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Elevation of Glutathione Levels by Phase II Enzyme Inducers: Lack of Inhibition of Human Immunodeficiency Virus Type 1 Replication in Chronically Infected Monocytoid Cells

HANS J. PROCHASKA, LEWIS RUBINSON, YONG YEH, PENNY BARON, and BRUCE POLSKY

Molecular Pharmacology and Therapeutics Program (H.J.P., L.R., Y.Y.) and Infectious Disease Service (P.B., B.P.), Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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SUMMARY

Supplementation of media with high concentrations of thiols (5–20 mM) inhibits human immunodeficiency virus type 1 (HIV-1) replication *in vitro*. Compounds that prevent carcinogenesis via induction of phase II enzymes also elevate intracellular GSH levels, thus raising the possibility that chemopreventive enzyme inducers may represent a more pharmacologically feasible method to inhibit viral replication. Previous studies revealed that oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione] was the only GSH inducer tested that could inhibit HIV-1 replication in acutely infected H9 cells. Because thiols are proposed to suppress transcription of the integrated HIV-1 genome by preventing the activation of nuclear factor-κB, experiments evaluating inducers of GSH levels in acutely infected H9 cells do not rule out the ability of these compounds to inhibit viral replication in chronically infected cells exposed to cytokines or mitogens. Therefore, we determined the antiviral effects of several inducers in phorbol-12-myristate-13-acetate-stimulated U1 cells, a monocytoid cell line that contains two integrated copies of the HIV-1 genome. Although 1,2-dithiole-3-thione, dimethyl fumarate, and oltipraz can elevate cytosolic thiol levels, only oltipraz inhibited HIV-1 replication. Moreover, decreased nuclear factor-κB binding activity could be correlated with increases in cytosolic thiols produced by various treatments ($r^2 = 0.8$) but not with suppression of viral replication ($r^2 = 0.01$). These data suggest that oltipraz-induced increases in GSH are not responsible for the antiviral action of oltipraz and that elevation of intracellular GSH levels by chemopreventive enzyme inducers does not inhibit viral replication.

Many compounds protect against the toxic and neoplastic effects of carcinogens by raising the levels of phase II detoxication enzymes, as well as the enzymes responsible for the synthesis and maintenance of GSH pools (1–8). Based on reports demonstrating that GSH levels are depleted in HIV-1-infected individuals (9, 10) and that low molecular weight thiols can inhibit viral replication *in vitro* (11, 12), we were interested in whether these compounds were inhibitors of HIV-1 replication. Our laboratories have recently tested a number of monofunctional inducers (13) as inhibitors of HIV-1 replication in acutely infected H9 human cutaneous T cell lymphoma cells (14). Although all inducers tested were able to effectively elevate GSH levels, only oltipraz was active (14).

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Oltipraz [5-(2-pyrazinyl)-4-methyl-DTT], developed and tested clinically as an antischistosomal agent, exerts many biochemical effects. The antiparasitic activity of oltipraz may be related to the disruption of GSH metabolism in the schistosomes. Bueding *et al.* (15) showed that oltipraz depletes levels of GSH in schistosomes but paradoxically elevates GSH and phase II enzyme activities (e.g., glutathione *S*-transferase, UDP-glucuronosyltransferase, and QR) in mammalian tissues (3, 5, 6, 16). Its ability to coordinately induce phase II enzymes and GSH levels led Bueding and colleagues (4, 16) to predict that oltipraz was an anticarcinogen. Numerous studies in rodents have proven this assertion to be correct and have prompted a clinical evaluation of oltipraz as a human anticarcinogen (8, 17). Oltipraz (18, 19) and its metabolites (20) inactivate key GSH-dependent schistosomal enzymes. The inactivation of target enzymes by oltipraz may be relevant to its antiretroviral mechanism, because oltipraz inactivates HIV-1 RT (14).

ABBREVIATIONS: HIV, human immunodeficiency virus; RT, reverse transcriptase; NF-κB, nuclear factor-κB; PMA, phorbol-12-myristate-13-acetate; DMSO, dimethylsulfoxide; DMF, dimethyl fumarate; DTT, 1,2-dithiole-3-thione; NAC, *N*-acetyl-L-cysteine; QR, quinone reductase (EC 1.6.99.2); EMSA, electrophoretic mobility shift assay; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

The inhibition of RT, as well as the ineffectiveness of other inducers of GSH levels to inhibit the basal replication of HIV-1, suggests that oltipraz acts through a GSH-independent mechanism. However, low molecular weight thiols are proposed to suppress oxidant- or cytokine-induced transcription of the integrated HIV-1 genome by preventing the activation of NF-κB (21). Thus, the inability of GSH inducers to inhibit HIV-1 replication in acutely infected H9 cells may not rule out their ability to inhibit viral replication in cytokine- or mitogen-stimulated cells. We chose to examine chemopreventive enzyme inducers in the U1 cell line, a chronically infected monocytoid cell line that can be stimulated to replicate HIV-1 in response to a variety of agents, including PMA (12, 22).

Experimental Procedures

Materials. PMA, dithioerythritol, leupeptin, antipain, phenylmethylsulfonyl fluoride, Nonidet P-40, and polyethylene glycol (average molecular weight, 3350 g/mol) were purchased from Sigma Chemical Co. (St. Louis, MO), acrylamide and bisacrylamide were obtained from Bio-Rad (Melville, NY), T4 kinase was purchased from Stratagene (La Jolla, CA), G25 columns and poly(dI·dC) were purchased from Boehringer Mannheim (Indianapolis, IN), 10× TBE (890 mM Tris·borate, 20 mM EDTA, pH 8.0) buffer (diluted 71-fold before use) was purchased from Sun Bioscience (Seymour, CT), and [^{32}P]ATP (3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Oligonucleotides were prepared at the Memorial Sloan-Kettering Cancer Center Microchemistry Facility. The remaining reagents were obtained from the sources cited previously (14).

Cell line. The U1 cell line was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Rockville, MD). The cells were carried in suspension cultures grown at 37° in RPMI 1640 medium containing 20% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine. The cells were split 3–4 days before plating in microtiter wells or flasks, to ensure that they were in logarithmic phase at the start of each experiment.

Assay for inhibition of HIV-1 replication in microtiter plates. U1 cells were plated in microtiter wells at a density of 50,000 cells/well, in medium containing 0.1% DMSO with or without test compounds (final volume, 200 μl). After 24 hr of growth, a 2.5 nM final concentration of PMA in 10 μl of medium was added to all wells with the exception of unstimulated controls. After an additional 48 hr of growth, the supernatants were collected and assayed for p24 antigen as described by the manufacturer (Coulter HIV-1 p24 antigen assay, Hialeah, FL). Viable cell densities were measured by MTT staining (23) of non-PMA-stimulated cells.

Growth and stimulation of U1 cells in 25-cm² flasks. U1 cells were placed in 25-cm² flasks at a density of 2.5 × 10⁶ cells/10 ml of medium containing 0.1% DMSO, with or without oltipraz, DMF, DTT, or 10 mM NAC (positive control) (12). PMA stimulation (2.5 nM final concentration) occurred 0, 24, or 72 hr after the cells were exposed to drugs. For some studies, matched control cells were incubated in the absence of PMA so that biochemical parameters could be measured. PMA-stimulated (and, if applicable, matched control) cells were harvested either 4 or 48 hr after the addition of PMA. Aliquots of cells were removed before harvest for cell count determinations by Coulter counter as well as viability determinations by trypan blue exclusion. There were three flasks in each group.

Assay for low molecular weight thiol and QR levels. U1 cells were harvested and assayed for cytosolic low molecular weight thiol, QR, and protein content as described (14), except that the cells were lysed by the addition of 400 μl of a hypotonic buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml antipain), followed 15 min later by the addition of 25 μl of 10% Nonidet P-40 (24). Cells were vigorously vortexed for 15 sec and the resulting cytosols were collected by centrifugation of the cell lysates at 14,000 rpm for 1 min, in an Eppendorf 5402 refrigerated centrifuge. The resulting nuclear pellets were assayed for NF-κB binding activity (see below).

Assay of NF-κB binding activity. EMSA was performed with nuclear pellets as described (24). Each nuclear extract was assayed in the presence or absence of a 1000-fold excess of unlabeled oligonucleotide. Resulting gels were quantified with a βetascope 603 gel analyzer (Betagen, Waltham, MA) or examined by autoradiography.

Assay for inhibition of HIV-1 replication. Supernatants from harvested cells grown in flasks were collected and aliquots were assayed for p24 antigen levels and RT activities. For the RT assay, viral particles were collected and lysed as described (25), by incubation of 1 ml of cell-free culture supernatants with 333 μl of 30% polyethylene glycol overnight at 4° and centrifugation of the resulting suspension at 14,000 rpm for 5 min in an Eppendorf 5415C centrifuge. The pellets were lysed by the addition of 100 μl of a 20% glycerol solution containing 0.74 M KCl, 1 mM dithioerythritol, 0.5% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, and 50 mM Tris·HCl, pH 7.9. Aliquots (10 μl) were assayed in triplicate according to the method of Flexner *et al.* (26), except that KCl and detergent were not added to the assay buffer (25 μl).

Statistical treatment of results. To adequately represent the data without bias, all results except those in Table 1 were pooled from multiple independent experiments. In the case of data from microtiter plates and thiol levels from cells grown in flasks, the absolute values from individual experiments were used for statistical analysis. For other parameters shown, all values (including controls) were normalized to the average control values from a particular experiment. These independent normalized values (including controls) were then used as individual points for statistical analysis. The number of points per experimental group ranged between 3 and 18. Statistical significance was determined by the unpaired *t* test, using the InStat software package (GraphPAD, San Diego, CA).

Results

We assessed whether exposure of U1 cells to typical monofunctional inducers (13) (DMF, DTT, or oltipraz) 24 hr before PMA stimulation would inhibit viral replication in cells cultured in 96-well plates. As demonstrated previously in H9 cells (14), only oltipraz was able to inhibit p24 antigen release into the culture supernatants. Although there is a narrow therapeutic index for oltipraz in U1 cells (Fig. 1) that is not observed in acutely infected H9 cells (14), HIV-1 replication was inhibited in PMA-stimulated cells at nontoxic concentrations that are achievable in human serum (8, 27, 28).

Cells were grown in flasks so that biochemical and viral parameters could be measured simultaneously. We used 5,5′-dithiobis(2-nitrobenzoic acid) to measure cytosolic low molecular weight thiol levels (14) so that we could assay QR and NF-κB levels in the same cells. We have determined that GSH levels in control and inducer-treated cells determined by the method of Hissen and Hilf (29) are comparable to thiol levels reported here (Table 1). Oltipraz, DMF, DTT, and NAC all significantly elevated cytosolic low molecular weight thiol levels (Fig. 2). The magnitude of elevation of thiol levels by inducers in U1 cells is similar to that of increases observed in human H9 T cell lymphoma cells (14) and that of increases in GSH levels observed in Hepa 1c1c7 murine hepatoma cells (6). QR, a phase II enzyme, was also effectively induced by DMF, DTT, and oltipraz (data not shown). NAC had no effect on QR activities, indicating that it acts as a direct thiol group donor and does not possess inductive activity.

Oltipraz inhibited PMA-induced viral replication, as measured by p24 antigen levels and RT activities in the culture

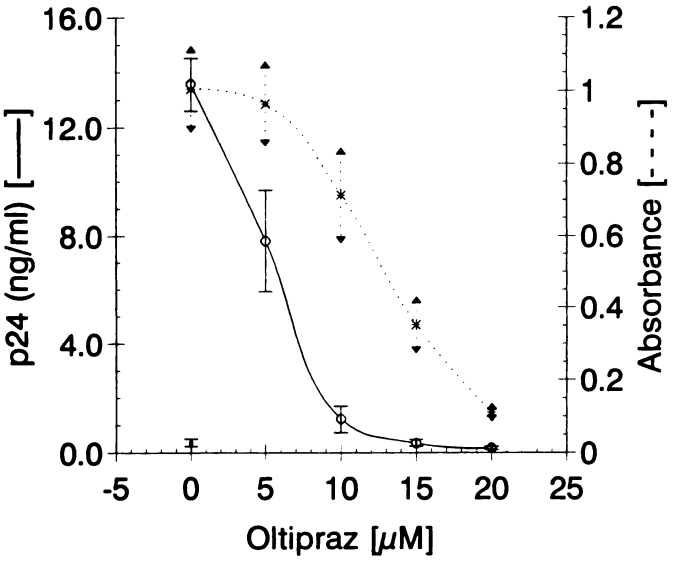

Fig. 1. Potency of inhibition of p24 antigen release (——) and cellular proliferation (- - -) by oltipraz in U1 cells. U1 cells were plated in microtiter wells at a density of 50,000 cells/well, in medium containing 0.1% DMSO with or without test compounds (final volume, 200 μl). p24 antigen levels were measured from culture supernatants collected at 72 hr from cells treated with 2.5 nM PMA at 24 hr (with the exception of unstimulated controls). p24 antigen levels in unstimulated cells were 0.368 ± 0.132 ng/ml (■). Viable cell densities were measured by MTT staining (23) in non-PMA-stimulated cells treated with oltipraz for 72 hr. The values shown are mean ± standard error for five and nine independent experiments for the p24 antigen and MTT stains, respectively.

TABLE 1

Comparison of cytosolic low molecular weight thiol levels (14) with GSH levels as determined by the method of Hissen and Hilf (29)

U1 cells were placed in flasks in medium containing 0.1% DMSO, with or without 30 μM DTT and were incubated for 24 hr, and the contents of the flasks were split into two fractions. One set of cells were assayed for thiol levels (14). The other set of cells were washed once in phosphate-buffered saline, and cellular proteins were precipitated with 5% trichloroacetic acid. The resulting supernatants were assayed for GSH levels (29). Results are typical of three independent experiments. Values are mean ± standard error (four flasks/group).

Treatment	Thiol levels	GSH levels
	nmol/mg of protein	*nmol/mg of protein*
Control	63.9 ± 4.9	60.0 ± 3.5
30 μM DTT	119 ± 14	114 ± 6

supernatants from cells grown in flasks. DMF and DTT were notable for their increases in both measures of viral replication. NAC inhibited release of RT into the culture suparnatants, as reported previously (12). Interestingly, p24 antigen release was much less impressively suppressed by NAC treatment. As has been demonstrated by Kalebic *et al.* (12), levels of RT in cell lysates are more effectively suppressed than are other viral antigens in stimulated U1 cells.

Although oltipraz exerted an antiproliferative effect on U1 cells grown in flasks, its antiviral activity was apparent under conditions where cell counts were not diminished, relative to controls (Fig. 3). Indeed, under several conditions cell counts of oltipraz-treated cells were increased, relative to controls, after 48 hr of PMA treatment. The antiviral effect of oltipraz was potentiated in a preincubation time-dependent manner.

Low molecular weight thiols are thought to exert antiretroviral activity by inhibiting the transcription of the integrated HIV-1 genome via retardation of oxidant- or cytokine-induced activation of NF-κB (21). As can be seen with EMSA (Figs. 4

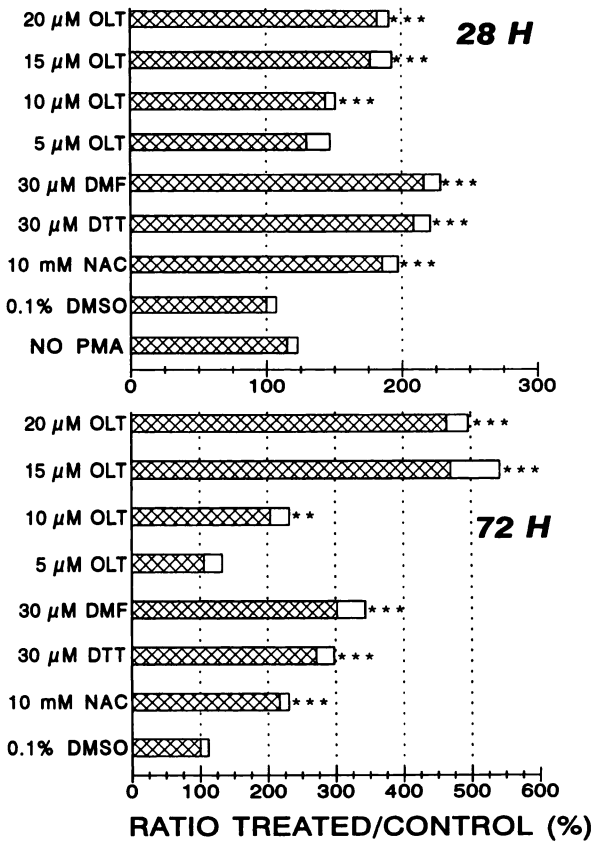

Fig. 2. Effect of oltipraz (*OLT*), DMF, DTT, and NAC on cytosolic low molecular weight thiol levels in the U1 cell line. Cells (2.5×10^5/ml) were placed in 10 ml of medium containing 0.1% DMSO, with or without test compounds, at 0 hr. *Upper*, cells were grown for 24 hr, 2.5 nM PMA was added, and the cells were harvested 4 hr later. *Lower*, cells were grown and harvested under identical conditions except that the cells were grown for 72 hr in the absence of PMA. ⊠, Mean values; □, standard error values. Control levels of cytosolic low molecular weight thiols at 28 and 72 hr were 58.1 ± 4.1 nmol/mg of protein (14 determinations) and 13.3 ± 1.6 nmol/mg of protein (14 determinations), respectively. Statistical analyses were carried out as described in Experimental Procedures. **, $p < 0.005$; ***, $p < 0.0005$.

and 5), treatment with GSH inducers and NAC decreased the PMA-induced activation of NF-κB (the upper band represents the transcriptionally active form of NF-κB) (21). The bands are specific for NF-κB, because unlabeled NF-κB probe could effectively compete with radiolabeled probe, whereas irrelevant oligonucleotides could not (Fig. 4). The relative levels of NF-κB after treatment of cells with inducers for 28 hr and with PMA for 4 hr before harvest are given in Table 2.

We compared levels of NF-κB binding (Table 2) with cytosolic thiol levels as well as RT activities in culture supernatants from cells cultured under identical conditions for an additional 44 hr. As can be seen in Fig. 6, a reasonably linear relationship can be seen for thiol levels versus NF-κB binding ($r^2 = 0.8$). However, RT activities are not correlated with NF-κB binding ($r^2 = 0.01$).

Discussion

We initially tested oltipraz and other chemopreventive enzyme inducers as inhibitors of HIV-1 replication because of their ability to elevate GSH levels (14). Such a therapeutic strategy might be a more desirable alternative to the direct

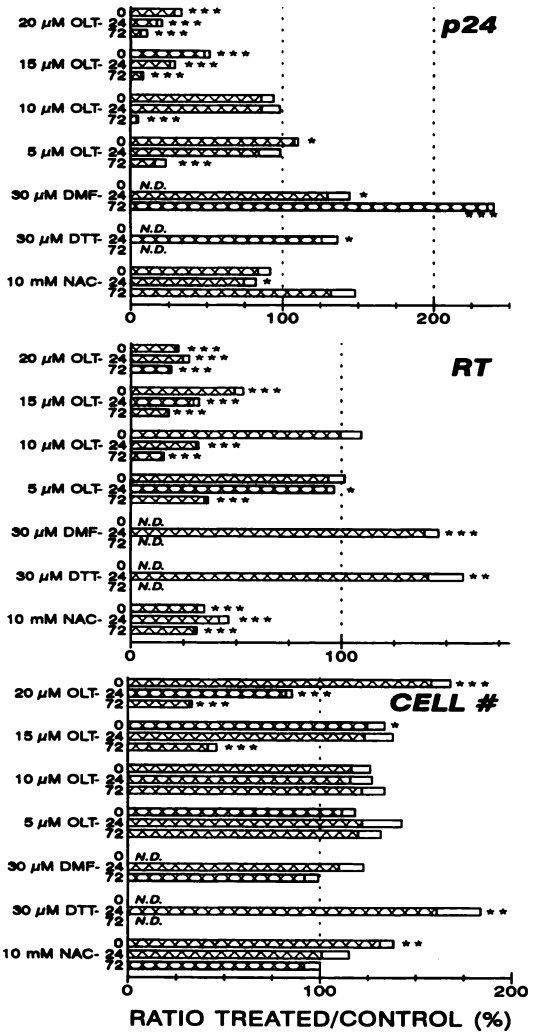

Fig. 3. Effect of oltipraz (*OLT*), DMF, DTT, and NAC on HIV-1 replication and cell growth in PMA-stimulated U1 cells. Cells (250,000 cells/ml of medium) were incubated in the presence of drug for the times indicated and then exposed to 2.5 nM PMA for an additional 48 hr. The cells were counted (*lower*) and culture supernatants were assayed for p24 antigen levels (*upper*) and RT activities (*middle*) as described in Experimental Procedures. Viabilities were scored by trypan blue exclusion and were >90% for all conditions shown. All data were normalized to PMA-stimulated cells grown in matched tissue culture flasks (three flasks/group). The data shown are the mean (⊠) and standard error (□) values derived from three to 18 experimental points (one to six independent experiments). The p24 antigen levels and RT activities from culture supernatants of unstimulated U1 cells cultured for 72 hr were 3.6 ± 0.5% (11 determinations) and 8.5 ± 3.1% (14 determinations), respectively, of levels measured from supernatants of U1 cells stimulated with PMA at 24 hr and harvested at 72 hr. Statistical analyses were carried out as described in Experimental Procedures. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. *N.D.*, not determined.

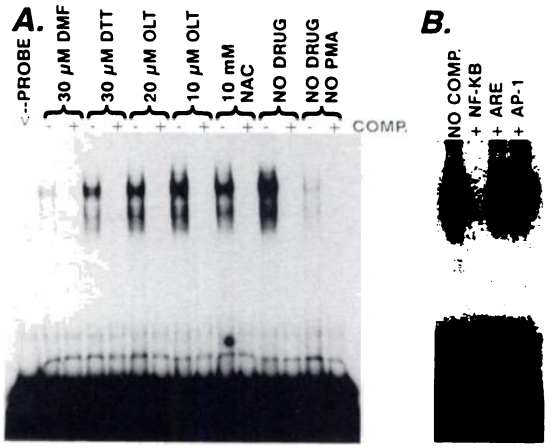

Fig. 4. Effect of inducers of GSH levels on NF-κB binding activity. A, NF-κB binding activity after 4 hr of PMA stimulation in U1 cells that were pretreated for 28 hr with DMF, DTT, oltipraz (*OLT*), or NAC. Cells (2.5×10^6) were placed in 10 ml of medium containing 0.1% DMSO, with or without test compounds, at 0 hr, exposed to 2.5 nM PMA at 24 hr (except no-drug/no-PMA cells), and harvested 4 hr later. Nuclear extracts were obtained and EMSA was performed as described in Experimental Procedures. + or − *COMP.*, presence or absence, respectively, of a 1000-fold excess of unlabeled NF-κB probe. B, NF-κB binding activity from PMA-stimulated U1 cells in the absence of unlabeled oligonucleotide (*NO COMP.*) or in the presence of a 1000-fold excess of unlabeled NF-κB (24), antioxidant response element (*ARE*) (also known as EpRE) (30), or AP-1 (30) oligonucleotides.

administration of thiols, because high concentrations (5–20 mM) of NAC or GSH in the medium are required to inhibit HIV-1 replication *in vitro* (11, 12). Our prior investigations showed that oltipraz was unique among inducers tested in its ability to inhibit HIV-1 replication, and we identified RT as a potential target for the antiviral action of oltipraz (14). Our results did not rule out a beneficial effect of raising GSH levels, because we examined only basal replication. The present study demonstrates that elevation of GSH levels by chemopreventive enzyme inducers does not play a significant role in the inhibition of HIV-1 replication, and it may indicate that the *in vitro* antiviral effects of low molecular weight thiols are more complex than has been proposed. Moreover, DMF and DTT, two inducers of GSH levels, stimulate viral replication. This phenomenon has been recently observed with another inducer of xenobiotic metabolism, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (31).

Although reasons for the poor correlation between the inhibition of NF-κB binding and antiretroviral activity are unclear (Fig. 6), two factors bear consideration. First, none of the drugs tested completely blocked the PMA-induced activation of NF-κB. Second, the compounds tested induce phase II enzymes and are therefore able to regulate transcription (32). The HIV-1 genome is known to interact with a number of *trans*-acting factors; perhaps one or more of these are affected by treatment with xenobiotics. In regard to this last point, it is interesting to note that the HIV-1 genome contains tandem and functionally active AP-1 recognition sites (33, 34). Because the transcriptional activation of glutathione *S*-transferase and QR by inducers is mediated via a novel regulatory element that contains two tandem AP-1-like sequences (30, 32), it is possible that PMA (as well as DTT and DMF) is able to activate HIV-1 replication through these sites.

Oltipraz exerts a significant time-dependent antiviral effect on U1 cells. Such a phenomenon was not observed in a basal replication model with H9 cells, and this may suggest multiple mechanisms for the inhibition of viral replication. In agreement with oltipraz possessing multiple pharmacological activities are observations we have made with a substituted DTT that is 5-fold more potent than oltipraz in our basal replication model. It shares with oltipraz the kinetic behavior of inactivating HIV-

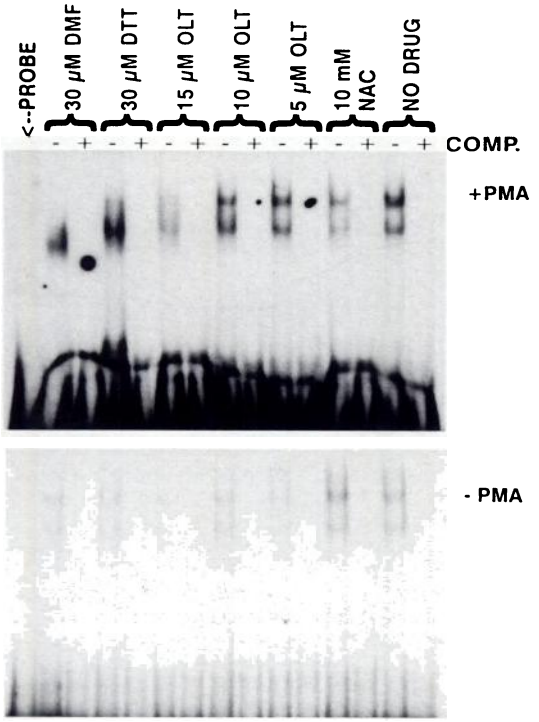

Fig. 5. Activity of NF-κB binding in U1 cells pretreated for 72 hr with DMF, DTT, oltipraz (*OLT*), or NAC, with (*upper*) or without (*lower*) PMA stimulation for 48 hr before harvest. Cells (2.5×10^6) were placed in 10 ml of medium containing 0.1% DMSO, with or without test compounds, at 0 hr. *Upper*, NF-κB binding of nuclear extracts from cells exposed to PMA at 24 hr and harvested at 72 hr. *Lower*, NF-κB binding activity of nuclear extracts from cells harvested 72 hr after exposure to drugs without PMA stimulation. EMSA was performed as described in Experimental Procedures. Incubation conditions for PMA-stimulated and unstimulated cells were identical, as were electrophoresis conditions and exposure to autoradiography. + or − *COMP.*, presence or absence, respectively, of a 1000-fold excess of unlabeled oligonucleotide NF-κB probe.

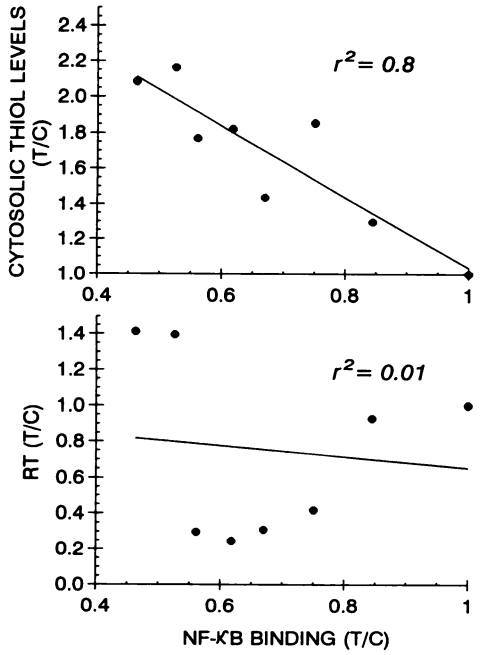

Fig. 6. Relationship of cytosolic low molecular weight thiol levels and RT activities to NF-κB binding activities. *Upper*, thiol levels as a function of NF-κB binding activities in U1 cells grown for 28 hr in the presence of test compounds and for 4 hr with 2.5 nM PMA before harvest. *Lower*, RT activities of culture supernatants from cells treated with test compounds for 72 hr and with 2.5 nM PMA for 48 hr before harvest as a function of NF-κB binding activities of cells exposed to test compounds for 28 hr and to PMA for 4 hr before harvest. *T/C*, treated/control.

TABLE 2

NF-κB binding activity in response to pretreatment with oltipraz, DMF, DTT, or NAC

Cells (2.5×10^6) in 10 ml of medium containing 0.1% DMSO, with or without test compounds, were placed in 25-cm² flasks. After 24 hr of growth, 2.5 nM PMA was added, and the cells were grown for an additional 4 hr. Cells were harvested and assayed for NF-κB binding by EMSA as described in Experimental Procedures. The radioactivity from the upper band of each lane was quantitated with a Betagen βetascope 603 gel analyzer and normalized to the PMA-stimulated control. Values are mean ± standard error.

Treatment	NF-κB binding activity (treated/PMA-stimulated control)	Inhibition	*n*[a]
		%	
20 μM Oltipraz	0.62 ± 0.05	54	2
15 μM Oltipraz	0.56 ± 0.03	63	3
10 μM Oltipraz	0.67 ± 0.09	47	4
5 μM Oltipraz	0.85 ± 0.25	21	3
30 μM DMF	0.53 ± 0.11	67	5
30 μM DTT	0.46 ± 0.03	77	4
10 mM NAC	0.75 ± 0.05	36	5
No drug (+ PMA)	Δ1[b,c]	Δ0	5
No PMA treatment	0.30 ± 0.04	Δ100	5

[a] *n*, number of experiments.
[b] Δ, defined as.
[c] For each experiment, the radioactivity measured for all groups was normalized to the radioactivity measured for the no-drug/+PMA group.

1 RT but does not inhibit HIV-1 replication in PMA-stimulated U1 cells.[1]

Oltipraz has already been used in humans to treat schistosomiasis. Its effectiveness as an inhibitor of experimental carcinogenesis has led to the clinical evaluation of oltipraz as a potential human anticarcinogen (8, 17). Although the narrow therapeutic index of oltipraz in U1 cells raises questions about its safety, it is clear that a significant antiviral effect can be exerted at nontoxic concentrations. It is notable that 5 μM oltipraz in microtiter wells and 5–10 μM oltipraz in flasks inhibited viral replication in U1 cells without evidence of cytotoxicity (Figs. 1 and 3). Concentrations of oltipraz that inhibit viral replication *in vitro* are achievable in humans (8, 27, 28). Moreover, plasma levels of oltipraz in rats chronically fed a diet supplemented with a nontoxic concentration of the drug (400 ppm) were ≈3 μM (7). Given that oltipraz has a greater antiviral effect with prolonged incubation time in U1 cells (Fig. 3), these plasma concentrations may be adequate to inhibit viral replication.

[1] 5-(2-Thienyl)-4-methyl-DTT was among the 70 dithiolethiones we screened for anti-HIV-1 activity in H9 cells using our immunofluorescence assay (14). The IC_{50} for viral replication was 2.2 ± 0.8 μM (seven experiments) (H. J. Prochaska and B. Polsky, unpublished observations).

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Send reprint requests to: Hans J. Prochaska, Laboratory for Chemical-Biological Interactions, Molecular Pharmacology and Therapeutics Program, RRL 601-E, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.