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Oltipraz, an inhibitor of human immunodeficiency virus type 1 replication

(1,2-dithiole-3-thiones/glutathione/chemoprotection/reverse transcriptase/AIDS)

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ABSTRACT **Glutathione depletion may play a pivotal role in the pathogenesis of human immunodeficiency virus type-1 (HIV-1) infection. Since certain compounds prevent experimental carcinogenesis by elevating the levels of glutathione and phase II detoxication enzymes, we compared the potencies of several inducers with their ability to inhibit basal levels of HIV-1 replication in H9 cutaneous T-cell lymphoma cells. All monofunctional inducers tested elevated the levels of glutathione and quinone reductase, a marker for phase II enzyme induction. However, only oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione] was effective at inhibiting HIV-1 replication (IC_{50} = 14.8 ± 3.1 μM). The antiviral effect of oltipraz was potentiated by 3′-azido-3′-deoxythymidine. Thus, 1,2-dithiole-3-thiones represent a hitherto unrecognized class of anti-HIV-1 agents. Oltipraz behaves kinetically as an irreversible inhibitor of HIV-1 reverse transcriptase in the template–primer binding domain. Oltipraz has been used to treat schistosomiasis in humans and is undergoing clinical evaluation as an anticarcinogen. Thus, oltipraz (and other 1,2-dithiole-3-thiones) may have therapeutic utility in HIV-1-infected individuals, not only because of their antiretroviral activity, but also by preventing the development of HIV-1-associated neoplasms.**

Reduced glutathione (GSH), a major antioxidant defense (1), is depleted in plasma and peripheral blood mononuclear cells of patients infected with human immunodeficiency virus type 1 (HIV-1). (2, 3). The role of oxidative stress in general and GSH in particular in modulating HIV-1 infection has been inferred from the following (reviewed in ref. 4). (*i*) GSH is required for a variety of immune functions. (*ii*) HIV-1 infection is associated with elevated levels of inflammatory cytokines which generate intracellular oxidants. (*iii*) HIV-1 transcription *in vitro* is dramatically increased by inflammatory cytokines as well as by H_2O_2. (*iv*) *N*-Acetylcysteine and a GSH ester inhibit HIV-1 replication in chronically and acutely infected cells (5–7). The inhibition of transcription by nuclear factor κB is thought to be the mechanism by which these thiols inhibit HIV-1 replication (8).

The administration of low molecular weight thiols to raise GSH levels has been advocated as a method to treat HIV-1-infected patients (9–13). Another unexplored strategy to raise GSH levels is the use of anticarcinogenic enzyme inducers. Compounds such as flavones, 1,2-dithiole-3-thiones, thiocarbamates, isothiocyanates, phenolic antioxidants, and α,β-unsaturated ketones block experimental carcinogenesis by inducing phase II detoxication enzymes {quinone reductase [QR; NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2], glutathione transferases, UDP-glucuronosyltransferases}, as well as enzymes responsible for the synthesis and maintenance of GSH pools (14–18). These compounds hold promise for the prevention of cancer in humans, especially since protection is not carcinogen- or organ-specific. Moreover, anticarcinogenic enzyme inducers are components of the human diet (19–22) or are consumed as food additives or medicines. One inducer, oltipraz [4-methyl-5-(2-pyrazinyl)-1,2-dithiole-3-thione], is under clinical evaluation as an anticarcinogen (23) because it potently inhibits experimental carcinogenesis (24–28) and has been used in humans as an antischistosomal agent (29–34).

These studies were undertaken to ascertain whether typical inducers of endogenous GSH synthesis could suppress the replication of HIV-1 in acutely infected cells.

MATERIALS AND METHODS

Materials. Oltipraz and 1,2-dithiole-3-thione (Fig. 1) were generous gifts of Thomas W. Kensler of the Johns Hopkins School of Hygiene and Public Health. Dimethyl fumarate, *tert*-butylhydroquinone (crystallized from ethyl acetate), Sudan III [1-(4-phenylazophenylazo)-2-naphthol], β-naphthoflavone, and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Aldrich. Test compounds were dissolved as 1000–2000× stocks in spectral-grade dimethyl sulfoxide (DMSO; Fisher, Fair Lawn, NJ) and were stored at −80°C until used. Bradford reagent was obtained from Bio-Rad. 3′-Azido-3′-deoxythymidine (AZT, zidovudine) and AZT 5′-triphosphate were from Burroughs Wellcome and Moravek Biochemicals (Brea, CA), respectively. Poly(A) and oligo(dT) (n = 10) were from Pharmacia. [α-^{32}P]dTTP (800 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Recombinant HIV-1 reverse transcriptase (RT) was from Worthington. DE81 paper was from Whatman. Centricon-10 microconcentrators were from Amicon (Danvers, MA). Goat anti-human IgG-fluorescein isothiocyanate conjugate was from ICN. Antiserum directed against HIV-1 was obtained from our patient population. Twelve-well HTC supercured 5-mm slides were from Cell Line Associates (Newfield, NJ). HIV-1 p24 antigen assay kits were from Coulter. Other reagents were from Sigma.

Cell Line and Virus. H9 cutaneous T-cell lymphoma cells and the HTLV-IIIB isolate of HIV-1 were obtained from R. Gallo, National Cancer Institute, Bethesda, MD. The cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 2 mM glutamine at 37°C in a humidified atmosphere of 5% CO_2.

Assay of GSH and QR. H9 cells (5 × 10^6) were grown in 25-cm^2 tissue culture flasks containing 10 ml of medium

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Abbreviations: HIV, human immunodeficiency virus; GSH, reduced glutathione; QR, quinone reductase; AZT, 3′-azido-3′-deoxythymidine; DMSO, dimethyl sulfoxide; $TCID_{50}$, 50% tissue culture infectious dose; IFA, indirect immunofluorescence assay; RT, reverse transcriptase.

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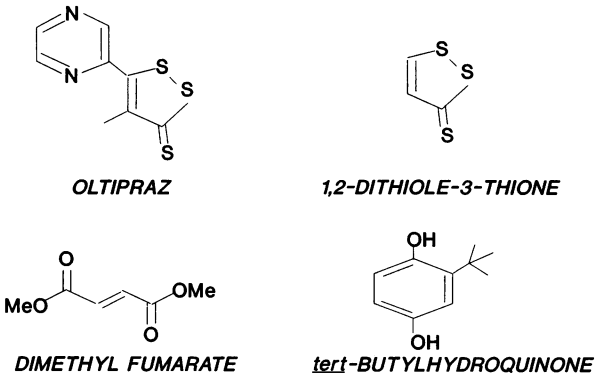

FIG. 1. Structures of GSH/phase II enzyme inducers tested for inhibition of HIV-1 replication.

containing 0.05% DMSO with or without test compounds until harvested (three flasks per group). After the cells were washed with ice-cold Dulbecco's phosphate-buffered saline (DPBS) three times, the cell pellets were lysed with 400 μl of ice-cold DPBS containing 0.5% Triton X-100. Supernatants were isolated by centrifugation (5000 × *g* for 20 min) and were kept at 0–4°C until the QR or GSH spectrophotometric assays were performed. QR levels were assayed in quadruplicate as described for human lymphocytes (35). Dicoumarol-inhibitable rates were <5% of the total rate. For GSH assay, a 300-μl sample was placed in a Centricon-10 microconcentrator and centrifuged (5000 × *g* for 45 min) until enough ultrafiltrate could be collected. Sample in quadruplicate (50 μl per well) was placed in a 96-well "half-area" plate and 20 μl of 1 mM 5,5'-dithiobis(2-nitrobenzoic acid) in DPBS was added. The yellow color (36) developed within 5 min and was measured at 405 nm with a microtiter-plate scanner. The optical densities of the specimens were calibrated to a GSH standard curve. Protein was determined as described (37); protein levels in the ultrafiltrates employed for GSH determinations were negligible (<0.01 mg/ml).

Assay for Inhibition of HIV-1 Replication. H9 cells were infected with HIV-1 at 1000 $TCID_{50}$ (50% tissue culture infectious doses) per 10^6 cells. Virus was allowed to adsorb for 1 hr, after which unadsorbed virus was removed by centrifugation. The cells were resuspended and washed twice with DPBS and centrifuged to collect the cell pellet (150 × *g* for 10 min). Cells were suspended in fresh medium and added at a final density of 500,000 cells per ml to a 96-well plate with the medium containing 0.1% DMSO and various concentrations of test compounds. The cells were refed freshly prepared medium containing 0.1% DMSO with or without test compounds on day 4 (for preinduction experiments, cells were exposed to inducers on days −4 or −2, 0, and +4 relative to the day of infection). After 7 days, cells were examined for cytotoxicity by trypan blue exclusion and for HIV-1 infection by p24 antigen release into culture supernatants according to the manufacturer's instructions (Coulter assay kit) or by indirect immunofluorescence assay (IFA). For IFA, cells were washed twice in DPBS and harvested onto 5-mm 12-well slides, air-dried, and fixed with acetone for 5 min. HIV-1-positive serum diluted 1:5 in DPBS was added to each well and incubated at 37°C for 30 min. After incubation, the slides were washed twice with DPBS, rinsed in deionized water, air-dried, and incubated at 37°C for 30 min with goat anti-human IgG-fluorescein isothiocyanate conju-

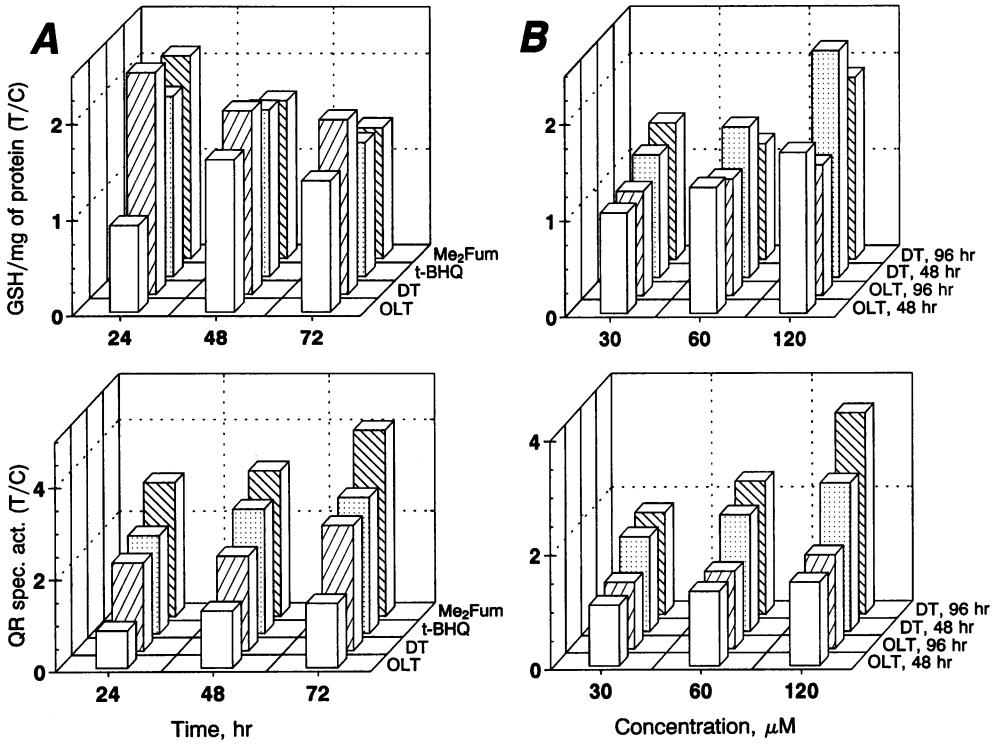

FIG. 2. Elevation of GSH levels (*Upper*) and QR specific activities (*Lower*) by monofunctional inducers in H9 cells. (*A*) QR and GSH in cells treated with 120 μM oltipraz (OLT), 120 μM 1,2-dithiole-3-thione (DT), 30 μM *tert*-butylhydroquinone (t-BHQ), or 30 μM dimethyl fumarate (Me_2Fum) for 24, 48, or 72 hr. (*B*) Induction of QR and GSH by oltipraz or 1,2-dithiole-3-thione as a function of concentration in H9 cells at 48 and 96 hr. Values are shown as treated/control (T/C) ratios. All values shown are significantly greater than control ($P < 0.05$) except for the following: in *A*, QR and GSH in cells treated with oltipraz for 24 hr ($P = 0.44$ and 0.18, respectively) and GSH in cells treated with dimethyl fumarate for 72 hr ($P = 0.057$); in *B*, QR and GSH in 30 μM oltipraz-treated cells at 48 hr ($P = 0.27$ and 0.53, respectively), GSH in 30 μM oltipraz-treated cells at 96 hr ($P = 0.57$), GSH in 30 μM 1,2-dithiole-3-thione-treated cells at 48 hr ($P = 0.12$), and GSH in 60 μM 1,2-dithiole-3-thione-treated cells at 96 hr ($P = 0.18$). Control QR specific activities and GSH levels for seven separate experiments (average of means ± standard error of the averages) were 131 ± 5.7 nmol/min per mg and 59.5 ± 4.8 nmol per mg of protein, respectively.

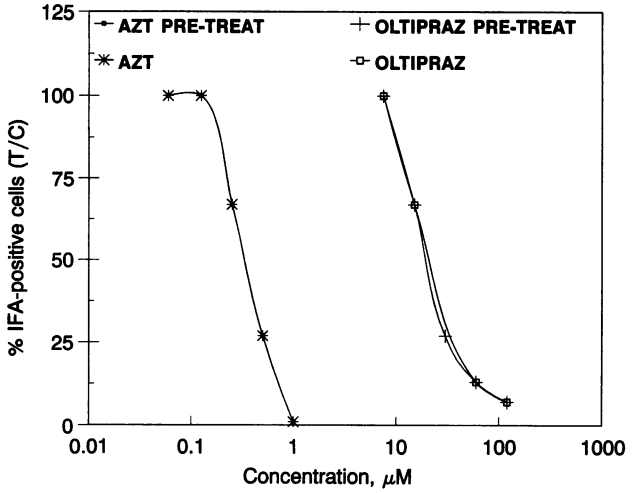

FIG. 3. Concentration dependence of inhibition of HIV-1 replication in H9 cells by oltipraz and AZT. H9 cells were grown, treated with compounds, exposed to virus, and assayed for inhibition of HIV-1 replication by IFA. The pretreated cells were exposed to drugs 96 hr prior to infection.

gate diluted 1:20 in DPBS containing 0.01% Evans blue. The slides were then washed twice with DPBS, rinsed with deionized water, dried, mounted with 50% glycerol in DPBS, and scored for the percentage of fluorescent cells by fluorescence microscopy.

Assay of RT. For RT assays (38) performed with oltipraz and 1,2-dithiole-3-thione, the assay mixture contained 0.13–0.29% DMSO, which had no effect on RT activity. All experimental points were measured in triplicate; SEMs were 1–8% of the mean values.

Data Analysis and Statistical Methods. Statistical significance for GSH and QR levels were established by the unpaired *t* test. The kinetic parameters for the inhibitory effects of oltipraz on RT were determined by performing Segal transformations (39) of Dixon plots or by Kitz–Wilson plots (40). The dose–effect relationships of oltipraz and AZT, alone or in combination, were determined by the median-effect method of Chou and Talalay (41). Calculations (including linear regression analyses) were performed on spreadsheets with Lotus 1-2-3 software (Cambridge, MA).

RESULTS AND DISCUSSION

Since thiols inhibit HIV-1 replication *in vitro*, we tested the proposal that inducers of endogenous GSH synthesis would be effective anti-HIV-1 agents. We first established the ability of inducers to increase GSH and phase II enzymes in H9 cells. Typical monofunctional inducers§ (Fig. 1; ref. 42) of GSH and QR levels are active (Fig. 2), and their potencies in H9 cells are similar to potencies found in murine cells (42–45). Bifunctional inducers§ (42) such as Sudan III and β-naphthoflavone, which require intact Ah (aryl hydrocarbon) receptor function, did not raise either QR or GSH levels

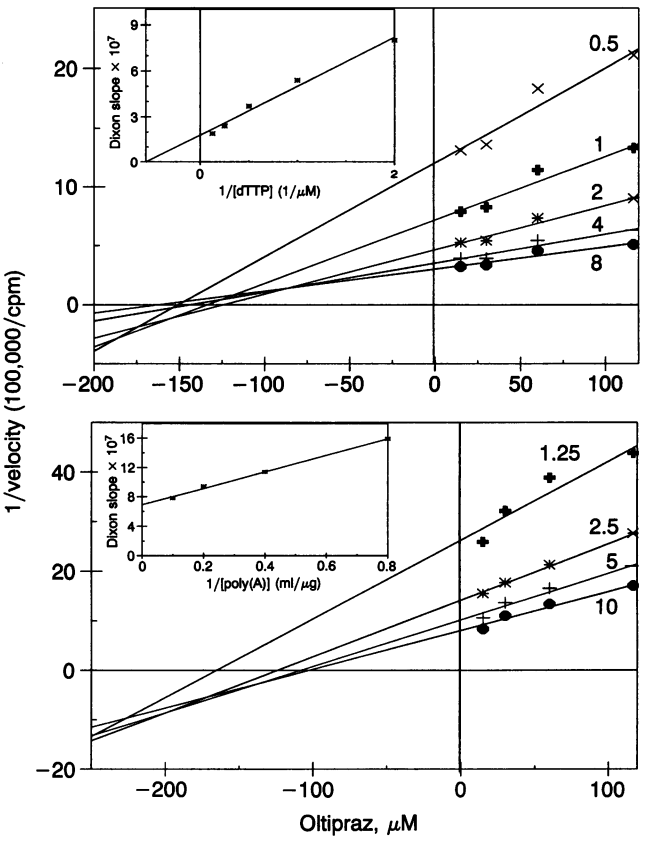

FIG. 4. Inhibition by oltipraz of HIV-1 RT with respect to dTTP (*Upper*) and poly(A)·oligo(dT) (*Lower*). Rates of dTTP incorporation into DNA were measured as described (38) with a range of concentrations of oltipraz with either fixed concentrations of dTTP (0.5, 1, 2, 4, and 8 μM) in the presence of poly(A) at 5 μg/ml and oligo(dT) at 2.5 μg/ml (*Upper*) or fixed concentrations of a 2:1 ratio of poly(A) to oligo(dT) [poly(A) at 1.25, 2.5, 5, and 10 μg/ml] in the presence of 4 μM dTTP (*Lower*). The K_i values were determined from the secondary Segal transformations (*Insets*), which were generated from the Dixon plots (1/velocity vs. [oltipraz]) as described (39).

at concentrations associated with induction in murine cells (1–5 μM; structures and data not shown). Since only monofunctional inducers are active inducers of QR in human lymphocytes (35), H9 cells represent a useful human lymphocyte model in which to study these chemoprotective agents.

Oltipraz was the only anticarcinogenic enzyme inducer tested that inhibited HIV-1 replication. The IC_{50} as determined by p24 assays was 14.8 ± 3.1 μM (n = 3 experiments). Oltipraz is 100- to 300-fold less potent than AZT (Fig. 3). Pretreatment of H9 cells with inducing agents 2 or 4 days prior to exposure to virus did not increase the antiviral effect of oltipraz (Fig. 3), and the other compounds tested remained inactive (data not shown).

Why should exogenous thiols inhibit HIV-1 replication when inducers of endogenous levels are inactive? First, the concentrations of thiols required to inhibit viral replication *in vitro* are high (5–30 mM) (5–7). This may explain why *N*-acetylcysteine did not alter surrogate markers for HIV-1 in infected patients in a recent pharmacokinetics study.¶ Second, in contrast to most studies with thiols as inhibitors of HIV-1 replication (5, 6), our model did not make use of inflammatory agents such as tumor necrosis factor α, inter-

§Anticarcinogenic enzyme inducers can be segregated into two families of compounds (16, 42). As defined by Prochaska and Talalay (42), *monofunctional* inducers (e.g., phenolic antioxidants, 1,2-dithiole-3-thiones, isothiocyanates, carbamates) elevate levels of phase II enzymes while exerting relatively minor and variable effects on phase I enzymes. *Bifunctional* inducers (e.g., azo dyes, flavones) elevate phase II enzyme levels as well selected phase I activities such as aryl hydrocarbon hydroxylase. The former compounds possess no obvious structural features in common, whereas the latter can all be characterized as flat planar polycyclic hydrocarbons and are potent ligands for the aryl hydrocarbon receptor. The molecular mechanisms for signaling the induction of phase II enzymes by these compounds has been recently reviewed (18).

¶Walker, R., Lane, H. C., Boenning, C. M., Pots, M. A., Kovacs, J. A., Falloon, J., Davey, R. T., Sussman, H., Gabel, L., Correa-Coronas, R., Masur, H. & Fauci, A. S. (1992) 8th International Conference on AIDS, July 19–24, Amsterdam, abstract MoB 0022.

leukin 6, or phorbol 12-myristate 13-acetate. Although *N*-acetylcysteine did inhibit HIV-1 replication in unstimulated MOLT-4 cells (IC_{50} = 5 mM; ref. 5), it might be anticipated that the inhibitory effect of elevated GSH levels on viral replication in our model would be minimized. Therefore, the finding that oltipraz could inhibit basal levels of HIV-1 replication in acutely infected H9 cells, while other inducers did not, suggests another mechanism for the observed antiviral effect. It is possible that elevated GSH levels may play a role in cytokine-stimulated systems.

Since oltipraz's ability to inhibit HIV-1 replication was unrelated to the induction of GSH in this model system, we evaluated it as an inhibitor of HIV-1 RT (Fig. 4). Oltipraz is a noncompetitive inhibitor of RT with respect to dTTP (K_i = 183 ± 50 μM; n = 2 experiments) and a mixed inhibitor with respect to poly(A)·oligo(dT) (K_i = 189 ± 29 μM; n = 3 experiments). Although oltipraz is ≈2500-fold less potent than AZT 5′-triphosphate, the closely related congener 1,2-dithiole-3-thione is inactive as an inhibitor of RT.

Oltipraz irreversibly inhibits glutathione transferases from *Schistosoma mansoni*, which may be responsible for its antiparasitic activity (46–48). Since oltipraz was a weaker inhibitor of RT in conventional enzyme assays than of HIV-1 replication, we considered the possibility that it is an irreversible inhibitor of RT. Preincubation of RT with oltipraz (Fig. 5 *Upper*) indicated that it behaves kinetically as an irreversible inhibitor of RT (K_i = 25.9 ± 3.4 μM; k_3 = 0.0518 ± 0.0089 hr^{-1}; n = 5 experiments). The binding site for oltipraz appears to be blocked by template–primer, since rates of RT inactivation in the presence of oltipraz are unrelated to its concentration and are indistinguishable from rates of RT inactivation in its absence (Fig. 5 *Lower*). Interestingly, high concentrations of phenylglyoxal and *N*-ethylmaleimide have been shown to irreversibly inhibit HIV-1 RT in the template–primer binding domain (49).

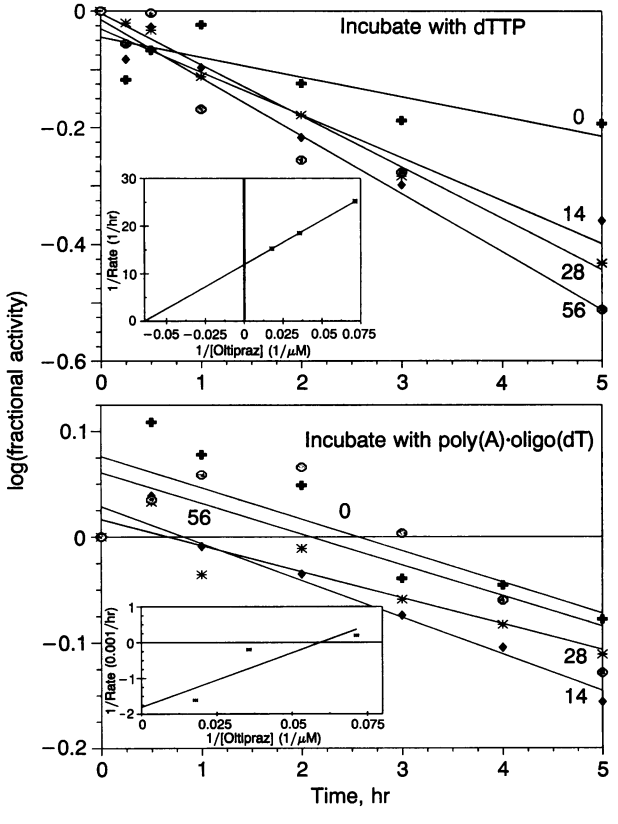

FIG. 5. Time- and concentration-dependent inhibition of HIV-1 RT by oltipraz in the presence of dTTP (*Upper*) but not poly(A)·oligo(dT) (*Lower*). RT was preincubated for the times indicated with oltipraz (0, 14, 28, and 56 μM) dissolved in DPBS containing bovine serum albumin (1 mg/ml), 0.13% DMSO, and either 4 μM dTTP or poly(A)·oligo(dT) (5 and 2.5 μg/ml). After incubation, 10-μl aliquots in triplicate were added to 25 μl of a reaction mixture containing 4 μM dTTP, poly(A) (5 μg/ml), and oligo(dT) (2.5 μg/ml) and assayed for dTTP incorporation into DNA over 30 min (38). *Insets* are Kitz–Wilson transformations (40). Kinetic parameters for oltipraz in the presence of dTTP for the experiment shown are k_3 = 0.0839 hr^{-1} and K_i = 15.6 μM. Kinetic parameters for oltipraz in the presence of poly(A)·oligo(dT) are inconsistent with irreversible inhibition [i.e., the limiting rate of inactivation (k_3; represented by the y intercept of the Kitz–Wilson plot) is a negative value].

Finally, we examined whether oltipraz was synergistic with AZT, since a multiple drug regimen may be advantageous in the treatment of HIV-1 (50). The effect of a combination of oltipraz and AZT (300:1 molar ratio) on HIV-1 replication was examined (Fig. 6) by utilizing the IC_{50} and the slope data of the median-effect plots to generate a combination index plot (41) (Fig. 6 *Inset*). At moderate to high levels of inhibition of p24 release, oltipraz and AZT were synergistic (n = 4 experiments). In analogy with the inhibition of HIV-1 replication, oltipraz was highly synergistic with AZT 5′-triphosphate at high levels of RT inhibition (data not shown).

In conclusion, inducers of GSH levels were not observed to inhibit the replication of HIV-1 in this *in vitro* system. Oltipraz is an exception since it inhibits HIV-1 RT. Although oltipraz is a weak inhibitor of HIV-1 RT in comparison to other inhibitors (51), several of its features favor further examination as an anti-HIV-1 agent. (*i*) Oltipraz behaves kinetically as an irreversible inhibitor of RT, unlike other compounds in clinical use (51). Thus, oltipraz represents a separate class of compounds for which derivatives can be synthesized and tested. (*ii*) Oltipraz acts synergistically with AZT in inhibiting the replication of HIV-1. (*iii*) Oltipraz has been used in the treatment of schistosomiasis, and extensive information on the human pharmacology and safety of oltipraz exists; of particular note, high doses of oltipraz (20–35 mg/kg) have been used to treat schistosomiasis (29, 31–34),

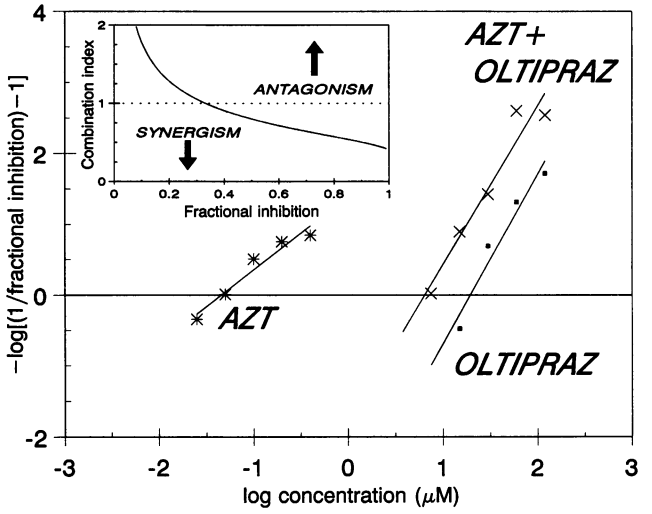

FIG. 6. Median-effect plot (41) showing the inhibition of p24 release into supernatants of H9 cell cultures by AZT, oltipraz, or their mixture (1:300 ratio). Cells were grown and treated with drugs, and after exposure to virus for 7 days the supernatants were assayed for HIV-1 p24 antigen. Coefficients of determination (r^2) for all plots are >0.94. (*Inset*) Drug combination index with respect to fractional decrease of p24 production relative to HIV-1-infected controls, assuming that the drugs act in a mutually exclusive manner. Note that the drugs are synergistic with fractional inhibitions > 0.37. Calculation of the combination index assuming mutually nonexclusive conditions shows synergy with fractional inhibitions > 0.46 (data not shown).

and a single 250–500 mg dose (3–6 mg/kg) of oltipraz in adults can raise peak serum levels to >1 μg/ml (4 μM) (52, 53). (*iv*) It is possible that oltipraz may exert an additional beneficial effect via the elevation of GSH levels under circumstances where viral replication is stimulated by oxidative stress. (*v*) Oltipraz is under consideration as an anticarcinogen in humans at high risk for cancer [i.e., in areas where aflatoxin exposure is endemic (23–28)]. Since two AIDS-defining illnesses are Kaposi sarcoma and lymphoma, it is possible that oltipraz could also prevent these diseases by virtue of its anticarcinogenic properties.

We dedicate this work to the memory of Ernest Bueding. We thank Professor Thomas W. Kensler for providing oltipraz and 1,2-dithiole-3-thione. These studies were supported by institutional grants from the Memorial Sloan–Kettering Cancer Center and the Society of Memorial Sloan–Kettering Cancer Center. H.J.P. is a recipient of an American Cancer Society Junior Faculty Research Award (JFRA-422).

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