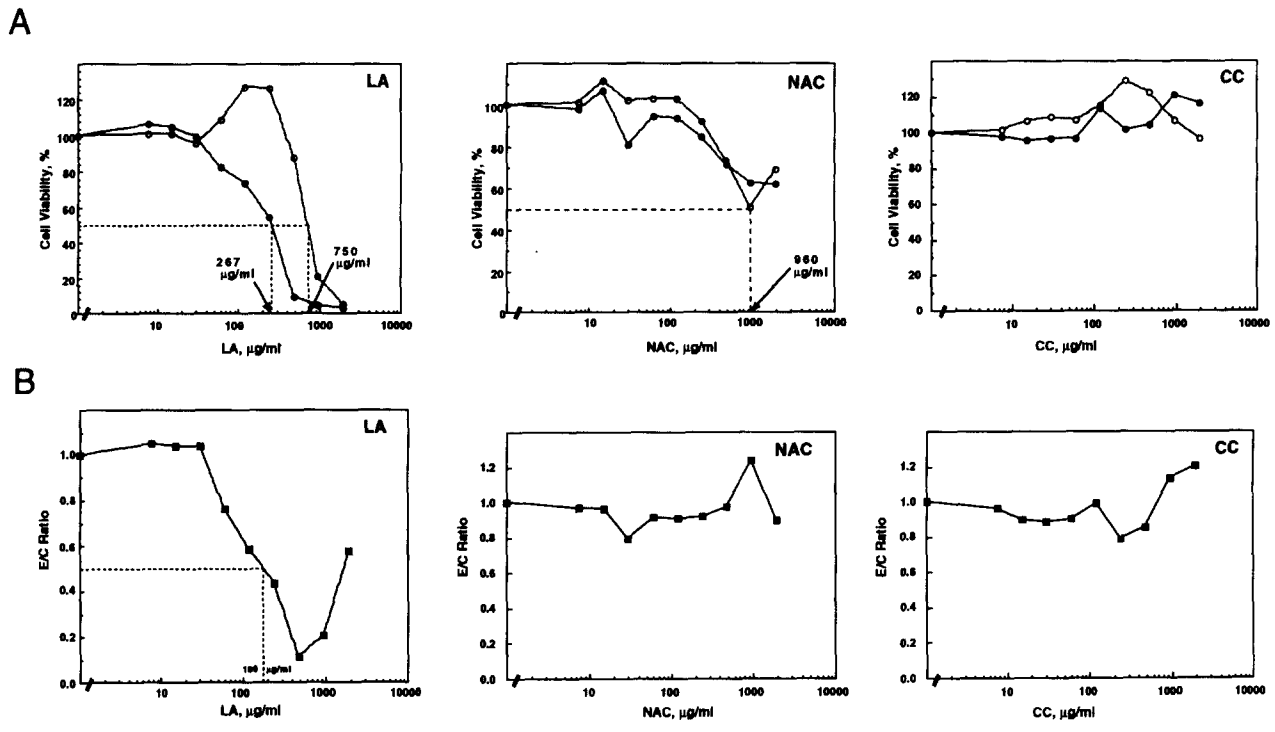

Fig. 2. Effects of LA and NAC on HIV-1 LTR-directed hygromycin resistance in T-Xba cells. (A) T-Xba cells were treated with varying concentrations of LA and NAC in the presence (●) or absence (○) of 500 µg/ml hygromycin B. Carbenicillin (CC) was used as a negative control. EC_{50} represents 50% effective concentration. (B) The effect-to-control (E:C) ratio values for each compound. The definition of E:C ratio was described previously as considering both the non-selective intrinsic cytotoxicity of a test compound and the selective effect of blocking the expression of hygromycin resistant gene [19]. An E:C ratio less than 1 is an indication of an effective inhibitory action of the drug on HIV-1 gene expression in this assay system.

possibly of other host transcription factors by measuring the level of cell viability in T-Xba recombinant host cells. By using 500 µg/ml hygromycin B, it was assured that the killing of T-Xba cells was due to the genuine effect of either LA or NAC since prior to the actual screening assay, such concentration was found to cause almost complete killing of THP-1 parental cells while approx. 80% of T-Xba recombinant cells remained viable.

To provide an additional measure of test validity, control cultures, that is, cells exposed to drugs but not to hygromycin B, were run in parallel with test cultures. Results represented the nonselective cytotoxicity of the drug and, therefore, were excluded in the calculation of E:C ratios [19]. EC_{50} values were also determined to compare the degree of anti-HIV-1 activities among the test drugs. We demonstrated here that LA exerted a stronger inhibitory effect against NF-κB-dependent HIV-1 gene expression compared to NAC. EC_{50} value was calculated to be 0.9 mM for LA, while it could not be determined for NAC because of its higher nonselective cytotoxicity. This was further confirmed by the results of the p24 antigen assay in which it required 50-times as much NAC to cause the same inhibitory action as LA.

There are several advantages of our recommended screening test compared to other methods. Specifically, it offers the advantages of being free from handling infectious virions and radioactive materials. It is also simple and gives reproducible results. The semi-automated spectrophotometric quantitation and subsequent computerized data processing makes the test easy to perform. These features make this assay suitable for complete automation for mass screening of potentially useful anti-HIV drugs.

Using this new system, we could confirm the earlier reports of the efficacy of LA and NAC [1–7,10–16]. LA and NAC have been shown to block the NF-κB-dependent gene expression by interfering with the signal transduction cascade leading to the activation of NF-κB [11–17]. It has been well established that the NF-κB activation cascade is redox regulated. There is a large body of evidence that ROI serve as common messengers in the activation of NF-κB. Both agents were found to exert an inhibitory effect on NF-κB-dependent HIV-1 gene expression as evidenced by the decreased resistance to hygromycin B (Fig. 2) and the inhibition of viral p24 induction upon TNF stimulation of OM 10.1 cells (Fig. 3). LA was found to have an advantage over NAC as its potency was evident at much lower concentrations. The mechanism of action of both compounds is known to be through direct free radical scavenging actions on ROI. However, while NAC is believed to inhibit NF-κB and HIV replication by replenishing intracellular cysteine required to produce GSH [10–12], LA, on the other hand, has the ability to increase the total intracellular thiol levels [4,6]. The intracellular build-up of the oxidized LA, DHLA and increase in GSH levels due to LA administration has been postulated to shift the redox status of the cell thus conceivably blocking the ROI-mediated NF-κB activation. These observations together with the earlier observations by Baur et al. [24] using the acute HIV-1 infection system suggested the clinical usefulness of LA in the treatment of AIDS and its related conditions.

In conclusion, we have reestablished the efficacy of LA and NAC, as measured by anti-NF-κB MTT and p24 antigen assays. Moreover, we have confirmed the applicability of the currently prescribed anti-NF-κB MTT assay. Thus, it can be

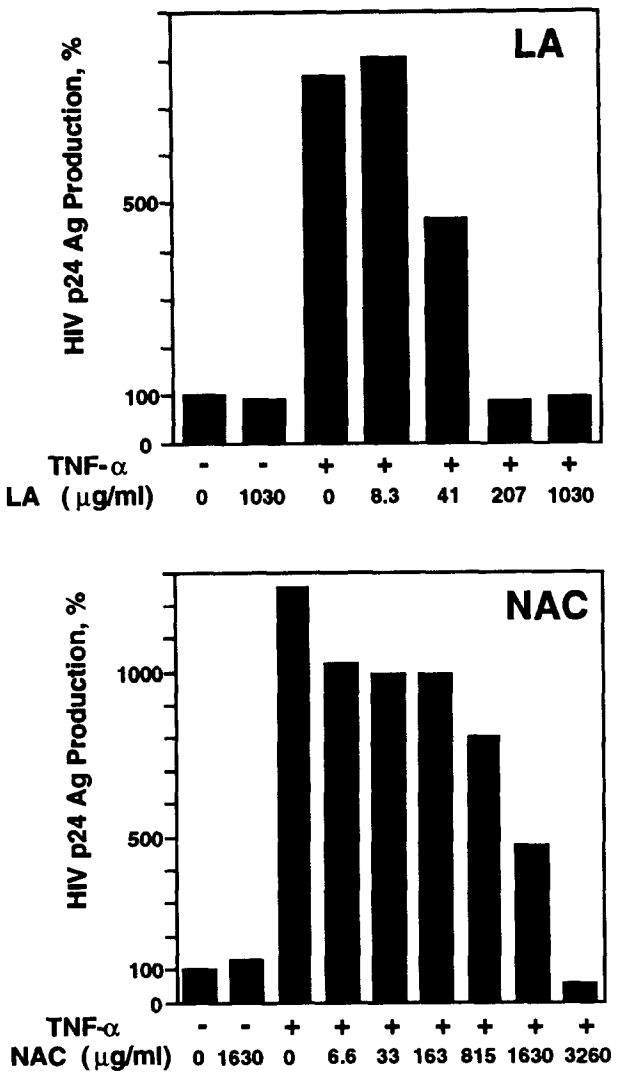

Fig. 3. Inhibitory activity of LA and NAC on HIV-1 p24 antigen expression in the TNF-α-stimulated OM 10.1 cultures. Cells were pretreated overnight with varying concentrations of LA or NAC. Following NF-κB stimulation with 2 ng/ml TNF-α for another 72 h, HIV-1 p24 antigen level was quantitated using solid phase enzyme immunoassay.

recommended for use in screening other candidate anti-NF-κB agents which can be used to prevent or to limit the progression of NF-κB-mediated diseases such as AIDS.

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