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2-Glycineamide-5-chlorophenyl 2-pyrryl ketone, a non-benzodiazepin Tat antagonist, is effective against acute and chronic HIV-1 infections in vitro

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Abstract

In the search for effective Tat-dependent transcription inhibitors using a screening assay system that has recently been developed, 2-glycineamide-5-chlorophenyl 2-pyrryl ketone (GCPK) has proved to be a potent and selective inhibitor of human immunodeficiency virus type 1 (HIV-1) replication in vitro. This compound was inhibitory to HIV-1 replication in both acutely and chronically infected cells. The 50% effective concentration (EC_{50}) of GCPK in acutely infected MOLT-4 and CEM cells was 0.62 and 0.13 μg/ml, respectively. These values were similar to those of the known Tat-dependent transcription inhibitors Ro5-3335 and Ro24-7429. Like these inhibitors, GCPK could inhibit HIV-1 replication in MOLT-4/III_B (MOLT-4 cells chronically infected with HIV-1) and tumor necrosis factor-α- (TNF-α)-induced viral activation in OM10.1 cells (a HL-60 clone latently infected with HIV-1). GCPK is distinct from Ro5-3335 and Ro24-7429 in that this novel Tat-dependent transcription inhibitor has no benzodiazepin ring.

Keywords: Anti-Tat; Anti-HIV; GCPK; Chronic infection; Ro5-3335; Ro24-7429

1. Introduction

A number of nucleoside and non-nucleoside derivatives have been reported as selective inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT). However,

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these RT inhibitors are only effective against HIV-1 replication in acutely infected cells. HIV-1 protease and Tat-dependent transcription inhibitors are effective in chronically infected cells as well as acutely infected cells. In fact, the Tat-dependent transcription inhibitors Ro5-3335 and Ro24-7429 proved to be inhibitory to both acute and chronic HIV-1 infections. Recovery of CD4 receptor on chronically infected cells has also been observed in the presence of the Tat-dependent transcription inhibitors (Shahabuddin et al., 1992). HIV-1 mutants resistant to the Tat-dependent transcription inhibitors have not been identified so far, though HIV-1 protease inhibitors generate drug-resistant mutants in vitro and in vivo (Condra et al., 1995).

HIV-1 Tat is produced as a 14 kDa protein from the HIV-1 genome and is located in the nucleus of HIV-1-infected cells (Arya et al., 1985; Sodroski et al., 1985). Tat binds *cis*-acting Tat-responsive element (TAR) that is mapped to a sequence immediately downstream from the cap site of viral transcripts (+ 1– + 59) (Okamoto and Wong-Staal, 1986; Rosen et al., 1985). Tat stimulates initiation of HIV transcription and stabilizes the elongation of transcription of HIV-1 mRNA (Berkhout et al., 1990; Feinberg et al., 1991; Laspia et al., 1989; Sharp and Marciniak, 1989). Thus, Tat plays an important role in the pathogenesis and progression of HIV-1 replication, and this prompted us to develop a rapid and sensitive colorimetric assay for screening effective Tat-dependent transcription inhibitors by using molecular biology techniques (Kira et al., 1995).

In this study, we have examined 2-glycineamide-5-chlorophenyl 2-pyrryl ketone (GCPK) for its anti-Tat activity and inhibitory effect on HIV-1 replication in acutely and chronically infected cells and found that GCPK is a selective inhibitor of HIV-1 as well as Ro5-3335 (Hsu et al., 1991) and Ro24-7429 (Hsu et al., 1993).

2. Materials and methods

2.1. Compounds

Ro5-3335 (MW 259.69), Ro24-7429 (MW 272.73), and GCPK (MW 277.70) were synthesized by the Yamanouchi Pharmaceutical Co., Tokyo, Japan. The chemical structure of GCPK is shown in Fig. 1. 3′-Azido-3′-deoxythymidine (AZT) was purchased from Sigma (St. Louis, MO). All compounds were dissolved in dimethyl sulfoxide (DMSO) at 50 mM (or higher) to exclude any antiviral or cytotoxic effect of DMSO and were stored − 20°C until use.

2.2. Cells and viruses

CEM, MOLT-4, MT-4, MOLT-4/III_B (MOLT-4 cells chronically infected with HIV-1) and OM10.1 (Butera et al., 1991) cells were used in the anti-HIV-1 assays. CEM-pKO cells, which are the plasmid pKO containing CEM cells, were used for the anti-Tat assay. The construction of the pKO plasmid was already reported (Kira et al., 1995). All cell lines were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 200 U/ml of penicillin G, and 200 μg/ml of streptomycin.

The laboratory HIV-1 strain HTLV-III_B was used in the anti-HIV-1 assays in acutely infected cells. The virus was propagated in MT-4 cells. Titer of the virus stocks was determined in MT-4 cells, and the stocks were stored at − 80°C until use.

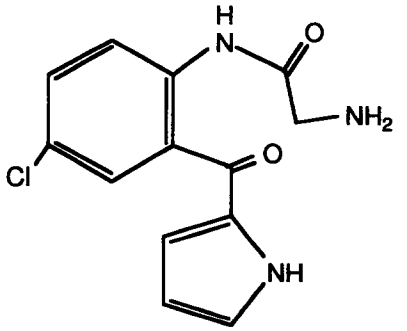

Fig. 1. Formula of GCPK.

2.3. Anti-Tat compound assay

The assay system for screening anti-Tat compounds has been described in our previous paper (Kira et al., 1995). Briefly, CEM-pKO cells were cultured in a flat-bottomed 96-well microtiter plate in the presence of varying concentrations of test compounds and 300 μg/ml of hygromycin B. After a 7-day incubation at 37°C, the number of viable cells was determined by the MTT method (Pauwels et al., 1988). For cytotoxicity evaluation, CEM-pKO cells were also cultured in the presence of varying concentrations of test compounds but in the absence of hygromycin B. All experiments were carried out in triplicate.

2.4. Anti-HIV-1 assays

Inhibitory effects of the compounds on HIV-1 replication in acute infection were determined in MOLT-4, CEM and MT-4 cells. These cells were suspended in culture medium at 1×10^5 cells/ml and infected with HIV-1 at a multiplicity of infection (m.o.i.) of 0.1 for MOLT-4 and CEM cells and 0.01 for MT-4 cells. Immediately after virus infection, the cell suspension (100 μg/ml) was brought into each well of a flat-bottomed microtiter tray containing varying concentrations of the test compounds. After a 4-day incubation at 37°C, MOLT-4 and CEM cells were subcultured at a ratio of 1:5 with fresh culture medium containing appropriate concentrations of the test compounds and further incubated. The number of viable cells was determined by the MTT method on day 4 (MT-4 cells) and day 8 (MOLT-4 and CEM cells) after virus infection. The cytotoxicity of the test compounds was evaluated in parallel with their antiviral activity. The evaluation was based on the viability of mock-infected cells, as monitored by the MTT method.

The inhibitory effects of the compounds on HIV-1 replication in chronic infection were determined in MOLT-4/III_B and OM10.1 cells. HIV-1 replication was determined by p24 antigen level and RT activity in culture supernatants of these cells. The assay procedure for activation of HIV-1 by tumor necrosis factor-α (TNF-α) in OM10.1 cells has been previously described (Feorino et al., 1993). The amount of p24 was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Abbott Laboratories, North Chicago, IL) according to the manufacturer's instructions. The RT assay was carried out by a conventional method, using poly(A)·oligo(dT) (Pharmacia Fine Chemicals, Piscataway, NJ) and [α-^{32}P]dTTP (Amersham Corporation, Arlington Heights, IL) as the template primer and substrate, respectively. Recovery of CD4 receptor in OM10.1 cells was assessed by direct immunofluorescence and fluorescence-activated cell scan (FACScan, Becton Dickinson, San José, CA) analysis.

3. Results

When the anti-Tat activity of GCPK was tested in CEM-pKO cells, where effective compounds can suppress the expression of hygromycin B phosphotransferase gene through the inhibition of Tat, the number of viable cells in the presence of hygromycin B became less than that in the absence of hygromycin B (Fig. 2A). At a concentration of 1.5–6.25 μg/ml, difference in viable cell number was obvious between the hygromycin (+) and hygromycin (−). GCPK achieved 30–40% reduction of viable cell number in the presence of hygromycin B compared with the absence of hygromycin B. Similar results were obtained with the known Tat-dependent transcription inhibitor Ro24-7429 (Fig. 2B). These results suggest that GCPK inhibits HIV-1 Tat activity.

In the next set of experiments, we examined whether GCPK could inhibit HIV-1 replication in acutely infected cells. Table 1 summarizes the anti-HIV-1 activities of GCPK, Ro5-3335, Ro24-7429 and AZT in MOLT-4, CEM and MT-4 cell cultures. The 50% effective concentration (EC_{50}) of GCPK was 2.24 μM in MOLT-4 cells, which was approximately 10-fold higher than the EC_{50} of Ro5-3335 and Ro24-7429 (0.26 and 0.19 μM, respectively). On the other hand, its cytotoxicity was approximately 5-fold less than those of Ro5-3335 and Ro24-7429, when their 50% cytotoxic concentration (CC_{50}) values were compared. The selective index (SI) of GCPK based on the ratio of

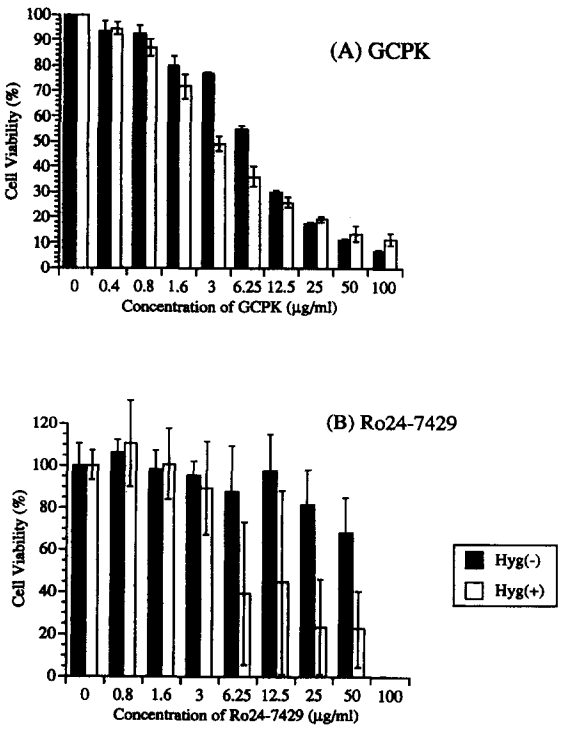

Fig. 2. Anti-Tat activity of GCPK (A) and Ro24-7429 (B) in CEM-pKO cells. The details of experimental procedures are described in Section 2.

CC_{50} to EC_{50} was 13. In CEM cells, GCPK was found to be a more potent inhibitor than Ro5-3335, and its EC_{50} was 0.47 μM (Table 1). In contrast, no anti-HIV-1 activity of GCPK was detected in MT-4 cells, as has previously also been shown for Ro5-3335 and Ro24-7429 (Witvrouw et al., 1992).

GCPK proved effective in inhibiting the production of HIV-1 in MOLT-4/III_B and TNF-α-treated OM10.1 cells. The former is a chronically HIV-1-infected T-cell line and continuously produces the virus. The latter is a chronically HIV-1-infected promyelocyte cell line but does not produce the virus without stimuli such as TNF-α and phorbol myristate acetate. GCPK could suppress the p24 level in culture supernatants of MOLT-4/III_B and OM10.1 cells at a concentration of 2 μg/ml, at which it did not suppress the cell viability (Fig. 3 and data not shown). The activity of GCPK in MOLT-4/III_B and OM10.1 cells was also confirmed by the experiments on HIV-1 RT activity in the culture supernatants. As shown in Fig. 4, GCPK inhibited the RT activity of culture supernatant in a dose-dependent fashion. The EC_{50} values of GCPK and Ro5-3335 were approximately 1 and 4 μg/ml in MOLT-4/III_B cells, respectively (Fig. 4A and B). However, in this experiment, GCPK seemed to be a weaker inhibitor than Ro5-3335 in OM10.1 cells (Fig. 4C and D).

It has been demonstrated that Ro5-3335 could recover surface CD4 receptor in HIV-1 chronically infected cells (Feorino et al., 1993; Shahabuddin et al., 1992). Like Ro5-3335, GCPK also increased the number of $CD4^+$ OM10.1 cells at a concentration of 5 μg/ml (Fig. 5). Nucleoside and non-nucleoside RT inhibitors did not show any effect on the expression of CD4 in OM10.1 cells (Feorino et al., 1993).

Table 1
Inhibitory effects of GCPK and other compounds on HIV-1 replication in acutely infected cells[a]

Compound	Cell	EC_{50}[b] (μg/ml)	CC_{50}[c] (μg/ml)	SI[d]
GCPK	MOLT-4	0.62 ± 0.04	7.9 ± 0.53	13
Ro5-3335	MOLT-4	0.067 ± 0.018	1.6 ± 0.04	24
Ro24-7429	MOLT-4	0.053 ± 0.021	1.8 ± 0.05	34
AZT	MOLT-4	0.089	> 10	
GCPK	CEM	0.13 ± 0.003	3.0 ± 0.08	23
Ro5-3335	CEM	0.25 ± 0.01	1.18 ± 0.19	7.2
Ro24-7429	CEM	0.058 ± 0.017	3.5 ± 0.29	60
AZT	CEM	0.072	> 10	
GCPK	MT-4	> 1.7	> 1.7	< 1
AZT	MT-4	0.0061	14.84	2432

[a] Except for AZT, all data represent means ± standard deviations for at least two separate experiments.
[b] Based on the inhibition of HIV-1-induced cytopathogenicity.
[c] Based on the reduction of viability of mock-infected cells.
[d] Based on the ratio of CC_{50} to EC_{50}.

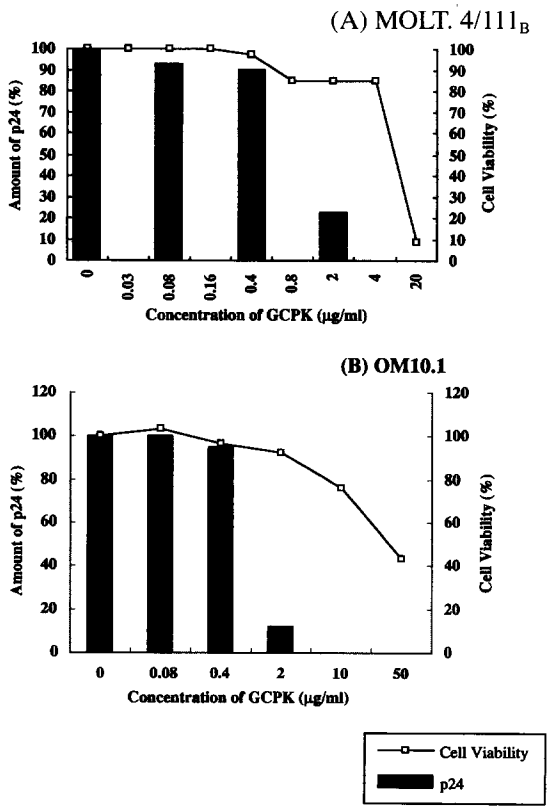

Fig. 3. Inhibitory effects of GCPK on HIV-1 replication of MOLT-4/III_B (A) and OM10.1 (B) cells. The amount of p24 antigen was measured by a sandwich ELISA kit.

4. Discussion

In this study, we have found GCPK is a selective inhibitor of HIV-1 replication through its interaction with Tat-dependent transactivation. Camptothecin, D-penicillamine, and DRB have also been reported to have anti-Tat activity in vitro (Chandra et al., 1988; Li et al., 1994; Marciniak and Sharp, 1991). However, in our anti-Tat assay in CEM-pKO cells, these compounds did not show any selective inhibitory effect of Tat activity or HIV-1 replication at their non-toxic concentrations (Kira et al., 1995). Ro5-3335 and Ro24-7429 are the only Tat 'antagonists' known to inhibit HIV-1 replication in cell culture. Ro5-3335 and Ro24-7429 belong to the family of benzodiazepin derivatives, yet GCPK dose not have a benzodiazepin ring (Fig. 1). Thus, the benzodiazepin structure is not necessary to exert the anti-Tat activity.

Ro5-3335 and Ro24-7429 are known to interact with a cellular protein which cooperates with Tat on TAR (Braddock et al., 1994). The cytotoxicity of these Tat-dependent transcription inhibitors may be attributed to this mechanism of action. Based on the similarities in chemical structure and antiviral activity profile, it is possible that GCPK also act interacts with the same cellular protein. Ro5-3335 was not inhibitory in the human T-lymphotropic virus type 1 (HTLV-1)-transformed $CD4^+$ cell lines MT-4 and MT-2 (Witvrouw et al., 1992), indicating that the efficacy of known Tat-dependent transcription inhibitors is strongly dependent on the host cell type. Clinical trials with Ro24-7429 failed to demonstrate efficacy (Bawen et al., 1995). Compounds that directly interact with Tat itself may be able to inhibit HIV-1 replication irrespective of the cell type.

GCPK, as well as Ro5-3335 and Ro24-7429, inhibit HIV-1 replication through the Tat-mediated pathway and they are not active in MT-4 cells. Luznik et al. have reported that Tat-defective HIV strains could proliferate in MT-2 and MT-4 cells, because these cell lines are transformed by HTLV-1 and additional cellular factors (high levels of nuclear factor-κB (NF-κB) etc.) are expressed in these cell lines by HTLV-1 tax (Luznik et al., 1995).

Another issue to be clarified is whether GCPK can sufficiently suppress the production of HIV-1 in chronically infected cells. In our experiments, GCPK could achieve approximately 80% inhibition of viral production at a concentration of 2 μg/ml (Fig. 3). It has been reported that the transcription of Tat-defective HIV-1 RNA occurs in the presence of NF-κB (Luznik et al., 1995; Popik and Pitha, 1993, 1994). TNF-α initiates the transcription of HIV-1 RNA through the activation of NF-κB, whereas Tat up-regulates the transcription and stabilizes the elongation of the HIV-1 RNA (Antoni et al., 1994). Thus, it may be necessary to combine Tat-dependent transcription inhibitors and NF-κB inhibitors to achieve sufficient suppression of HIV-1 replication in chronically infected cells. In fact, such combinations have been shown to afford cooperative inhibition in vitro (Biswas et al., 1993).

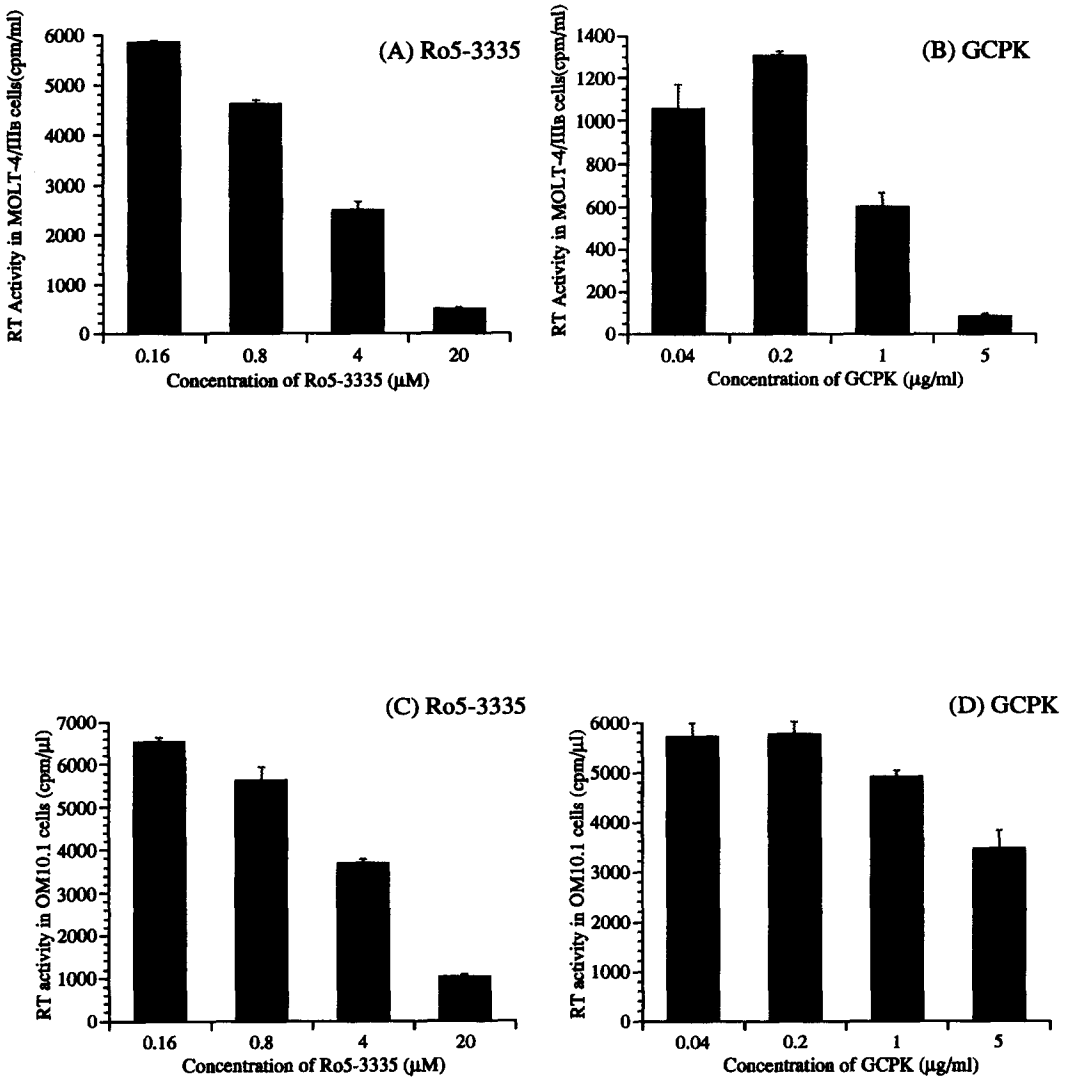

Fig. 4. Inhibitory effects of Ro5-3335 (A, C) and GCPK (B, D) on HIV-1 replication of MOLT-4/III_B (A, B) and OM10.1 (C, D) cells. The production of HIV-1 in culture supernatants was determined by the RT activity.

In HIV-1-infected cells, the expression of CD4 receptor is down-regulated in general (Geleziunas et al., 1991; Hoxie et al., 1985, 1986). The Tat-dependent transcription inhibitors including GCPK could recover the expression of CD4 (Fig. 5). If the compounds could also recover impaired functions of the infected cells, they might have beneficial effects on immune reconstruction in HIV-1-infected patients. Furthermore, Tat-dependent transcription inhibitors might favor conditions in the patients that are similar to those in long-term non-progressors, who have a low level of HIV-1 replication through some unknown mechanisms. In conclusion, the Tat-dependent transcription inhibitor GCPK is a novel anti-HIV-1 agent and should be further pursued for its potential.

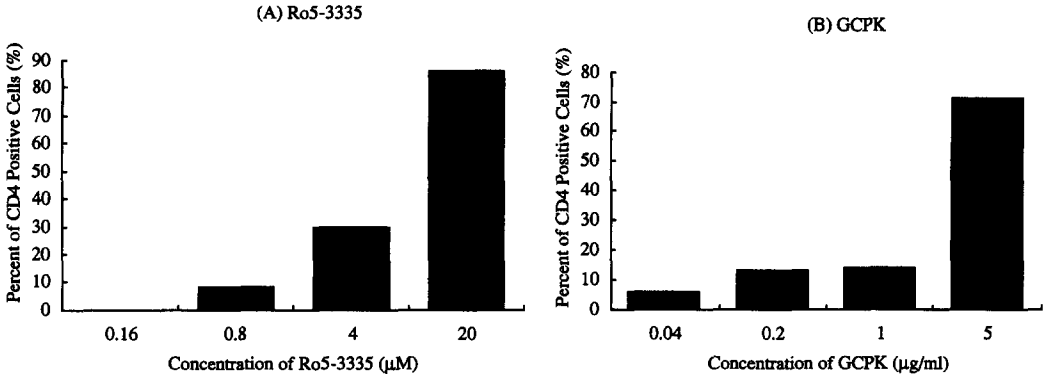

Fig. 5. Recovery of CD4 in OM10.1 cells by Ro5-3335 (A) and GCPK (B). The number of $CD4^+$ cells was assessed by direct immunofluorescence and FACScan analysis.

References

Antoni, B.A., Stein, S.B. and Rabson, A.B. (1994) Regulation of human immuno-deficiency virus infection: implications for pathogenesis. Adv. Virus Res. 43, 53–145.

Arya, S.K., Guo, C., Josephs, S.F. and Wong-Staal, F. (1985) Trans-activator gene of human T-lymphotropic virus type III (HTLV-III). Science 229, 69–73.

Bawen, E.F., Atkins, M., Weller, I.V.D. and Johnson, M.A. (1995) In: D.J. Jeffries and E. De Clercq (Eds), Antiviral Chemotherapy, pp. 65–79. John Wiley & Sons, Chichester.

Berkhout, B.B., Gatignol, A., Rabson, A.B. and Jeang, K. (1990) TAR-independent activation of HIV-1 LTR: evidence that Tat requires specific regions of the promoter. Cell 62, 757–767.

Biswas, D.K., Ahlers, C.M., Dezube, B.J. and Pardee, A.B. (1993) Cooperative inhibition of NF-κB and Tat-induced superactivation human immunodeficiency virus type 1 long terminal repeat. Proc. Natl. Acad. Sci. USA 90, 11044–11048.

Braddock, M., Cannon, P., Muckenthaler, M., Kingsman, A.J. and Kingsman, S.M. (1994) Inhibition of human immunodeficiency virus type 1 Tat-dependent activation of translation in *Xenopus* oocytes by the benzodiazepine Ro24-7429 requires *trans*-activation response element loop sequence. J. Virol. 68, 25–33.

Butera, S.T., Perez, V.L., Wu, B.-Y., Nabel, G.J. and Folks, T.M. (1991) Oscillation of the human immunodeficiency virus surface receptor is regulated by the state of activation in a $CD4^+$ cell model of chronic infection. J. Virol. 65, 4645–4653.

Chandra, A., Demirhan, I., Arya, S.K. and Chandra, P. (1988) D-Penicillamine inhibits trans activation of human immunodeficiency virus type-1 (HIV-1) LTR by transactivator protein. FEBS Lett. 29, 282–286.

Condra, J.H., Schleif, W.A., Blahy, O.M., Gabryelski, L.J., Graham, D.J., Quintero, J., Rhodes, A., Robbins, H.L., Roth, E., Shivaprakash, M., Titus, D., Yang, T., Teppler, H., Squires, K.E., Deutsch, P.J. and Emini, E.A. (1995) In vivo emergence of HIV-1 variants resistant to multiple protease inhibitors. Nature 374, 569–571.

Feinberg, M.B., Baltimore, D. and Frankel, A.D. (1991) The role of Tat in the human immunodeficiency virus life cycle indicates a primary effect on transcriptional elongation. Proc. Natl. Acad. Sci. USA 88, 4045–4049.

Feorino, P.M., Butera, S.T., Folks, T.M. and Schinazi, R.F. (1993) Prevention of activation of HIV-1 by antiviral agents in OM-10.1 cells. Antiviral Chem. Chemother. 4, 55–63.

Geleziunas, R., Bour, S., Boulerice, F., Hiscott, J. and Wainberg, M.A. (1991) Diminution of CD4 surface protein but not CD4 messenger RNA level in monocytic cells infected by HIV-1. AIDS 5, 29–33.

Hoxie, J. A., Haggarty, B.S. and Rackowski, J. (1985) Persistent noncytopathic infection of normal human T lymphocyte with AIDS-associated retroviruses. Science 229, 1400–1402.

Hoxie, J.A., Alpers, J.D., Rackowski, J.L., Huebner, K., Haggarty, B.S., Cedarbaum, A.J. and Reed, J.C. (1986) Alteration in T4 (CD4) protein and mRNA synthesis in cells infected with HIV. Science 234, 1123–1127.

Hsu, M., Schutt, A.D., Holly, M., Slice, L.W., Sherman, M.I., Richman, D.D., Potash, M.J. and Volsky, D.J. (1991) Inhibition of HIV replication in acute and chronic infections in vitro by a Tat antagonist. Science 254, 1799–1802.

Hsu, M., Dhingra, U., Earley, J.V., Holly, M., Keith, D., Nalin, C.M., Richou, A.R., Schutt, A.D., Tam, S.Y., Potash, M.J., Volsky, D.J. and Richman, D.D. (1993) Inhibition of type 1 human immunodeficiency virus replication by a Tat antagonist to which the virus remains sensitive after prolonged exposure in vitro. Proc. Natl. Acad. Sci. USA 90, 6395–6399.

Kira, T., Merin, J.P., Shigeta, S., Baba, M. and Okamoto, T. (1995) Anti-Tat MTT assay: a novel anti-HIV drug screening system using the viral regulatory network of replication. AIDS Res. Hum. Retrovir. 11, 1359–1366.

Laspia, M.F., Rice, A.P. and Mathews, M.B. (1989) HIV-1 Tat protein increases transcriptional initiation and stabilizes elongation. Cell 59, 283–292.

Li, C.J., Wang, C. and Pardee, A.B. (1994) Camptothecin inhibits Tat-mediated transactivation of type 1 human immunodeficiency virus. J. Biol. Chem. 269, 7051–7054.

Luznik, L., Kraus, G., Guatelli, J., Richman, D.D. and Wong-Staal, F. (1995) Tat-independent replication of human immunodeficiency viruses. J. Clin. Invest. 95, 328–332.

Marciniak, R. and Sharp, P. (1991) HIV-1 Tat protein promotes formation of more-processive elongation complexes. EMBO J. 10, 4189–4196.

Okamoto, T. and Wong-Staal, F. (1986) Demonstration of virus-specific transcriptional activator(s) in cells infected with HTLV-III by an in vitro cell-free system. Cell 47, 29–35.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and De Clercq, E. (1988) Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. J. Virol. Methods 20, 309–321.

Popik, W. and Pitha, P. (1993) Role of tumor necrosis factor alpha in activation and replication of the *tat*-defective human immunodeficiency virus type 1. J. Virol. 67, 1094–1099.

Popik, W. and Pitha, P. (1994) Differential effect of tumor necrosis factor-α and herpes simplex virus type 1 on the Tat-targeted inhibition of human immunodeficiency virus type 1 replication. Virology 202, 521–529.

Rosen, C.A., Sodroski, J.G. and Haseltine, W.A. (1985) The location of *cis*-acting regulatory sequences in the human T cell lymphotropic virus type III (HTLV-III/LAV) long terminal repeat. Cell 41, 813–823.

Shahabuddin, M., Volsky, B., Hsu, M. and Volsky, D.J. (1992) Restoration of cell surface CD4 expression in human immunodeficiency virus type 1-infected cells by treatment with a Tat antagonist. J. Virol. 66, 6802–6805.

Sharp, P.A. and Marciniak, R.A. (1989) HIV TAR: an RNA enhancer? Cell 59, 229–230.

Sodroski, J., Patarca, R. and Rosen, C. (1985) Location of the *trans*-activating region on the genome of human T-cell lymphotropic virus type III. Science 229, 74–77.

Witvrouw, M., Pauwels, R., Vandamme, A., Schols, D., Reymen, D., Yamamoto, N., Desmyter, J. and De Clercq, E. (1992) Cell type-specific human immuno-deficiency virus type 1 activity of the transactivation inhibitor Ro5-3335. Antimicrob. Agents Chemother. 36, 2628–2633.