ELSEVIER

Antiviral Research 24 (1994) 305–314

Antiviral Research

Inhibition of human immunodeficiency virus by *N*-methylisatin-β4′:4′-diethylthiosemicarbazone and *N*-allylisatin-β-4′:4′-diallythiosemicarbazone

Y. Teitz[a,*], D. Ronen[a], A. Vansover[b], T. Stematsky[a,b], J.L. Riggs[c]

[a]*Biochemical Virology, Department of Human Microbiology, Sackler School of Medicine, Tel Aviv University, Tel Aviv 69978, Israel*
[b]*Central Virology Lab., Tel Hashomer, Israel*
[c]*Viral and Rickettsial Disease Lab., Department of Health Services, Berkeley, CA, USA*

(Received 6 October 1993; accepted 14 March 1994)

Abstract

N-methylisatin-β4′:4′-diethylthiosemicarbazone(M-IBDET) and *N*-allylisatin-β-4′:4′-diallylthiosemicarbazone(A-IBDAT) inhibit the production of Human Immunodeficiency virus (HIV). Virus inhibition was related to the thiosemicarbazone derivative (TSCD) concentrations and time of treatment. Inhibition of HIV production was confirmed by various parameters of virus assay employing reverse transcriptase activity, plaque forming units (PFU) and levels of viral structural proteins. Effective antiviral TSCD concentrations ranged from 0.17 μM to 2.04 μM for M-IBDET, and from 1.45 μM to 17.4 μM for A-IBDAT. Treatment of the chronic HIV-infected cells for 48 h with 0.34 μM M-IBDET or 2.9 μM A-IBDAT caused about 50% inhibition in as virus yield ED_{50} as assayed by the PFU method. Almost 2 logs of virus infectivity (PFU) was suppressed after 48 h of treatment with 17.4 μM A-IBDAT. Therapeutic index values of 20 and 30 were found for M-IBDET and A-IBDAT, respectively. A significant selective inhibition of HIV structural protein synthesis was shown by both M-IBDET and A-IBDAT.

Key words: HIV; Antiretroviral compound; Thiosemicarbazone derivative

*Corresponding author. Fax: +972 3 6409160.

Elsevier Science B.V.
SSDI 0166-3542(94)00028-7

1. Introduction

Human immunodeficiency virus (HIV) infection can be divided into three stages: an acute stage representing primary infection; a chronic stage where minimal but detectable clinical and immunologic abnormalities are present; and a crisis stage where profound immunodeficiency is extant and opportunistic infections take place. This clinical observation of the disease show a pathogenesis of a chronic viral disease in which the virus is persistently integrated into host cell chromosomes and potentially can be expressed. In the effort to develop anti-AIDS drugs we adapted a strategy to test anti-AIDS compounds for their potential to suppress chronic HIV infection. Thiosemicarbazone derivatives (TSCD) are synthetic antiviral compounds that act as inhibitors of virus production (Bauer, 1972). Our previous studies showed that these TSCD are effective against other retro viruses that cause chronic cell infections (Sherman et al., 1980; Ronen et al., 1984; Teitz et al., 1993; Teitz et al., 1994a,b). It was found that the target for inhibition of the virus by the TSCD is viral protein synthesis and the affected step of this inhibition is not at the level of viral RNA transcription but a result of direct interference with RNA translation. In this study we report the inhibition of HIV by two TSCD that suppress virus production in chronically HIV-infected cells. The specific inhibition of virus production was found to parallel the selective suppression of viral protein synthesis.

2. Materials and methods

Cells and viruses. The H_9/HIV cell line (Gallo et al., 1984; Levy et al., 1984), a human established T-cell line chronically infected with HIV, and MT_2 (Harada et al., 1985) cell line, a human established T-cell line carrying the $HTLV_1$, were used in this study. The cells were grown in RPMI 1640 medium containing 10% fetal calf serum (FCS).

Reverse transcriptase (RT) activity assay. Reverse transcriptase activity was assayed by the micro method as described before (Teitz, 1971; Harada et al., 1985). Samples of 0.05 ml of H_9/HIV culture fluid containing the HIV particles released into the media were used for the assay. After removal of cells by centrifugation – the fluid samples were suspended in Tris-NaCl buffer (pH 8.3), preincubated with 10 μl of 2.2% Nonidet P-40 for 15 min and mixed with equal amount of reaction mixture. The exogenous reaction was performed as described before (Teitz et al., 1971) in the presence of 10 mM Mg^{2+} in the reaction mixture. The results are expressed as counts per min (usual yield, 220 cpm/pmol).

Thiosemicarbazone derivatives (TSCD). Methylisatin-β-4′:4′-diethylthiosemicarbazone (M-IBDET) and allylisatin-β-4′:4′-diallylthiosemicarbazone (A-IBDAT) were synthesized by Dr. A. Abramoff from the Department of Organic Chemistry, Weizmann Institute of Science, Rehovot, Israel (Edelstein et al., 1980) and are protected by patents (Teitz 1989, 1990). Stock solutions (10 mg/ml) were made in DMSO and kept in the dark. The concentrations of the thiosemicarbazones were determined, before use, by reading the absorbance at λmax 345–350 mm: ε was in

the range of 17 000–20 000. Control, untreated TSCD cultures contained 0.01% DMSO.

Cytotoxicity studies. H_9/HIV cell cultures were exposed to multiple concentrations of the TSCD. Cell viability and number was determined at indicated times by the trypan blue exclusion method (Hu et al., 1989).

Viral plaque forming units (PFU) assay. The amount of virus particles released from the infected cells into the culture medium was determined by the PFU assay as previously described (Harada et al., 1985). Briefly, samples of 25 μl supernatant fluids of H_9/HIV treated and untreated cells were diluted from 10^{-1} to 10^{-6} in DMEM medium containing 10% fetal calf serum and 2 μg/ml of polybrene. Each of the virus dilutions was mixed with 6×10^6 MT_2 cells (a human established T-cell line carrying the $HTLV_1$) (Harada et al., 1985). Cell cultures were incubated at 37°C for 1 h and then transferred to separate wells on a microtiter plate, previously treated with poly-1-lysine. Three wells were used for each dilution point. After 2 h of incubation that allowed cell attachment to the wells, the cultures were overlayed with 1.5% agarose in medium, and incubated for 10–12 days for plaque development. The number of plaques were counted after staining with neutral red.

Statistics. Data are presented as means $\pm$ standard deviation. A one-way analysis of variance and multiple range test – Scheffe procedure – were used to assess statistical significance. Differences were considered to be significant if P was < 0.01.

Cell labelling and radio-immunoprecipitation. Cell labelling and the immunocomplexes prepared from cell lysates were as described previously (Ronen et al., 1992). Briefly, 5×106 H_9/HIV cells were label led with 100 μCi of [^{35}S]methionine 1000 Ci/mmol (Amersham) in methionine-deficient medium. Labelled cells were collected by centrifugation, washed with cold phosphate-buffered saline (PBS), pH 7.5 and lysed in 2.0 ml lysis buffer (50 mM Tris-HCl (pH 7.5), 0.15 M Nacl, 0.5% Np-40, 0.5% deoxycholate and 0.05% SDS, 0.03 M caproic acid, and 2 mM phenylmethylsulfonyl fluoride (PMSF). Equal amounts of TCA-insoluble radioactive material were reacted with serum from AIDS patients obtained from the Viral and Rickettsial Disease Laboratory (California State Department of Public Health, Berkeley, CA 94704, USA) for 18 h at 4°C. The immune complexes were precipitated with 10% fixed Staphylococcus aureus and washed three times in immune buffer pH 7.5 (20 mM Tris-HCl (pH 7.5), 140 mM Nacl, 10 mM EDTA 0.5% NP_{40} 0.1% SDS). The immune complexes were separated on 10% SDS-PAGE (Laemmli, 1970).

3. Results

Inhibition of HIV production by TSCD. The ability of M-IBDET and A-IBDAT to inhibit HIV production in the chronically-infected H_9/HIV cells was examined. Rates of inhibition of virus production by various concentrations of M-IBDET and A-IBDAT were followed for 72 h. HIV reverse transcriptase activity served as an assay for virus production. Fig. 1 describes the kinetics of inhibition of HIV by various concentrations of the TSCD. The results show that virus production is inhibited proportionally to the concentration of the TSCD. Concentrations ranged

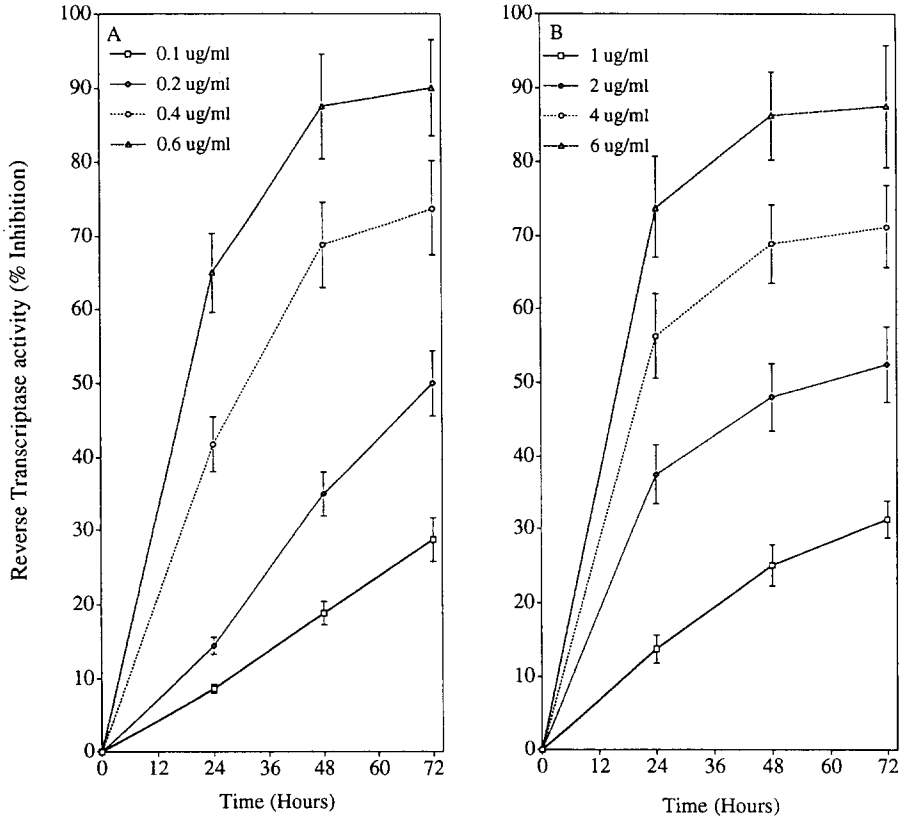

Fig. 1. Kinetics of HIV inhibition at different concentrations of M-IBDET (A) and A-IBDAT (B). H_9/HIV cells at concentration of 5×10^5 cells/ml were incubated with different concentrations of the drugs at 37°C and 7.5% CO_2. Cell count after 72 h reached values of 2.5×10^6–3×10^6/ml. Control, untreated drug cultures contained 0.01% DMSO. At indicated times, virus production was assayed by reverse transcriptase activity. Results are expressed as percent inhibition of enzyme activity. A value of 2.75×10^5 C.P.M. of RT activity was measured after 72 h in the control cells. Each value given represents means ± S.D. for three separate experiments.

from 0.1 to 0.6 μg/ml (0.34 μM to 2.04 μM) of M-IBDET (Fig. 1A). A similar pattern of inhibition was noted after treating the H_9/HIV cells with A-IBDAT, using drug concentrations ranging from 1 to 6 μg/ml (2.9 μM to 17.4 μM) (Fig. 1B). As can be seen from Fig. 1, 0.3 μg/ml M-IBDET and 2 μg/ml A-IBDAT caused 50% of RT activity after treatment of the cells for 48 h. The significant differences between control and TSCD treated cells was confirmed by statistical analysis ($P < 0.01$).

Inhibition of infectious virus production by M-IBDET and A-IBDAT. The effect of different concentrations of M-IBDET and A-IBDAT on HIV production was examined. Virus infectivity was measured by the plaque forming unit (PFU) assays. After 48 h exposure to different concentrations of M-IBDET or A-IBDAT, virus yield in supernatant fluids from untreated control and TSCD-treated cells was assayed by determining the number of plaques appearing on MT_2 target cell layers.

Table 1.
Effect of M-IBDET and A-IBDAT on HIV infectivity (PFU)

M-IBDET μg/ml	PFU × 10^3/ml	% Inhibition
Control[a]	183 ± 19.5	0
0.1	88 ± 8.5	52
0.2	59 ± 5.7	68
0.3	40 ± 3.8	46
0.4	22 ± 2.0	88
0.6	0	100
A-IBDAT μg/ml	**PFU × 10^3/ml**	**% Inhibition**
Control[a]	183 ± 19.5	0
0.5	141 ± 7.9	23
1.0	82 ± 8.5	55
2.0	55 ± 5.6	70
4.0	27 ± 2.6	85
6.0	2 ± 1	99

H_9/HIV cell cultures were treated with different M-IBDET and A-IBDAT concentrations and incubated for 48 h at 37°C and 7.5% CO_2. Virus production in the supernatants was assayed for PFU, as described in Section 2. Results shown are means ± S.D. for 3 separate experiments, $P<0.10$. [a]Control untreated drug cultures contained 0.01% DMSO.

Table No. 1 summarizes the results. Treatment for 48 h with 0.1 μg M-IBDET caused 52% of HIV inhibition, while 0.6 μg blocked HIV production entirely. 1 μg/ml of A-IBDAT caused 55% of HIV inhibition, while 6 μg/ml of the TSCD blocked effectively the infective virus production. Isatin β thiosemicarbazone (IBT) and methyl/isatinthiosemicarbazone (M-IBT) had no effect on virus production (data not shown).

Determination of therapeutic indexes (T.I.). To assess the activity of TSCD against HIV we have examined their T.I. values. H_9/HIV cells were exposed to various concentrations of M-IBDET or A-IBDAT and the T.I. values were determined after exposure to the TSCD for 48 h. The number of viable cells was estimated, and virus RT activity was assayed for each TSCD concentration. The therapeutic indexes for both TSCD were calculated from the responsive curves obtained using a calculation method previously described (Hu et al., 1989). The effect of the TSCD on HIV production and cell viability in H_9/HIV cells is described in Fig. 2. As can be seen, the drug concentrations that caused 50% inhibition of virus yield (EC_{50}) after 48 h of treatment were 0.4 μg/ml and 2 μg/ml for M-IBDET and A-IBDAT, respectively, and 50% inhibiton in cell viability (CC_{50}) was found at 8 μg/ml and 60 μg/ml M-IBDET and A-IBDAT, respectively. The T.I. values (CC_{50}/EC_{50}) for each TSCD were calculated and found to be 20 for M-IBDET (Fig. 2A) and 30 for A-IBDAT (Fig. 2B). The concentration response curves obtained show a T.I. value of 20 for M-IBDET (Fig. 2A) and a T.I. value of 30 for A-IBDAT (Fig. 2B).

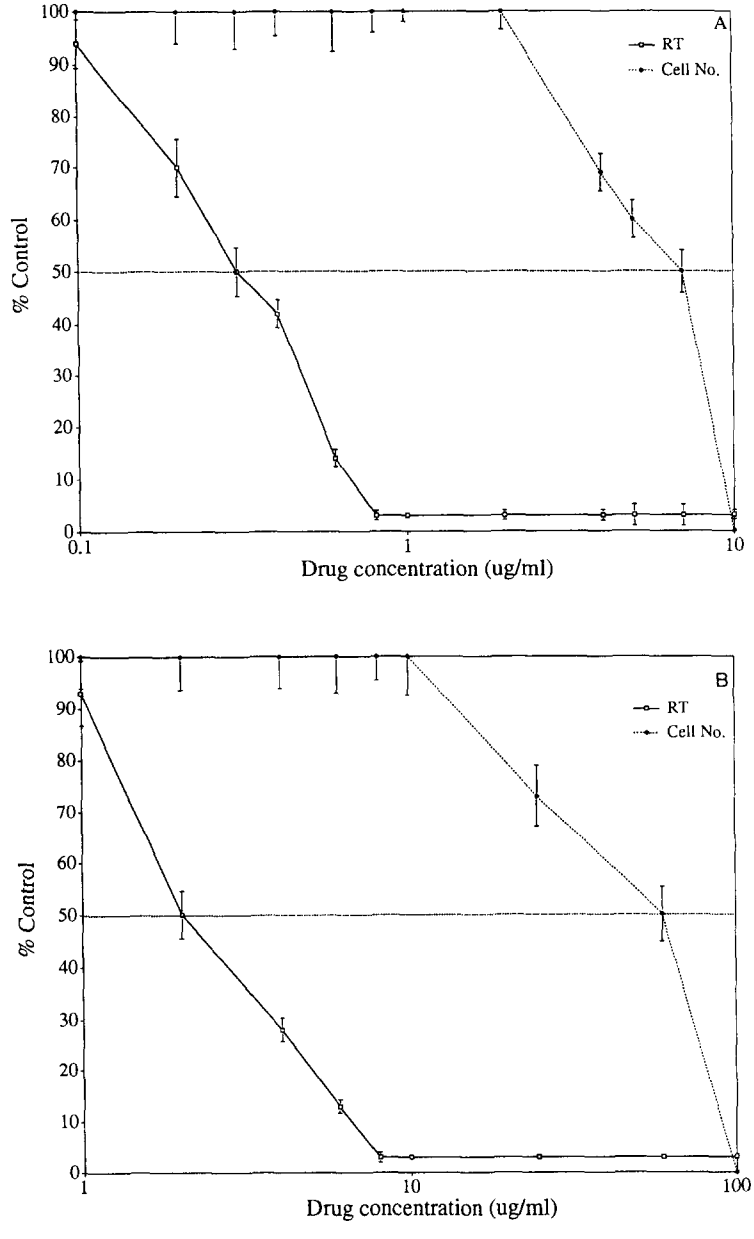

Fig. 2. Determination of Therapeutic Index (T.I.) values of M-IBDET and A-IBDAT in H_9/HIV cells. H_9/HIV cells (5×10^5/ml) were treated with increasing concentrations of M-IBDET (A) or A-IBDAT (B). Virus production (RT activity), cell viability and number were determined in untreated and treated cells for 48 h after exposure to the TSCD, and the percent of inhibition was calculated. Results represent means $\pm$ S.D. of three separate experiments ($P < 0.01$). The T.I. values were determined from the ratio between the drug concentration that causes 50% inhibition of cell viability (CC_{50}) and the drug concentration that causes 50% inhibition of virus production (EC_{50}) (see Section 2).

Selective inhibition of HIV's structural proteins by TSCD. The effect of HIV inhibition by the TSCD on virus structural protein synthesis was examined. H_9/HIV cells were treated with 0.5 μg/ml of M-IBDET or 5 μg/ml of A-IBDAT. 9 h later the cells were pulse-labelled with [^{35}S]methionine for 2 h in the presence of the TSCD. Radiolabelled cell lysates from treated and untreated cells were prepared and collected as described in Section 2. The cell lysate was reacted with anti-HIV sera. The resulting immunoprecipitates were analysed on SDS-PAGE. The effect of M-IBDET and A-IBDAT on HIV protein synthesis is shown in Fig. 3. Both TSCD, at the indicated concentrations, caused significant suppression of all virus structural proteins, including viral envelope glycoproteins (gp 160, gp 120, gp 41) as well as

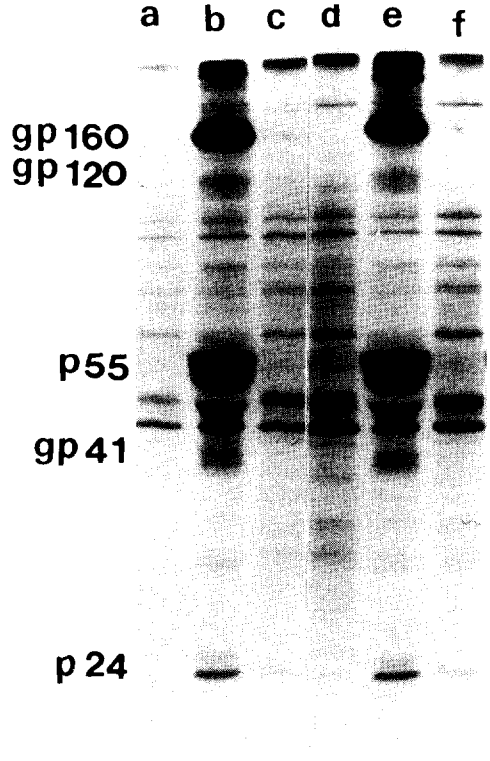

Fig. 3. Effect of M-IBDET and A-IBDAT on HIV structural protein synthesis in H_9/HIV cells. H_9/HIV cells (5×10^5/ml) were incubated with 0.01% DMSO (control) or with 0.5 μg/ml of M-IBDET or 0.5 μg/ml of A-IBDAT. After 9 h of incubation at 37°C and 7.5% CO_2, the cells were radiolabelled with 100 μCi [^{35}S]methionine for 2 h in the presence of the TSCD. Cell lysates were obtained from treated and untreated cells and reacted with HIV patient serum. The immunoprecipitates obtained were analyzed in 10% SDS PAGE (see Section 2). Lane a,d: Immunoprecipitates from control untreated H_9/HIV cells after reaction with normal human serum. Lane b,e: Immunoprecipitates from control untreated H_9/HIV cells after reaction with HIV patient serum. Lane c: Immunoprecipitate from M-IBDET (0.5 μg/ml) treated H_9/HIV cells after reaction with HIV's patient serum. Lane f: Immunoprecipitate from A-IBDAT (5 μg/ml) – treated cells H_9/HIV after reaction with HIV's patient serum.

viral core proteins (p 55 and p 24). At these TSCD concentrations, no effect on the general cellular protein synthesis, as reflected by the [^{35}S]methionine incorporation, was found. Moreover, at these conditions, synthesis of p45 actin and p53 nuclear oncogene, serving as cellular indicators, was not affected (Teitz et al., 1993).

4. Discussion

This work presents the first report on the inhibitory effect of M-IBDET and A-IBDAT on chronically HIV-infected cells. Our previous studies performed towards finding the relation between the anti-retroviral activity of TSCD and their chemical structure showed that M-IBDET and A-IBDAT were the most effective TSCD in suppression of Moloney leukemia virus in B lymphocytes chronically infected with the retrovirus (Teitz et al., 1994a, 1994b). We have also found that these compounds were effective in inhibiting feline immunodeficiency virus (FIV) in chronically-infected feline T lymphocytes (Teitz et al., 1994a). On the basis of these results, we examined in this study the potential of M-IBDET and A-IBDAT as anti-HIV compounds. Our experimental results with the H_9/HIV cells showed a similar concentration-response curve, based on inhibition of RT activity. M-IBDET at 0.6 μg/ml or A-IBDAT at 6 μg/ml suppressed 90% of HIV production after 3 days of treatment.

Thus, for A-IBDAT a 10-fold higher concentration was needed to exert the same antiviral effect as obtained with M-IBDET (a similar result was obtained with the same drug in the FL_4/FIV system (Teitz et al., 1994a)). Inhibition of virus production by the TSCD was also confirmed by testing virus infectivity. The PFU assay served as a more sensitive method for testing HIV infectivity. Comparison of the EC_{50} values obtained by the RT and the PFU method indicates that the EC_{50} of M-IBDET is 0.34 μM by the PFU and 1.02 μM by the RT test. For A-IBDAT the EC_{50} obtained was 2.9 μM by the PFU test and 5.8 μM by the RT assay. Higher T.I. values would have been obtained if the EC_{50} values were obtained by the PFU method instead of the RT activity. Values of 80 and 60 would have been obtained for M-IBDET and A-IBDAT, respectively. However, the RT test is simpler, much quicker and suitable for large scale screening of potential antiviral drugs.

Treatment of infected cells for 48 h with A-IBDAT at 6 μg/ml resulted in almost 2-log decrease of virus infectivity. Our therapeutic index (T.I.) analyses indicated values of 20 and 30 for M-IBDET and A-IBDAT, respectively. In general, as shown by us and others, β-thiosemicarbazone derivatives vary in their effect on cell growth and cellular DNA synthesis (Sherman et al., 1980; Teitz et al., 1994b). The chosen compounds, when employed at their optimal antiviral concentrations, do not markedly affect cellular DNA synthesis and thus are specific inhibitors of retrovirus protein synthesis. Regardless of the TSCD used, viral infection is inhibited, while cellular growth rate is unaffected, over a relatively long period (4 weeks) (Teitz et al., 1994a,b).

Preliminary experiments carried out in our laboratory showed a delay in the appearance of Abelson lymphoma in mice treated with M-IBDET (Sponaro, 1988). This effect was noted at the same dosage range as that employed for M-IBT (Mar-

boran) in humans against smallpox (Bauer, 1965). Our results aiming at understanding the mechanism of inhibition of retroviruses by TSCD showed that the target of inhibition is virus protein synthesis (Ronen et al., 1987). Inhibition of the synthesis of HIV structural proteins by TSCD is consistent with this conclusion.

Acknowledgements

The authors wish to thank Ms. E. Burstein for her excellent technical assistance. This work was supported by a grant from the Chief Scientist's Office,Ministry of Health, Jerusalem.

References

Bauer, D.J. (1965) Clinical experience with the antiviral drug Marboran (*N*-methyl isatin *β* thiosemicarbazone). Ann. NY Acad. Sci. 130, 110–117.

Bauer, D.J. (1972) In: Thiosemicarbazone in Chemotherapy of Virus Diseases, Vol. 1, Pergamon Press, Oxford.

Edelstein, F., Avramoff, M., Stacher, G. and Teitz, Y. (1980) New thiosemicarbazones of mono and bicyclic alpha ketolactam. Eur. J. Med. Chem. 15, 566.

Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.I., Redfield, R., Oleske, J., Safai, B., White, G., Foster, P. and Markham, P.D. (1984) Frequent detection and isolation of cytopathic retrovirus ($HTLV_{III}$) from patients with AIDS and at risk for AIDS. Science 224, 500–502.

Harada, S., Koganagi, Y. and Yamamoto, N. (1985) Infection of $HTLV_{III}$/LaV in HTLV-1-carrying cells MT-2 and MT-4 and application in a plaque assay. Science 229, 563–566.

Hu, J.M. and Hsiung, G.D. (1989) Evaluation of new antiviral agents. In vitro perspectives. Antivral Res. 11, 217–232.

Laemmli, U.K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature (London) 227, 680–685.

Levy, J.A., Hoffman, A.D., Kramer, S.M., Landis, J.A. Shimabukurd, J.M. and Oshior, L.S. (1984) Isolation of Lymphocytopathic retroviruses from San Francisco patients with AIDS. Science 225, 840–842.

Ronen, D. and Teitz, Y. (1984) Inhibition of the synthesis of Moloney leukemia virus structural proteins by *N*-methylisatin-*β*-4′:4′-diethylthiosemicarbazone.Antimicrob. Agents Chemother. 26, 913–916.

Ronen, D., Nir, E. and Teitz, Y. (1985) Effect of *N*-methylisatin-*β*-4′:4′-diethylthiosemicarbazone on intracellular Moloney leukemia virus constituents. Antiviral Res. 5, 249–254.

Ronen, D., Sherman, L., Bar-Nun, S. and Teitz, Y. (1987) *N*-methylisatin-*β*-4′:4′-diethylthiosemicarbazone an inhibitor of Moloney leukemia virus protein production: Characterization and in vitro translation of viral mRNA. Antimicrob. Agents. Chemother. 31, 1798–1802.

Ronen, D., Teitz, Y., Goldfinger, N. and Rotter, V. (1992) Expression of wild-type and mutant p53 proteins by recombinant Vaccinia viruses. Nucleic Acid Res. 20, 3435–3441.

Schupbach, J., Popovic, M., Gilden, R., Gonda, M.A., Sarngadharan, M.G. and Gallo, R.C. (1984) Serological analysis of a subgroup of human T-lymphocyte retroviruses ($HTLV_{III}$) associated with AIDS. Science 224, 503–505.

Sherman, L., Edelstein, F., Stacher, G., Avramoff, M. and Teitz, Y. (1980) Inhibition of Moloney leukemia virus production by *N*-methylisatin-*β*-4′:4′-diethylthiosemicarbazone. J. Gen. Virol. 46, 195–203.

Sponaro, R. (1988) The effect of M-IBDET on infections with different leukemia viruses belonging to the Retroviridae in vitro and in vivo. Tel Aviv University, Tel Aviv, Israel.

Teitz, Y. 'Thiosemicarbazone derivatives – potential drugs against retroviruses". Patent granted: Israel No. 77496 (1989). UK No. GB2184944B (1989). USA No. 4,827,843 (1990).

Teitz, Y. (1971) RNA dependent DNA polymerase in C-type particles from normal rat thymus cultures. Nature New Biology 232, 250–252.

Teitz, Y., Ladizensky, E., Barko, N. and Burstein, E. (1993) Selective repression of v-abl coded protein by *N*-methylisatin-β-4′:4′-diethylthiosemicarbazone and *N*-allylisatin-β-4′:4′-diallylthiosemicarbazone.Antimicrob. Agents Chemother. 37, 2483–2485.

Teitz, Y., Barko, N., Torten, M. and Ronen, D. (1994a) Inhibition of Feline immunodeficiency virus production and HIV Tat activity by thiosemicarbazone derivatives. Antiviral Chem. Chemother., in press.

Teitz, Y., Barko, N., Abramoff, M. and Ronen, D. (1994b) The relationship between structure and antiretroviral activity of Thiosemicarbazone derivatives. Chemotherapy 76, 1–6.