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Oltipraz, a Novel Inhibitor of Human Immunodeficiency Virus Type 1 (HIV-1) Replication

Hans J. Prochaska, MD, PhD[1], Surendra J. Chavan, PhD[1], Penny Baron, MS[2], and Bruce Polsky, MD[2]

[1] Molecular Pharmacology and Therapeutics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021
[2] Infectious Disease Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

Abstract Glutathione (GSH) levels are markedly depleted in patients infected with human immunodeficiency virus type 1 (HIV-1) and supplementation of media with high concentrations (5–20 mM) of low-molecular weight thiols prevents HIV-1 replication in cultured cells. We were intrigued whether chemopreventive enzyme inducers might represent a more pharmacologically feasible method to inhibit HIV-1 replication since these compounds elevate intracellular concentrations of GSH at nontoxic doses *in vivo*. After establishing that all inducers surveyed were able to elevate GSH levels in human T-cell and monocytoid cell lines, we were surprised to find that oltipraz (5-pyrazinyl-4-methyl-1,2-dithiole-3-thione) was uniquely able to inhibit HIV-1 replication (IC_{50} = 5–15 μM). Oltipraz and other antiviral 1,2-dithiole-3-thiones (DTTs) appear to inhibit acute HIV-1 replication by inactivating reverse transcriptase (RT). However, among DTTs that inhibit HIV-1 replication in acutely infected cells, only oltipraz was able to inhibit HIV-1 replication in a chronic infection model. Thus, in addition to inactivating RT, oltipraz appears to have an additional antiviral mechanism distal to viral integration. Our laboratories are attempting to determine the mechanism by which oltipraz inhibits HIV-1 replication in chronically infected cells; we are also attempting to determine the bioorganic mechanism for the inactivation of RT. Since the covalent modification of schistosomal proteins and transcription factor(s) are thought to be responsible for the antiparasitic and chemopreventive activities of DTTs, respectively, our studies should be relevant to understanding the diverse medicinal properties of DTTs. Oltipraz, an antischistosomal drug undergoing clinical evaluation as an anticarcinogen, inhibits HIV-1 replication at concentrations achievable in human serum. It is intriguing to consider oltipraz as a therapeutic agent not only for its antiretroviral activity, but also for the prevention of HIV-1 associated neoplasms.

Key words: AIDS, anticarcinogen, antiviral, chemoprevention, dithiolethiones, glutathione, reverse transcriptase

In the thirteen years since the first description of acquired immunodeficiency syndrome (AIDS), an impressive understanding of the human immunodeficiency virus 1 (HIV-1) life cycle and HIV-1-host cell interactions has been accrued [1,2]. Theoretically, any step in the HIV-1 life cycle is susceptible to pharmacologic attack and many targets for antiviral intervention have been investigated [3,4] (Fig. 1). The virus-encoded

Address correspondence to Hans J. Prochaska, MD, PhD, Laboratory for Chemical-Biological Interactions, 601-E Rockefeller Research Laboratories, Molecular Pharmacology and Therapeutics Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

reverse transcriptase (RT) remains the most accessible target under investigation. To date, four nucleoside analog inhibitors of reverse transcription have received approval for marketing in the US, with other nucleoside and non-nucleoside compounds in advanced clinical trials. However, none of these drugs are ideal antiviral agents; they can cause toxicity and the development of drug-resistant HIV-1 strains. The efficacy of zidovudine (3'-azido-3'-deoxythymidine, AZT), the first approved anti-HIV-1 drug, is time-limited and its use in asymptomatic patients is questionable [5]. Examples of other prominent targets (and proposed therapeutics) are initial attachment of viral gp120 to CD4 receptors on the host cell (recombinant soluble CD4, polysulfonic acids); inhibition of factors known to up-regulate HIV-1 replication (*tat* inhibitors and inhibitors of cytokine production [*e.g.*, thalidomide, pentoxyphylline]); inhibition of post-transcriptional events (interferons, antisense RNA, and ribozymes); assembly of viral proteins (protease inhibitors, inhibitors of myristoylation); and viral budding/release (interferons).

The role of oxidative stress in the replicative life cycle of HIV-1 has received a great deal of scrutiny. Release of cytokines, especially from B cells which are in a chronic state of activation in HIV-1 infected individuals, may play an important pathogenetic role by sustaining HIV-1 replication [2,6]. Cytokines (*i.e.*, tumor necrosis factor-α, interleukin-1) and mitogens (*i.e.*, phorbol-12-myristate-13-acetate, or PMA) stimulate the production of reactive oxygen intermediates (ROI), which activate nuclear factor-κB (NF-κB). This transcription factor, initially identified by binding to enhancer elements of the κ-light chain, binds to regulatory elements of a number of cellular genes as well as to two enhancer elements within the long terminal repeat (LTR) of HIV-1. The

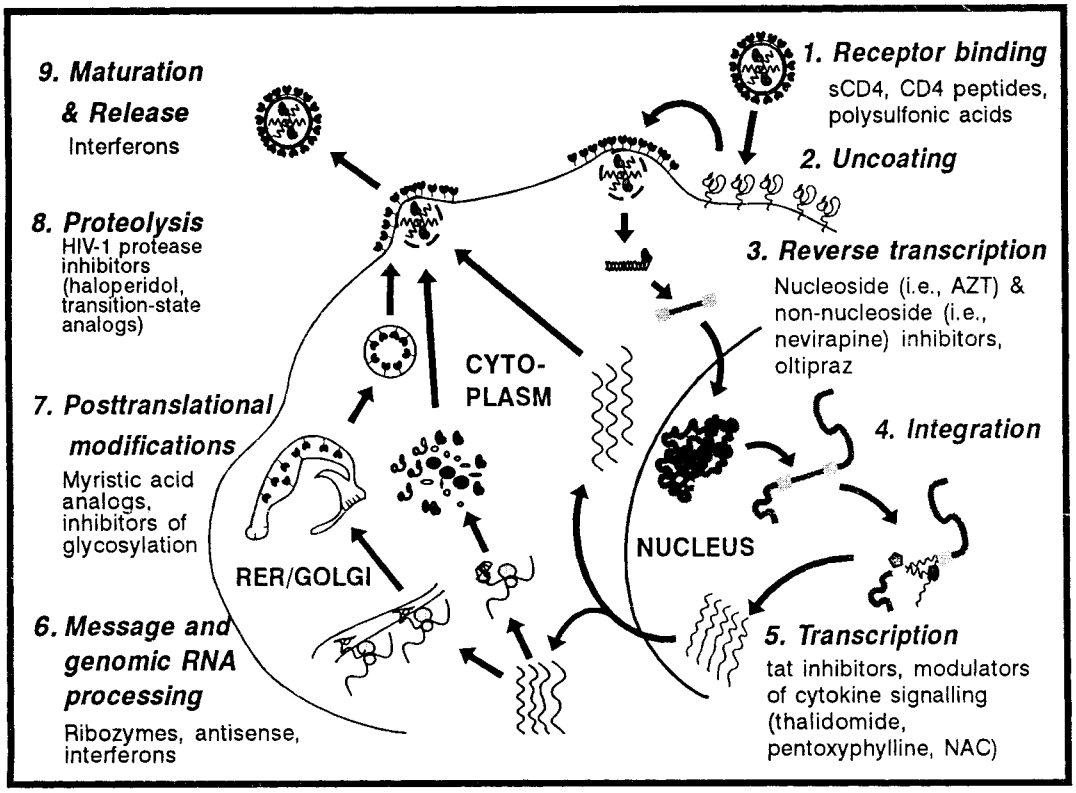


Fig. 1. Replicative life cycle of HIV-1 and pharmacologic strategies to inhibit viral replication. See references [1–4] for details. Oltipraz appears to inhibit HIV-1 replication by inactivating HIV-1 RT as well as disrupting another point of the replicative lifecycle that is distal to viral integration.

activation of NF-κB thus greatly enhances the transcriptional activity of the integrated HIV-1 genome. Direct exposure of cells to oxidants such as H_2O_2 results in NF-κB activation and increased HIV-1 transcription, establishing the role of ROI as an important pathogenetic mechanism [reviewed in 7,8].

In 1989, two reports demonstrated that reduced glutathione (GSH) was markedly depleted in the plasma and peripheral blood mononuclear cells (PBMCs) of HIV-1-infected patients [9,10]. Plasma GSH levels were particularly affected; symptom-free HIV-1-infected patients had plasma GSH levels that were 30% of those in uninfected controls. Treatment with zidovudine had the effect of partially restoring plasma GSH levels [9].

The observation that HIV-1 infection depleted GSH levels raised the possibility that this critical antioxidant defense played an important role in the pathophysiology of HIV-1 infection. GSH is known to modulate a number of immune functions, including T-cell proliferation, T- and B-cell differentiation, cytotoxic T-cell activity, and natural killer cell activity [7,8]. Decreasing GSH by 10–40% with the GSH synthesis inhibitor *l*-buthionine-(*S*,*R*)-sulfoximine (BSO) completely inhibits T-cell activation *in vitro* [11]; a similar magnitude in the reduction of GSH is observed in $CD4^+$ and $CD8^+$ subsets of T-cells from patients with symptomatic HIV-1 disease [8]. Thus, GSH is an important modulator of immune function, and its depletion may play an important role in the development of AIDS.

Shortly after these clinical studies were published, Herzenberg and colleagues [12,13] tested low-molecular weight thiols as inhibitors of HIV-1 replication *in vitro*. They found high concentrations (5–20 mM) of *N*-acetyl-*l*-cysteine (NAC) could block NF-κB activation and NF-κB-driven transcription of reporter genes, as well as HIV-1 itself, in cytokine and non-cytokine challenged models. Subsequently, Kalebic *et al.* [14] showed that NAC, GSH, and GSH ester could inhibit HIV-1 replication in chronically infected monocytoid (U1) cells challenged with PMA or cytokines. These data suggested that GSH precursors such as NAC or *l*-2-oxothiazolidine-4-carboxylic acid (procysteine) might be effective at inhibiting HIV-1 replication. Several clinical trials with these compounds are underway [15–17]. Unfortunately, clinical studies have yet to demonstrate a significant antiviral effect, in part because increases in plasma GSH are resistant to these maneuvers (even at intravenous doses of 100 mg/kg). Moreover, a lack of antiviral efficacy might be anticipated since the concentrations of thiols that inhibit HIV-1 replication *in vitro* are nonphysiological (normal plasma GSH levels are on the order of 5–15 μM versus the millimolar concentrations required to inhibit HIV-1 replication in cultured cells [18]).

Many compounds that prevent the toxic, mutagenic, and neoplastic effects of chemical carcinogens induce electrophile-processing phase II detoxication enzymes as well as enzymes concerned with the synthesis and maintenance of GSH pools. Since these compounds can elevate intracellular GSH levels by up to two-fold (in contrast to plasma, millimolar concentrations of GSH are found intracellularly [18]), we were intrigued by the possibility that chemopreventive enzyme inducers would be a more pharmacologically feasible method to elevate GSH levels and block viral replication. Studies by Gordon *et al.* [19] indicated that anticarcinogenic enzyme inducers were pharmacologically active in PBMCs.

STUDIES IN ACUTELY INFECTED CELLS

Our initial studies examined the ability of several inducers [1,2-dithiole-3-thione (DTT), 5-pyrazinyl-4-methyl-1,2-dithiole-3-thione (oltipraz), *tert*-butylhydroquinone, and dimethyl fumarate) to block HIV-1 replication in acutely infected H9 T-cell lymphoma cells [20]. As in PBMCs, these compounds effectively elevated quinone reductase, also known as NAD(P)H:(quinone-acceptor)oxidoreductase (QR, a phase II enzyme) and GSH levels in H9 cells. We were surprised, therefore, that oltipraz was the only inducer tested that could inhibit HIV-1 replication ($IC_{50} \approx 10$ μM), especially since it was the weakest inducer of QR and GSH. Pretreatment of the cells with inducers to elevate intracellular levels of GSH prior to infection did not potentiate the antiviral activity of oltipraz; other inducers tested remained inactive [20].

The antiparasitic activity of oltipraz is thought to result from its ability to inactivate schistosomal GSH *S*-transferases [21], and the induction of phase II enzymes by chemopreventive DTTs is thought to be via the covalent modification of

transcription factor(s) [22]. Since the ability of oltipraz to inhibit HIV-1 replication was unrelated to the elevation of GSH, we evaluated it as an inactivator of HIV-1 RT. We found that oltipraz inactivates RT by binding to a site that is blocked by template:primer (K_i = 26 μM; k_3 (limiting rate of inactivation) = 0.052 hr^{-1}) [20] (Fig. 2).

We have screened seventy DTTs as inhibitors of RT and of HIV-1 replication in acutely infected H9 cells. As illustrated with selected examples in Table I, we have drawn two major conclusions from these studies. First, we have identified several congeners that are nontoxic inhibitors of acute replication in H9 cells; only antiviral DTTs behaved kinetically as inactivators of RT. In addition to providing evidence that RT is the presumptive target for antiretroviral DTTs, we found large differences in K_i and k_3. These results indicate that it may be possible to design DTTs that bind to RT with high affinity (low K_i) and productively inactivate the enzyme (high k_3). Second, the various medicinal properties of DTTs are dissociable, implying that DTTs with specific therapeutic activities can be developed (see Table I). For example, DTT is inactive as an inhibitor of HIV-1 replication and RT, yet is more potent than oltipraz as an inducer of phase II enzymes and as a chemoprotector [22,24]. The compound 5-(2-thienyl)-4-methyl-DTT is devoid of antischistosomal activity but is five-fold more potent than oltipraz as an inhibitor of HIV-1 replication in H9 cells. In contrast, 5-(3-pyridazinyl)-4-methyl-DTT possesses antischistosomal activity but does not inhibit viral replication or inactivate RT.

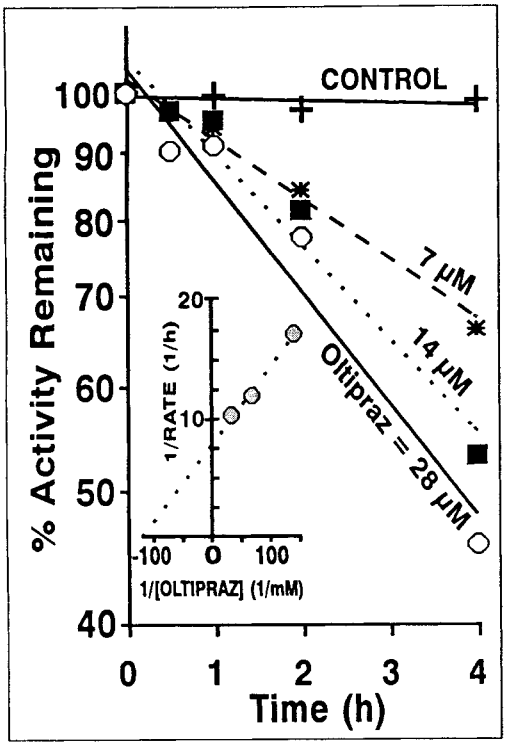


Fig. 2. Time-dependent inhibition of HIV-1 RT by oltipraz. RT was incubated with the concentrations of oltipraz indicated in the figure as described [20] except that the buffer was supplemented with 25 mM $MgCl_2$. At the times indicated, 10 μl aliquots were removed and assayed for RT activity using poly(A)·oligo(dT). The *inset* is the corresponding Kitz-Wilson transformation. The kinetic parameters determined from the plot shown were K_i = 8.51 μM and k_3 = 0.130 hr^{-1} (r^2 = 0.991).

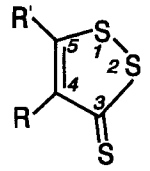
STUDIES IN A CHRONIC INFECTION MODEL

Although our results with H9 cells suggested that oltipraz inhibited viral replication through a GSH-independent mechanism, we did not rule out the potential benefit of raising GSH levels when HIV-1 replication was induced by cytokines or mitogens. We therefore examined the ability of oltipraz, DTT, dimethyl fumarate and NAC to inhibit viral replication and NF-κB activation in PMA-stimulated U1 cells. The U1 cell line, cloned from HIV-1-infected U937 promonocytic leukemia cells, contains two copies of integrated HIV-1 in its genome. HIV-1 replication can be stimulated by a variety of agents, including PMA [14].

Supplementing the culture medium with these inducers or NAC increased intracellular thiol levels by two-fold within 24 hr [25]. In contrast to the enzyme inducers tested, NAC had no effect on QR, indicating that it acts directly as a thiol donor and not as an enzyme inducer. As expected, PMA-induced NF-κB activation was also decreased by pretreating the cells with these agents; inhibition of NF-κB binding activity as estimated by electrophoretic mobility shift assays (EMSA) was inversely correlated to GSH levels (r^2 = 0.8) [25].

The antiviral activities of the inducers were more complex (Fig. 3). Although oltipraz inhibited HIV-1 replication (as measured by release of RT activity and p24 antigen into culture

TABLE I. Effect of Selected 1,2-Dithiole-3-thiones (DTTs) on HIV-1 Replication in Acutely Infected H9 T-cell Lymphoma Cells, the Inactivation of HIV-1 Reverse Transcriptase (RT), the Induction of Quinone Reductase (QR) in Hepa 1clc7 Murine Hepatoma Cells, and Schistosomiasis

 Compound	IC_{50} for acute HIV-1 replication in H9 T-cell lymphoma cells[a]	Inhibitory constants for RT[b]		Concentration to double (CD) QR levels in Hepa 1clc7 murine hepatoma cells[c]	Antischistosomal activity[d]
		Inhibitory constant (K_i)	Limiting rate of inactivation (k_3)		
	(μM)	(μM)	(hr^{-1})	(μM)	
1,2-Dithiole-3-thione (DTT)	Inactive	Inactive	Inactive	1.5	N.D.[e]
5-Pyrazinyl-4-methyl-DTT (Oltipraz)	10	26	0.05	22	Active
5-(2-Thienyl)-4-methyl-DTT	2.2	19	0.03	N.D.	Inactive
5-(2-Pyridyl)-4-methyl-DTT	7.5	740	2.4	N.D.	N.D.
5-(3-Pyridazinyl)-4-methyl-DTT	Inactive	Inactive	Inactive	N.D.	Active

[a] determined by the immunofluorescence assay described in [20] (N ≥ 4 independent experiments); [b] determined as described in [20]; [c] from [22]; [d] from [23]; [e] N.D., Not Determined.

supernatants), dimethyl fumarate and DTT were notable for their increase in viral replication. Although NAC (10 mM) inhibited HIV-1 replication as measured by RT activity in culture supernatants, it was largely ineffective in reducing p24. Kalebic *et al.* [14] evaluated the antiviral activity of low molecular weight thiols in U1 cells by measuring RT activity in the culture supernatants, not by p24 antigen release. These workers also showed that RT levels from the cell lysates of stimulated cells were more effectively suppressed by NAC than other viral antigens. Thus, in contrast to the excellent inverse correlation between GSH levels and NF-κB binding activities, correlations between either of these parameters and viral replication were poor.

How can these results be rationalized? First, the differential effect of NAC on RT and p24 suggests that the antiviral effect of thiols is probably more complex than has been proposed. Second, none of the treatments completely reduced the PMA-induced increase in NF-κB binding [25]. Third, chemopreventive enzyme inducers regulate the transcriptional activity of many genes (*i.e.*, phase II enzymes). The HIV-1 genome is known to interact with a number of *trans*-acting factors; perhaps one or more of these factors are affected by xenobiotic treatment. With respect to this last point, it is notable that the HIV-1 genome contains tandem and functionally active AP-1 recognition sites [26]. Because the induction of phase II enzymes is mediated via a regulatory element that contains two tandem AP-1-like sites [27], it is possible that chemopreventive enzyme inducers (and PMA) are able to activate HIV-1 replication through these sites [25].

The antiretroviral mechanism for oltipraz in this chronic infection model differs from the mechanism of inhibition in acutely infected cells. First, antiviral activity of oltipraz is potentiated by the length of time the cells are preincubated

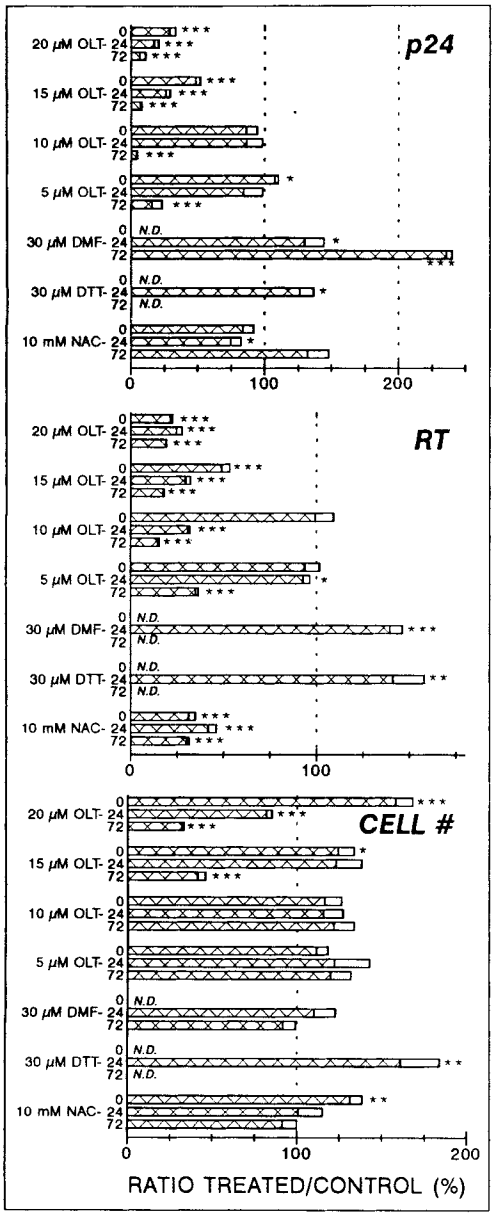


Fig. 3. Effect of oltipraz (OLT), dimethyl fumarate (DMF), 1,2-dithiole-3-thione (DTT), and NAC on HIV-1 replication and cell growth in PMA-stimulated U1 cells. Cells (250,000/ml of media) were incubated in the presence of drug for the indicated times and then exposed to 2.5 nM PMA for an additional 48 hr. The cells were counted (*lower*) and culture supernatants were assayed for p24 antigen levels (*upper*) and RT activities (*middle*). 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 (N = 3 flasks per group). The data shown are the mean (hatched bars) and SEM values (clear bar extensions) derived from 3 to 18 experimental points (1–6 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 (N = 11) and 8.5 ± 3.1 % (N = 14), respectively, of levels measured from supernatants of U1 cells stimulated with PMA at 24 hr and harvested at 72 hr. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. Reproduced with permission from [25].

with the drug before adding PMA. Indeed, HIV-1 replication is inhibited by 65–80% if U1 cells are exposed to 5 μM oltipraz 72 hr prior to treatment with 2.5 nM PMA (Fig. 3). Second, with the exception of oltipraz, DTT analogues that inhibit HIV-1 replication in acutely infected H9 cells are inactive as inhibitors of HIV-1 replication in PMA-stimulated U1 cells. It is our view that oltipraz exerts an antiviral effect at two points in the replicative lifecycle of HIV-1, one at the reverse transcription step and the other occurring distal to integration of the HIV-1 genome.

STUDIES IN PROGRESS

Our laboratories are working on two fronts. First, we are interested in establishing the bioorganic mechanism for the inactivation of HIV-1 RT. We are now undertaking studies to determine the amino acid residue(s) targeted by the drug and the nature of the adduct formed. It is our objective to model the binding event based on the known crystallographic structure of RT [28,29]. This information should provide us with the opportunity to rationally design a DTT analog that will bind with high affinity. Moreover, elucidating the chemistry for how DTTs modify target proteins will not only be important in developing drugs with potent antiretroviral activity, but should also provide an understanding for the other medicinal properties of DTTs. Second, we are attempting to determine the mechanism for inhibiting viral replication in chronically infected cells. Oltipraz may act as a prodrug in schistosomiasis [30]; it is our view that metabolite(s) of oltipraz may be responsible for inhibiting HIV-1 replication at a stage in the lifecycle distal to viral integration.

ARE DTTs WORTHY OF CONSIDERATION AS CLINICAL ANTI-HIV-1 AGENTS?

Although oltipraz is a modest inhibitor of HIV-1 replication in comparison to others, we believe several of its features render it worthy of consideration as an antiretroviral drug. First, oltipraz can inactivate RT, a mechanism different from other compounds in clinical use. Oltipraz therefore represents a new class of compounds for which derivatives can be synthesized and tested. Second, oltipraz can inhibit HIV-1 replication in a chronic infection model via a mechanism unrelated to the inhibition of RT. Since viral replication is ongoing in lymphoid tissues, even in the asymptomatic phase of viral infection when plasma viremia is difficult to detect [31,32], inhibitors of HIV-1 replicative lifecycle distal to reverse transcription might offer greater promise as inhibitors of disease progression. Third, oltipraz has been used to treat schistosomiasis, and extensive information on the human pharmacology and safety of oltipraz exists [33]. In particular, high doses of oltipraz (20–35 mg/kg) have been used to treat schistosomiasis and a single 250–500 mg dose (3–6 mg/kg) of oltipraz in adults can raise peak serum levels to 5 μg/ml (20 μM) [33,34]. These concentrations were shown to be highly effective in inhibiting HIV-1 replication in the U1 cell line [25] (see Fig. 3). Third, oltipraz is undergoing Phase I and II clinical evaluation as a potential human anticarcinogen [reviewed in 33]. Thus, oltipraz is thought to have an adequate safety profile for use over protracted periods of time in patients at risk for cancer who are otherwise healthy.

Finally, it is interesting to speculate that HIV-1 infection could be an appropriate setting to study the drug's chemopreventive activity. Early in the epidemic, Kaposi's sarcoma and non-Hodgkin's lymphoma were recognized as common (and AIDS-defining) illnesses. However, epidemiologic studies have found several other malignancies that occur more frequently in HIV-1-infected patients, including invasive cervical cancer, Hodgkin's disease, non-melanoma skin cancers, and cancers of the rectum, anus, and nasal cavity [35,36]. Although many of these are "opportunistic cancers" that develop because the profound immunosuppression of advanced HIV-1 disease results in decreased tumor surveillance and activation of other viruses (*e.g.*, Epstein-Barr virus, human papilloma virus), it is possible that the biochemical effects of repleting antioxidant defenses by oltipraz could preserve immune function and reduce the risk of HIV-1-associated malignancies. Studies suggest that $CD4^+$ T-cells may be lost because HIV-1 gene expression decreases levels of antioxidant enzymes (thioredoxin, superoxide dismutase, and glutathione peroxidase) and renders the cells sensitive to apoptosis from lipid hydroperoxides [37]. In addition to raising GSH levels, oltipraz can induce α- and μ-class glutathione *S*-transferases [38] that detoxify products of lipid peroxidation [39,40]. Thus,

in addition to exerting a direct antiviral effect [20,25], the ability of oltipraz to raise GSH levels may restore T-cell function, and the induction of electrophile-processing phase II enzymes may lessen the rate of T-cell loss through apoptosis. It is intriguing to consider that oltipraz could be used to prevent the development of AIDS and AIDS-related malignancies.

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