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THIAMINE DISULFIDE AS A POTENT INHIBITOR OF HUMAN IMMUNODEFICIENCY VIRUS (TYPE-1) PRODUCTION [1]

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SUMMARY: Thiol and disulfide compounds were tested as an anti-HIV drug against transactivator (Tat)-mediated transactivation of HIV-1. Of all the compounds tested, thiamine disulfide, α-lipoic acid, and *N*-acetylcysteine significantly depressed HIV-1 Tat activity. Thiamine disulfide alone in these compounds possessing anti-HIV-Tat activity markedly inhibited production of progeny HIV-1 in acute and chronic HIV-1-infected CEM at nontoxic concentrations of 500~1000 μM. Thiamine disulfide (500 μM) blocked 99.7 % of HIV-1 production after 96 hr culture in acute HIV-1 (LAV-1) infection (m.o.i. = 0.002), whereas it inhibited 90~ 98 % of HIV-1 production in chronic-infected cells (CEM/LAV-1, H9/MN, and Molt-4/IIIB). The results suggest that thiamine disulfide may be important for AIDS chemotherapy.

Attempts to overcome HIV-1 infection have been made on the basis of the points of attack of the HIV-1 life cycle (1). HIV-1 RT inhibitors (AZT, TIBO) (2, 3), uncoating reagents (4), HIV-integrase inhibitor (5), HIV-1 protease inhibitors (6), and *N*-myristoyl inhibitor (7), have already been found. AZT and its derivatives have been used for clinical treatment of AIDS patients. Unfortunately, we have not yet found the anti-HIV reagents for use in chemotherapy. An

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Abbreviations used are: HIV-1, human immunodeficiency virus type-1; TDS, thiamine disulfide; IBTDS, O-isobutyrylthiamine disulfide; FST, fursultiamine; TAD, allithiamine; AIDS, acquired immunodeficiency syndrome; SeAP, secreted alkaline phosphatase; Tat, transactivator; RT, reverse transcriptase; AZT, 3'-azido-3'-deoxythymidine; TIBO, tetrahydroimidazo [4,5,1-jk] [1,4] benzodiazepin-2 (1H)- one and -thione; HTLV-I, human T cell lymphotropic virus-I; PBS(-), Ca^{2+}, Mg^{2+} free phosphate buffered saline; $TCID_{50}$, 50 % tissue culture infectious dose; m. o. i., multiplicity of infection; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; kD, kilo dalton; LTR, long terminal repeat; Pr, precursor.

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important feature of the replicative cycle of HIV is the ability of the virus to establish a latent infection of $CD4^+$ T-cells as a provirus integrated in the chromosome of the host cell.

Tat (transactivator of transcription), a regulatory protein of HIV-1, is of particular interest in the context of antiviral chemotherapy. Virally encoded Tat plays an important role in the replication of several members of the human retroviruses (8).

Full-length Tat protein has a number of interesting structural features that may ultimately be significance for the design of specific antagonists. Tat protein appears to be localized in the nucleus of the cell. The basic amino acids in the carboxy-terminal half of the molecule may serve as a nuclear localization signal. HIV-1-Tat also contains four Cys-X-X-Cys sequences that participate in the binding with metal ions to form a dimer in vitro (8). Mutation of the cysteine residues markedly reduces Tat function and abolishes virus replication but does not appear to affect metal binding (8).

Our interest is focussed on the thiol group of cysteine residues in the Tat molecule, and various disulfide compounds which are used in clinical treatments were tested for their anti-HIV activity. First, anti-Tat activity was measured using COS-7 cells co-transfected with the reporter plasmid, pBC12/HIV/SeAP, and the Tat expression plasmid, pBC12/HIV/Tat (9). Second, the compounds possessing anti-Tat activity were tested in acute and chronic-infected CEM on the basis of determination of antiviral activity ($TCID_{50}$/ml). Finally, the amount of a viral structural protein (p24) was determined by Western immunoblotting, and it was found to be markedly decreased upon treatment with thiamine disulfide. Thiamine disulfide alone is active against both acute and chronic infections and has a broad activity against different strains of HIV-1 in laboratory cell lines.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: *N*-acetylcysteine, diallyl disulfide, diallyl sulfide, thiamine, glutathione, and α-lipoic acid from Wako Chemical Co., (Osaka, Japan); *N*-acetyl-D-penicillamine, L-penicillamine, D-penicillamine acetone adduct hydrochloride, and D-penicillamine disulfide from Funakoshi Co., (Tokyo, Japan); thiamine disulfide (TDS), O-isobutyrylthiamine disufide (IBTDS), fursultiamine (FST), allithiamine (TAD), and RPMI-1640 medium from Nissui Seiyaku Co., (Tokyo, Japan); HTLV-I-producing cell line MT-4 (10), chronic HIV-1-infected laboratory cell lines CEM/LAV-1, H9/MN, Molt-4/IIIB, U937/MN, and U937/RF, and human T-cell lines CEM were maintained at 37°C in RPMI-1640 medium supplemented with 10 % fetal calf serum (100 IU/ml of penicillin and 100 mg/ml of streptomycin) in 5 % CO_2 (11). The reporter plasmid, pBC12/HIV/SeAP, and the Tat expression plasmid, pBC12/HIV/Tat, were kind gifts from Dr. M. -C. Hsu (Hoffmann - La Roche Inc., Nutley, NJ 07110).

Anti-Tat assay

Anti-Tat assay was carried out as described previously (9). The following quantities of plasmids were used per 1 x 10^6 cells (COS-7) in 1 ml of DEAE-dextran transfection solution: 800 ng of pBC12/HIV/SeAP and 400 ng of pBC12/HIV/Tat. Assays were performed with 96-well microtiter plates. The compound in the same medium was added 24 hr after cells were transfected, and alkaline phosphatase activities in culture medium were measured 72 hr after drug addition by

colorimetry (9). Control experiments were carried out under the same conditions in the absence of the compounds.

Cytopathic effect inhibition assay

The procedure for measuring anti-HIV-1 activity in HIV-1-infected cell lines was performed as follows. The various thiol and disulfide compounds were separately dissolved in PBS(-) containing 0.1 % hydrogenated castor oil (60) as a mild detergent, which is not cytotoxic at this concentration. The detergent is important for dispersing these compounds in the medium. An aliquot (100 μl) of these compound solutions (0~20 mM in PBS(-) with 0.1 % detergent) was individually added to HIV-1-infected cell solution (2 x 10^5 cells in 9.9 ml of the medium). The culture flask was incubated at 37 ℃ in 5 % CO_2 for 0, 24, 48, 72, 96, and 120 hr, and the number of viable cells at 24-hr intervals was counted by trypan blue dye exclusion (using microscopic examination). Control experiments were carried out under the same conditions without the compound.

Titration of infectious HIV-1 from treated cells

HIV-1-infected cells (2 x 10^5 cells, 10 ml in the medium) were cultured at 37 ℃ in 5 % CO_2 with or without the compounds (0~1 mM) under identical conditions as described previously in this paper. Mock experiments were carried out under the same conditions after by addition of PBS(-) containing the detergent alone instead of the compounds. After 96 hr incubation, the cultured fluid was collected by centrifugation (260 x g, 5 min), filtered by a syringe (0.45 μm), and HIV-1 infectivity was measured by titration with MT-4 (12). Titers of virus are expressed as 50 % tissue culture infectious dose/ml ($TCID_{50}$/ml, 13).

Immunoblot analysis

The cell lysates were separated on SDS-PAGE (10 %, 14), followed by immunoblotting as described (15). The filter was incubated with HIV-1-positive sera and bands were visualized with human IgG-horseradish peroxidase conjugates.

RESULTS

Effects of thiol and disulfide compounds on HIV-1-Tat activity

The transient transfection assay employing the indicator gene (secreted alkaline phosphatase, SeAP) was designed as a high throughput screening for compounds that specifically inhibited Tat-mediated transactivation of HIV-1 LTR (16). SeAP activity was measured with a colorimetric assay. Of all the thiol and disulfide compounds tested, α-lipoic acid, thiamine disulfide, and *N*-acetylcysteine significantly depressed the SeAP activities at noncytotoxic concentration ranges of 125 to 500 μM. Alpha-lipoic acid (250 μM), thiamine disulfide (500 μM), and *N*-acetylcysteine (500 μM) caused loss of 92 %, 64 %, and 18 % in the original activity, respectively.

The other thiol and disulfide compounds, diallyl sulfide, diallyl disulfide, L- or D-penicillamine derivatives, glutathione, and thiamine had no effect on the SeAP activity under the experimental conditions used.

Effects of TDS, α-lipoic acid, and *N*-acetylcysteine on both CEM and CEM/LAV-1 cell proliferation

The cytotoxicities of the three compounds, which inhibited significantly HIV-1-Tat-mediated SeAP activity, were measured by trypan blue dye exclusion (Fig. 1). TDS significantly suppressed CEM/LAV-1 cell proliferation at a concentration of 500 μM, but not CEM cell proliferation (Fig. 1 A and B). Alpha-lipoic acid and *N*-acethylcysteine also depressed CEM/LAV-1 (Fig. 1 C and D).

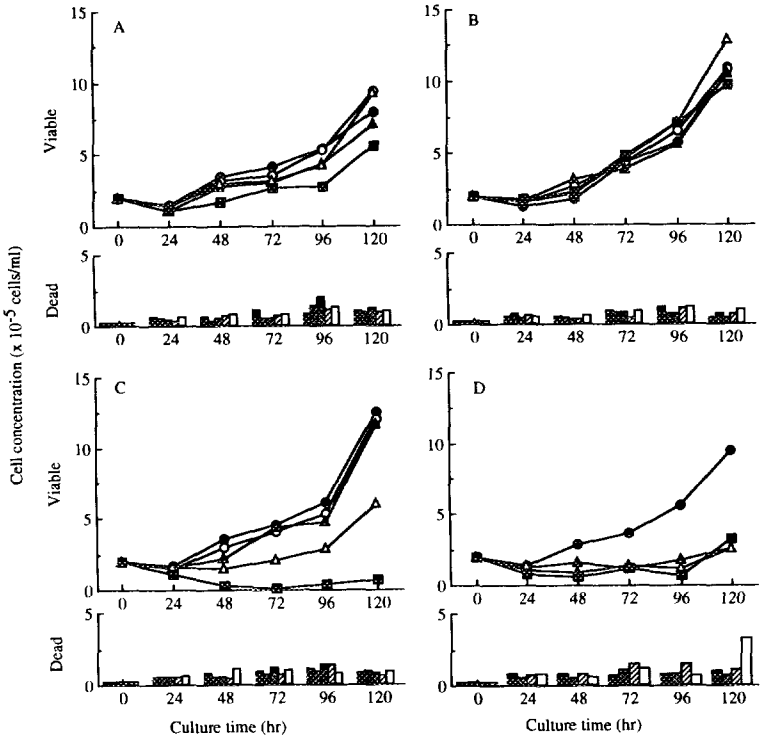

Figure 1. Effects of TDS, α-lipoic acid, and *N*-acetylcysteine on both CEM and CEM/LAV-1 cell proliferation.

CEM and CEM/LAV-1 (2 x 10^5cells, 10 ml) were separately cultured at 37 ℃ in 5 % CO_2 for 0~120 hr after a single addition of the test compound. Viable or dead cells were counted after every 24 hr cultivation as described in "Materials and Methods".

A: TDS, CEM/LAV-1; B: TDS, CEM; C: α-Lipoic acid, CEM/LAV-1; D: *N*-acetylcysteine, CEM/LAV-1. Curved lines indicate cell proliferation (viable cells) : —●—, control (0 μM) ; —○—, 62.5 μM; —▲—, 125 μM; —△—, 250 μM; —■—, 500 μM. Columns indicate dead cells: ▩, control (0 μM); ▨, 62.5 μM; ▩, 125 μM; ▨, 250 μM; □, 500μM.

The latter was cytotoxic against CEM at a concentration range of 250 ~ 500 μM, but the former was not at the same concentration (data not shown).

Effects of TDS and TDS-derivatives on cell proliferation of the chronic HIV-1-infected laboratory cell lines

HIV-1-infected laboratory cell lines (H9/MN, Molt-4/IIIB, U937/IIIB, and U937/RF) and CEM were used. The cytotoxicities of TDS and TDS-derivatives (structures are shown in Fig. 2) to the cells were measured as described above. No effects were observed at increasing concentrations (0 to 1000 μM) of TDS on cell proliferation of both these HIV-1-infected cells and CEM (data not shown because the results were close to that of TDS against CEM cell proliferation shown in Figure 1 B). On the other hand, TDS-derivatives were more cytotoxic to the cells. Especially, TAD depressed to approximate 2 ~ 10 % of the control cell viability at concentration of 125 μM for 96-

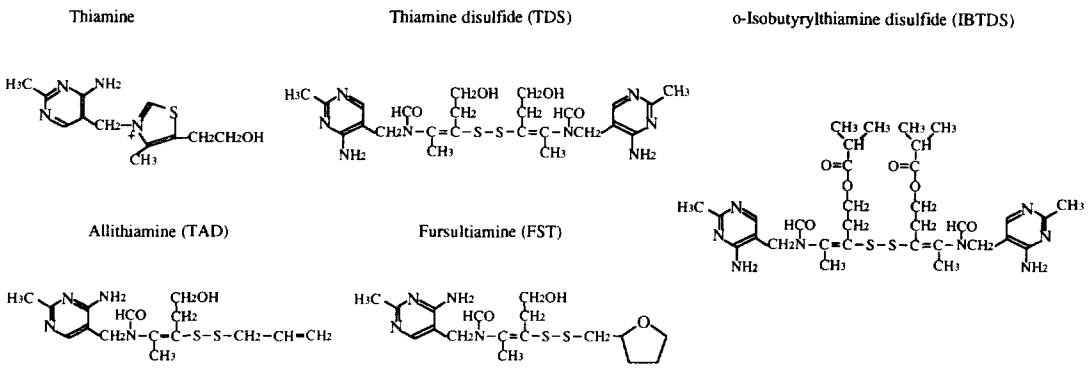

Figure 2. Structures of TDS and TDS-derivatives.

hr cultivation. IBTDS and FST were also cytotoxic to the cells in proportion to increasing concentrations (125 ~ 1000 μM).

Antiviral effects of TDS on acute and chronic HIV-1-infected cells

First, antiviral effect of TDS on acute HIV-1 (LAV-1)-infected CEM was investigated. CEM cells (2 x 10^5/ml, 10ml) were infected with HIV-1 (m.o.i. = 0.002) at 37 ℃ for 60 min, washed twice with the medium, and cultured at 37 ℃ for 0~96 hr in the presence and absence of TDS (0~1000 μM). At 24-hr intervals, viable or dead cells were counted by trypan blue dye exclusion .

After 96-hr culture, cells were removed from the medium by centrifugation (260 x g, 5min). The supernatant was filtered using 0.45 μm filter, and titrated as described in "Materials and Methods".

As shown in Table I, the values of $TCID_{50}$/ml were decreased with increasing concentrations of TDS, and these $TCID_{50}$/ml are 2 and 0.32 % of that of control (6.01 x 10^6 $TCID_{50}$/ml) at concentrations of 250 and 500 μM, respectively.

Second, antiviral effects of TDS on chronic HIV-1-infected cells were determined. The chronic HIV-1-infected cells (CEM/LAV-1, H9/MN, U937/RF, Molt-4/IIIB) were separately cultured at 37 ℃ in medium with TDS or TDS-derivatives at noncytotoxic concentrations. The resulting supernatants were harvested at 96 hr, and their antiviral activities were determined with virus progeny ($TCID_{50}$/ml method) as described in "Materials and Methods". Virus progeny ($TCID_{50}$/ml) was increasingly depressed by increasing concentrations of TDS in various chronic HIV-1-infected cells (Table I, chronic column). Virus progeny in all these cells treated with TDS at concentration of 500 μM was strongly depressed to approximate 1~20 % of untreated virus production. Especially, HIV-1 production in H9/MN was strongly inhibited with TDS at a concentration of 500 μM. Antiviral activities of TDS-derivatives (IBTDS, FST, and TAD) were omitted since the compounds were relatively toxic in the cultured cells at a concentration range of 125~1000 μM as described above.

Table I. Antiviral effects of TDS on the acute and chronic HIV-1-infected cell lines

TDS (μM)	TCID50/ml				
	acute [a]	chronic [b]			
		CEM/LAV-1	H9/MN	U937/RF	Molt4/IIIB
0 [c]	6.01×10^6 (100) [d]	1.58×10^6 (100)	1.00×10^5 (100)	6.30×10^4 (100)	7.94×10^5 (100)
50	1.30×10^6 (26)	1.58×10^6 (100)	6.31×10^4 (63)	3.98×10^4 (63)	7.94×10^5 (100)
125	1.00×10^6 (20)	7.80×10^5 (49)	1.00×10^4 (10)	6.30×10^4 (100)	1.58×10^5 (20)
250	1.00×10^5 (2)	3.95×10^5 (25)	1.00×10^4 (10)	2.51×10^4 (39)	6.31×10^3 (1)
500	1.60×10^4 (0.32)	1.58×10^5 (10)	1.26×10^3 (1)	1.26×10^4 (20)	2.51×10^4 (3)

a) MT-4 cells (2×10^5/ml, 10 ml) were infected with HIV-1 (m.o.i. = 0.002) at 37 ℃ for 60 min, washed twice with the culture medium, and cultured at 37 ℃ in 5 % CO_2 for 0 ~ 96 hr in the presence and absence of TDS (0 ~ 500 mM). Cytotoxicity was measured by trypan blue dye exclusion after every 24 hr culture. Values of TCID50/ml were determined by the methods described in "Materials and Methods".

b) The cells (2×10^5/ml, 10 ml) were cultured at 37 ℃ in 5 % CO_2 for 0 ~ 120 hr after a single addition of the test compounds. The values of TCID50/ml in the supernatant of 96hr-cultured medium was determined as described in "Materials and Methods".

c) Control experiments were carried out under the same conditions in the absence of the compound.

d) Relative activities (percent) are represented in parentheses.

Analyses of HIV-1 viral proteins obtained from acute HIV-1-infected CEM

The cell lysates treated with or without the compounds (α-lipoic acid, TDS, and *N*-acetylcysteine) were analyzed quantitatively by SDS-PAGE, and followed by Western immunoblotting (Fig. 3). HIV-1 viral structural proteins (Pr55, p41, and p24, shown by arrow) were detected in acute HIV-1-infected CEM cells with HIV-1 positive sera (lane 1, control). The major proteins (p24 and p41) were also found in the cells treated with α-lipoic acid (lanes 2 and 3) and *N*-acetylcysteine (lanes 6 and 7). The p24 protein is, however, hardly detectable in the cell lysate treated with TDS (lane 4, 500 μM).

DISCUSSION

Inhibition of HIV-1-Tat activity

HIV-1-Tat, the regulatory protein of HIV-1, is shown to play an important role in the process of HIV-1 replication. Therefore, Tat inhibitors are expected for use in AIDS chemotherapy. The assay for Tat-mediated transactivation of HIV-1 LTR has been established with the reporter plasmid, pBC12/HIV/SeAP, and the Tat expression plasmid, pBC12/HIV/Tat, as described previously (9).

Anti-Tat activities are measured using COS-7 cells co-transfected with the reporter and Tat expression plasmids. Tat activities have been significantly inhibited with TDS, α-lipoic acid, and *N*-acetylcysteine.

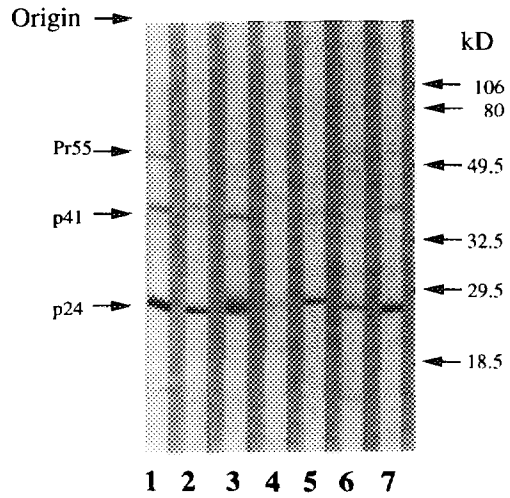

Figure 3. Analyses of HIV-1 viral proteins obtained from acute HIV-1-infected CEM treated with TDS.

The CEM cells (2 x 10^5/ml, 10 ml) were infected with HIV-1 (LAV-1, m.o.i. = 0.011), incubated at 37 ℃ for 24 hr, washed twice with the culture medium, and cultured again for 72 hr at 37 ℃ in 5 % CO_2. The cells were pelleted by centrifugation (260 x g, 5 min), rinsed twice with PBS(-), and lysed in lysis buffer (7).

The lysate was analyzed quantitatively by SDS-PAGE, and followed by Western immunoblotting analysis. Molecular weight markers are on the right. Arrows show HIV-1 viral proteins. Control experiments (lane 1) were carried out under the same conditions in the absence of the compounds. Healthy human serum was used instead of HIV-1 positive sera.

Control: lane 1; α-lipoic acid: lane 2 (250 μM), lane 3 (125 μM); TDS: lane 4 (500 μM), lane 5 (250 μM); *N*-acetylcysteine: lane 6 (250 μM), lane 7 (125 μM).

Alpha-lipoic acid inhibited the replication of HIV-1 in cultured lymphoid T-cells as described previously (16). It has also been reported that *N*-acetylcysteine depressed the cytokine-enhanced HIV LTR-directed expression of β-galactosidase in an *in vitro* HIV model system (17).

TDS inhibited expression of the SeAP gene controlled by the HIV-1 LTR promoter mediated with Tat. Its inhibition may be attributable to the interaction of TDS with either HIV-1 LTR Tat promoter gene or Tat protein. Therefore, the compound has been expected for practical use due to its anti-HIV activity in the same way as α-lipoic acid or *N*-acetylcysteine, which have been already reported as anti-HIV reagents (16,17).

Cytotoxicity and Antiviral activity

TDS possessing anti-HIV Tat activity was employed for measurement of cytotoxicity by trypan blue dye exclusion. The cytotoxicities of TDS to both five chronic HIV-1-infected laboratory cell lines (CEM/LAV-1, H9/MN, Molt-4/IIIB, U937/IIIB, and U937/RF) and human T-cell line (CEM) were not observed at the concentration range of 0 to 1000 μM as described above. On the other hand, the other TDS-derivatives (IBTDS, FST, and TAD) were cytotoxic to both five chronic HIV-1- infected cell lines and HIV-1-uninfected cell lines (data not shown) at

concentrations of 125, 250, 500 and 1000 μM. Isobutyryl, furylmethylthiol, and allyl groups of thiamine might be responsible for cytotoxicity.

The inhibitory mechanism of HIV-1 production with TDS remains unknown. TDS may inhibit the physiological system through a variety of mechanisms, because of its ability to block both acute and chronic HIV-1-infected CEM. Thiamine is an essential compound which exists naturally in physiological systems as a co-factor for pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and transketolase reactions. The disulfide bridge of TDS is shown to be essential for anti-HIV activity, because thiamine alone, the reduced form of TDS, neither inhibited HIV-1 production (data not shown) nor HIV-1-Tat activity. Some agents inhibited HIV-1 infection by interference with the thiol-disulfide interchange upon virus receptor interaction (18); thus, it is possible that TDS causes the blockage of adsorption of HIV-1 on its target cells by disulfide exchange and thiol-disulfide interchange. Furthermore, TDS or its reduced metabolites in cells are shown to participate in the cooperative inhibition of nuclear factor κB (NF-κB) and Tat-induced superactivation of HIV-1 (19) in a manner similar to that of α-lipoic acid (20) and a Tat antagonist (9). Hence, it was of interest to investigate whether TDS inhibited acute and chronic HIV-1 infection. Further work is ongoing in our laboratory to clarify such a mechanism in order to more effectively use this compound as a chemotherapeutic agent for AIDS.

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