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Inhibition of Replication and Cytopathic Effect of Human T Cell Lymphotropic Virus Type III/Lymphadenopathy-Associated Virus by 3′-Azido-3′-Deoxythymidine In Vitro

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Human T cell lymphotropic virus type III (HTLV-III)/lymphadenopathy-associated virus is the etiologic agent of the acquired immune deficiency syndrome (AIDS) and AIDS-related complex. The effect of 3′-azido-3′-deoxythymidine (AZT) on the HTLV-III/lymphadenopathy-associated virus infection was quantitatively studied with HTLV type I-carrying MT-4 cells. The AZT compound inhibited HTLV-III-induced cytopathic effect and virus-specific antigen expression in MT-4 cells at concentrations of 5 and 10 μM. In addition, a plaque-forming assay was performed to assess the effect of AZT on virus replication in MT-4 cells freshly infected with HTLV-III and in continuous HTLV-III-producing Molt-4/HTLV-III cells. Results showed that AZT efficiently and effectively inhibited the replication of HTLV-III in infected MT-4 cells. AZT is a strong inhibitor of reverse transcriptase activity of HTLV-III as a triphosphate, to such a degree that even 1.0 pM azido-TTP inhibits 50% of reverse transcriptase activity. However, it did not show any effect in the HTLV-III-producing cell line Molt-4/HTLV-III. Thus, AZT has no effect on virus replication of an already integrated virus. When 5 μM AZT was added to HTLV-III-infected MT-4 within 20 h after infection, a striking suppressive effect was noticed. This concentration was much lower than that which inhibits the growth of MT-4 cells. These results confirm those found in a previous report (H. Mitsuya, K. J. Weinhold, P. A. Furman, H. S. Clair, S. N. Lehrman, R. C. Gallo, D. Bolognesi, D. W. Barry, and S. Broder, Proc. Natl. Acad. Sci. USA 82:7096–7100, 1985) and suggest that AZT might be used as an experimental antiviral agent for AIDS and AIDS-related complex.

Human T cell lymphotropic virus type III (HTLV-III) (16), also named lymphadenopathy-associated virus (LAV) (1), is a newly recognized retrovirus which is cytopathic for helper-inducer T cells in vitro. This virus is most probably the etiologic agent of the acquired immune deficiency syndrome (AIDS) and AIDS-related complex (2, 3, 5, 11, 19, 20).

Previously, we reported that HTLV-III/LAV efficiently infected and propagated in a large number of HTLV type I-carrying cell lines, such as MT-4 (9). As a result, infected cells showed remarkable cytopathic effect, leading to the inability of further subcultivation of the cell line. Subsequently, we developed quantitative assay systems for HTLV-III. One such system is a plaque-forming assay in MT-4 cells (10, 15a).

Although a number of antiviral agents are now being considered for the experimental therapy of AIDS (13, 14, 17, 18), to date no therapy has been shown to cure clinical manifestations of AIDS or restore the underlying immune deficiency. Moreover, the chronicity of infection and the propensity of the virus to infect the brain (21, 22) make it necessary to explore new classes of drugs which have the potential for oral administration and the ability to penetrate across the blood-brain barrier. Very recently, Mitsuya et al. (15) reported that a nucleoside analog, 3′-azido-3′-deoxythymidine (AZT), had an inhibitory effect on both the infectivity and cytopathic effect of HTLV-III in vitro. They evaluated the AZT effect by using an immunofluorescence (IF) method for determination of HTLV-III *gag* protein expression, an assay for cytopathic effect of HTLV-III, and a reverse transcriptase (RT) assay. In this study, using our quantitative bioassay system, we found that AZT inhibited the replication and cytopathic effect of HTLV-III in vitro. In addition, the time of AZT treatment was investigated to assess the inhibitory effect of the compound on virus spreading.

MATERIALS AND METHODS

Cells. An HTLV type I-carrying cell line, MT-4, and an HTLV-III-producing cell line, Molt-4/HTLV-III (9), were used in this study. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml at 37°C in a CO_2 incubator.

Nucleosides. A nucleoside analog, AZT (molecular weight, 267.24) was prepared essentially as described by Glinski et al. (6) and was phosphorylated by phosphorus oxychloride in triethyl phosphate by the method of Yoshikawa et al. (24). The 5′-triphosphate (3′-azido-TTP) was prepared from the 5′-monophosphate as described by Maeda et al. (12) and was purified by DEAE-cellulose chromatography. The purity of the AZT and azido-TTP used in this study was more than 99.5%.

Virus and virus infection. HTLV-III was obtained from culture supernatants of Molt-4/HTLV-III as previously described (9). The titer of this virus preparation was 6×10^4 PFU/ml. Infection of MT-4 cells with HTLV-III was made at a multiplicity of infection of 0.002. Briefly, the cells were mixed with virus solution and incubated for 1 h at 37°C. After adsorption, infected cells were washed and resus-

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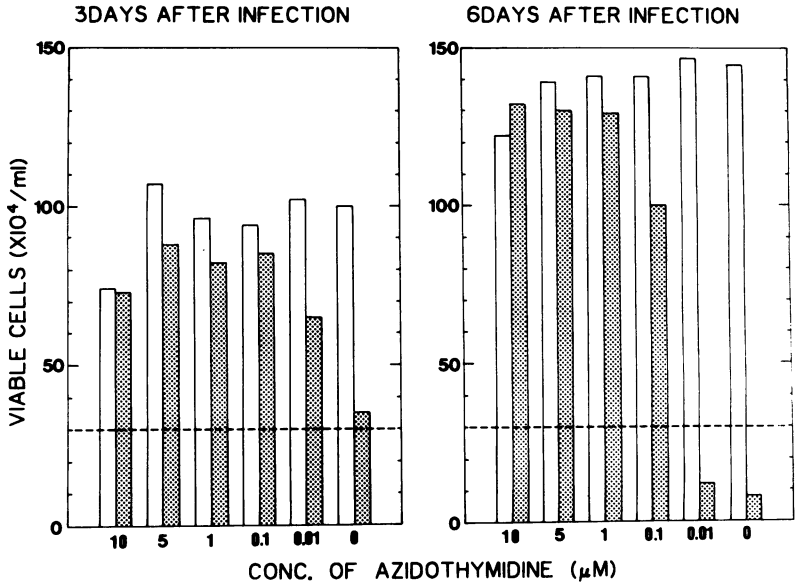

FIG. 1. Inhibitory effect of AZT on cytopathic effect of HTLV-III/LAV. MT-4 cells (open bars) and HTLV-III-infected MT-4 cells (dotted bars) were cultured in the presence of various concentrations of AZT. The viable cells were determined by the trypan blue dye exclusion staining method.

pended in fresh medium to a concentration of 3×10^5 cells per ml. This concentration was cultured in both the presence and absence of various concentrations of AZT in a CO_2 incubator.

Assay for HTLV-III/LAV-induced cytopathic effect. HTLV-III/LAV-induced cytopathic effect was analyzed by measuring the decrease in the number of viable cells. The viable cells were counted by the trypan blue exclusion staining method.

Assay for HTLV-III/LAV antigen expression. HTLV-III-infected MT-4 cells with virus-specific antigens were counted by an indirect IF method. Briefly, methanol-fixed cells were incubated with 1:1,000-diluted anti-HTLV-III positive human serum (IF titer, 1:4,096) for 30 min at 37°C. The preparations were then washed for 15 min with phosphate-buffered saline. The cells were then incubated with fluorescein isothiocyanate-conjugated rabbit anti-human immunoglobulin G (Dakoppatts A/S, Copenhagen, Denmark) for 30 min at 37°C and washed again with phosphate-buffered saline. More than 500 cells were counted under a fluorescence microscope, and the percentage of IF-positive cells was calculated.

Plaque-forming assay. To determine the suppressive effect of AZT on HTLV-III/LAV replication quantitatively, we performed a plaque-forming assay. The viruses were obtained from supernatants of HTLV-III-infected MT-4 cells or Molt-4/HTLV-III cells which were cultured with various concentrations of AZT for 3 days. The plaque-forming assay has been previously described (10, 15a). Briefly, to bind MT-4 cells onto culture vessels, 35-mm polystyrene tissue culture dishes were coated with poly-L-lysine (molecular weight, 120,000; Sigma Chemical Co., St. Louis, Mo.). A 1.5-ml sample of MT-4 cells (1.50×10^6/ml) was dropped onto each poly-L-lysine-coated dish and incubated for 1 h at room temperature. The dishes were gently washed with phosphate-buffered saline to remove unbound cells, and 100 μl of appropriately diluted virus preparation was slowly added over the cells. The cells were incubated for 1 h at room temperature for virus adsorption. After incubation, 1 ml of agarose overlay medium consisting of RPMI 1640 medium with 10% fetal calf serum, antibiotics, and 0.6% agarose (SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine) was poured onto each dish. The dishes were incubated in a CO_2 incubator at 37°C. After 3 days, 1 ml of agarose overlay medium containing neutral red was added and incubated for another 3 days. To evaluate the effect of AZT on infection of HTLV-III in MT-4 cells, a plaque assay was conducted in the presence of various concentrations of AZT in agarose overlay medium. All experiments were carried out in triplicate.

RT assay. Supernatants of Molt-4/HTLV-III were concentrated 100 times by sucrose gradient ultracentrifugation. The viral pellets were suspended in 100 μl of suspension buffer containing 5 mM Tris hydrochloride (pH 8.1), 0.5 M KCl, 0.1 mM dithiothreitol, and 0.1% Triton X-100. The assay for RT activity was performed at 37°C for 1 h with 10 μl of the disrupted HTLV-III in a final volume of 50 μl containing 50 mM Tris hydrochloride (pH 8.4), 2 mM dithiothreitol, 100 mM KCl, 10 mM $MgCl_2$, 0.01% Triton X-100, 1 μCi of [^{3}H]TTP (57 Ci/mmol; Radiochemical Centre, Amersham, England), and 50 μg of poly(rA)/oligo(dT) per ml (P-L Biochemicals, Inc., Milwaukee, Wis.). The reaction was stopped with 5% trichloroacetic acid. Precipitates were collected on glass fiber filters, and radioactivity was counted in a liquid scintillation counter (9). The assays were carried out in triplicate.

RESULTS

Inhibition of HTLV-III/LAV-induced cytopathic effect by AZT. Figure 1 illustrates the effect of AZT on HTLV-III-induced cytopathic effect in MT-4 cells. As a result of HTLV-III infection, the number of viable MT-4 cells decreased, and under the present experimental conditions (multiplicity of infection of 0.002), the number of viable cells dropped to 1.0×10^5 cells per ml, while the number of uninfected MT-4 cells increased to 1.45×10^6/ml 6 days after infection. At the same time, when 1, 5, and 10 μM of AZT

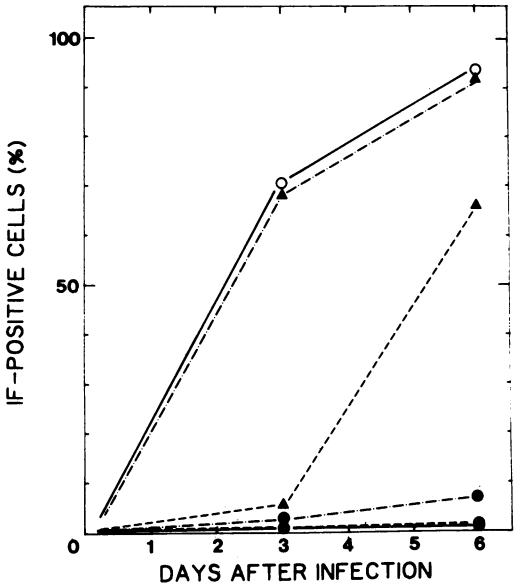

FIG. 2. Inhibitory effect of AZT on the expression of HTLV-III/LAV-specific antigen. More than 500 cells were counted, and the percentage of IF-positive cells was calculated on days 3 and 6 after infection. HTLV-III-infected MT-4 cells were cultured in the presence of 10 μM (—●—), 5 μM (--●--), 1 μM (·—·●·—·), 0.1 μM (--▲--), or 0.01 μM (·—·▲·—·) AZT and in the absence of AZT (—○—) as a control.

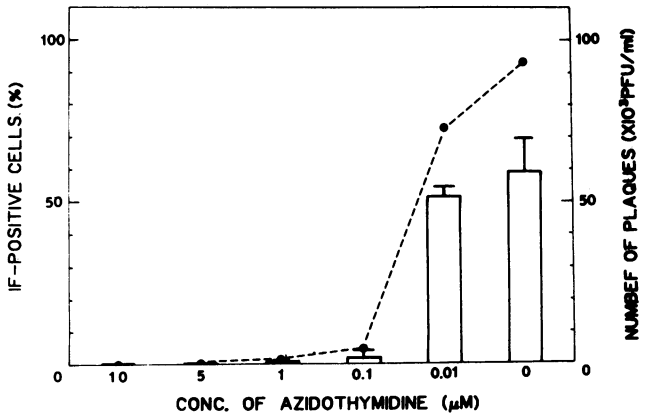

FIG. 3. Suppression of HTLV-III/LAV replication by AZT. HTLV-III-infected MT-4 cells were cultured in the presence of various concentrations of AZT for 3 days. The percentage of cells expressing HTLV-III-specific antigen was determined by the indirect IF method (●). The virus preparation was obtained from the supernatant of cell cultures, and the number of infectious virions was estimated by the plaque-forming assay. Experiments were carried out in triplicate.

was added, the growth inhibition of infected cells was not significant when compared with that of the uninfected MT-4 cells. Moreover, higher concentrations of AZT were not as toxic to the cells. For instance, 100 and 500 μM AZT caused only 15 and 50% growth inhibition, respectively (data not shown). These results suggest that AZT has a strong protective effect against the HTLV-III-induced cytopathic effect at concentrations of 1 to 10 μM.

Effect of AZT on expression of HTLV-III/LAV antigens. When MT-4 cells were infected with HTLV-III, 72% of the cells became positive for virus antigens 3 days after infection, and 95% were positive 6 days after infection, as determined by an indirect IF technique (Fig. 2). A significant inhibition was observed when the HTLV-III-infected MT-4 cells were cultured in the presence of 1 μM AZT, especially in the early stages of infection. Complete inhibition was observed at concentrations of 5 and 10 μM. Ten days after infection, although almost all HTLV-III-infected MT-4 cells were dead, MT-4 cells were still alive and viral antigens were not observed in the cell cultures with 5 and 10 μM AZT.

Suppression of HTLV-III/LAV replication by AZT in plaque-forming assay. To evaluate the effect of AZT on HTLV-III replication quantitatively, the number of infectious virus particles released from 3-day-old cultures of HTLV-III-infected MT-4 cells in the presence of various concentrations of AZT were counted by the plaque-forming assay. As shown in Fig. 3, 59×10^3 plaques per ml were formed with the control virus preparation obtained from HTLV-III-infected MT-4 cells. However, the addition of AZT resulted in a dose-dependent decrease in the number of plaques. Doses of 5 and 10 μM AZT completely inhibited the formation of plaques. Figure 3 also shows that the inhibitory effect of AZT on the plaque-forming activity of HTLV-III exactly coincides with the suppressive effect of AZT on virus antigen expression.

The effects of AZT on the production of viruses in an HTLV-III-producing cell line were also studied. The viruses were prepared from a culture supernatant of 3-day-old Molt-4/HTLV-III cells in both the presence and absence of AZT (Table 1). Results showed that there was no significant difference in the number of plaques in the supernatant from the cultures with or without AZT. This result implies that AZT has no effect on virus replication once HTLV-III has been integrated into cellular DNA.

Next, we performed a plaque assay with agarose overlay medium containing various concentrations of AZT (Table 2). Although 64×10^3 PFU/ml were calculated in control dishes, 52×10^3, 11×10^3, and 0.3×10^3 PFU/ml were calculated in dishes containing 0.01, 0.05, and 0.1 μM AZT, repectively. Furthermore, when more than 0.5 μM AZT was present, no plaques were detected at all in these dishes.

Effect of time of AZT treatment on HTLV-III-infected MT-4 cells. When MT-4 cells were only pretreated for 18 h with AZT and infected with HTLV-III, significant inhibition of virus-induced cell damage and virus antigen expression was observed by 3 days after infection. However, this inhibition was not seen at 6 days after infection. When AZT was added to the cells 18 h before, at 0 h, and 1 h after infection, and cells were cultured in the continuous presence of AZT thereafter, complete suppression of virus antigen expression was observed. When the compound was added 8 and 20 h after infection, striking suppression was still observed. How-

TABLE 1. Effect of AZT on the production of HTLV-III in Molt-4/HTLV-III cells detected by plaque-forming assay

Concn of AZT (μM)	No. of plaques per dish (10^3 PFU/ml)[a]
10	153 ± 6.7
5	159 ± 5.0
1	159 ± 4.2
0.1	163 ± 9.5
0.01	157 ± 4.2
0	168 ± 10.6

[a] Experiments were carried out in triplicate. Number represents the mean ± standard deviation.

TABLE 2. Inhibitory effect of AZT on the infectivity of HTLV-III detected by plaque formation in MT-4 cells

Concn of AZT (μM)	No. of plaques per dish (10^3 PFU/ml)[a]
1	0
0.5	0
0.1	0.3 ± 0.6
0.05	11 ± 4.6
0.01	52 ± 8.5
0	64 ± 2.3

[a] Experiments were carried out in triplicate. Number represents the mean ± standard deviation.

ever, when the cells were treated with AZT 30 and 50 h after infection, the inhibitory effect of AZT was no longer evident (Fig. 4).

Effect of azido-TTP on RT activity of HTLV-III. Figure 5 demonstrates the inhibitory effect of azido-TTP on the RT activity of HTLV-III in vitro. Azido-TTP showed strong inhibition of RT; 50% inhibition was observed at a concentration of 1 pM. In this assay, 22 pM of [^{3}H]TTP was added to each well. AZT in the nonphosphate form did not inhibit RT activity of HTLV-III.

DISCUSSION

Since the initial report of AIDS was made in 1981 (8, 23), the number of cases reported each year has increased dramatically. The most frequent clinical expression of AIDS is the occurrence of severe opportunistic infections, reflecting a profound defect of cellular immunity. Laboratory analysis demonstrates a broad spectrum of immune abnormalities in AIDS patients (21). Among these is a drastic reduction in the numbers of T_4 lymphocytes in peripheral blood and in secondary lymphoid organs as well as an impairment of their functions (4, 7). This may be accounted for by the biological properties of HTLV-III/LAV, which displays a selective tropism for T_4 lymphocytes both in vitro and in vivo (22), leading to a killing of T_4 lymphocytes.

It is urgently necessary to develop antiviral drugs with the ability to destroy virus particles and infected cells or which at least are able to stop the virus from spreading. Recently, a nucleoside analog, AZT, was reported to block the in vitro infectivity and cytopathic effect of HTLV-III while the in vitro immune functions of normal T cells remained essentially intact (15). In this study, we attempted to evaluate the effect of AZT on HTLV-III replication using our bioassay methods which could quantitate the HTLV-III/LAV virus.

The results reported here indicate that the replication of HTLV-III was completely inhibited by 5 and 10 μM AZT in MT-4 cells. By day 10 after infection, HTLV-III-infected MT-4 cells were still alive and viral antigens were not observed in the cell cultures with 5 and 10 μM AZT, while almost all infected MT-4 cells were dead in the absence of AZT. This effective concentration was much lower than that which inhibited the growth of uninfected MT-4 cells. When 5 μM AZT was added to HTLV-III-infected MT-4 cells 1 h after infection, there was a complete inhibitory effect. Furthermore, when AZT was added to the cells even 20 h after infection, an inhibitory effect was still evident. All these data suggest that AZT blocks the spreading of HTLV-III.

The data presented in this report suggest that AZT, which is converted to the triphosphate form in the cellular cytoplasm, directly inhibits the RT activity of HTLV-III. AZT is a competitive inhibitor of RT as a triphosphate, but the unphosphorylated compound does not inhibit RT. It has been reported that radioactively labeled AZT was anabolized by cells to the triphosphate form (P. A. Furman, M. St. Clair, K. Weinhold, J. A. Fyfe, S. Nusinoff-Lehrman, and D. W. Barry, Program Abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 440, 1985). Thus, it is most conceivable that AZT is converted to azido-TTP in cells, which inhibits reverse transcription of viral RNA into DNA, resulting in the inability of the viral genome to integrate into cellular DNA.

It is important to evaluate possible therapeutic compounds which might be effective at concentrations much lower than their cytotoxicity concentrations. Well-known RT inhibitors such as suramin and phosphonoformic acid have also been reported to inhibit HTLV-III, but they are effective only at concentrations of half or concentrations above those causing cellular toxicity in our bioassay system (data not shown). Thus, it is extremely important to test the potential efficacy of AZT against HTLV-III/LAV infection leading to AIDS and AIDS-related complex.

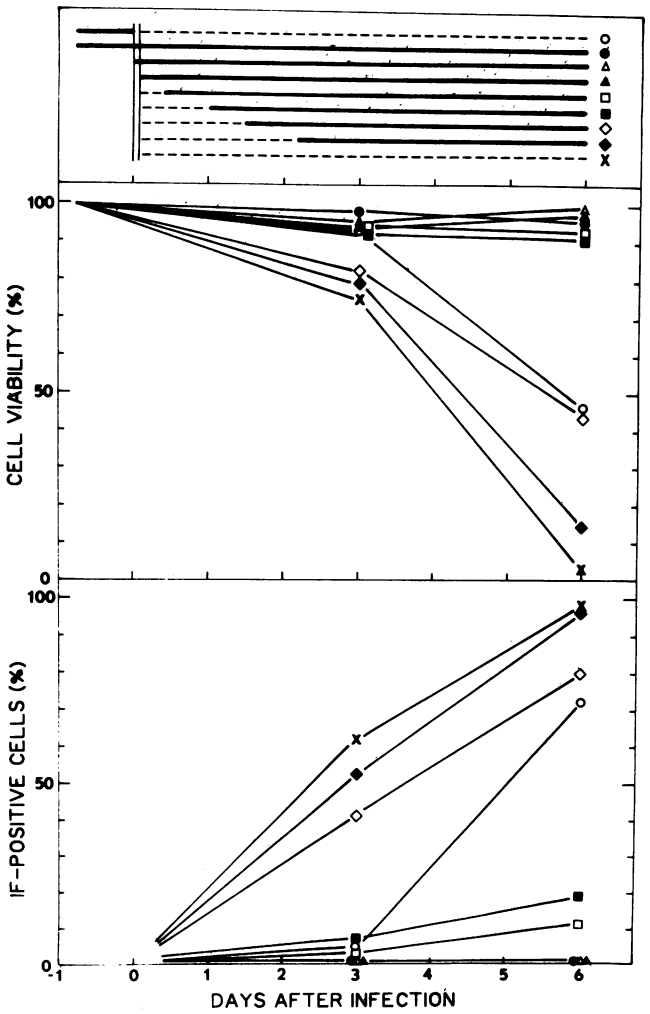

FIG. 4. Effect of time of AZT treatment on HTLV-III-infected MT-4 cells. MT-4 cells were infected with HTLV-III at a multiplicity of infection of 0.002. The cells were cultured in the presence (——) or absence (-----) of 5 μM AZT. On days 3 and 6 after infection, the viability of cells and the percentage of virus-specific antigen-expressing cells were determined by the trypan blue dye exclusion method and IF method.

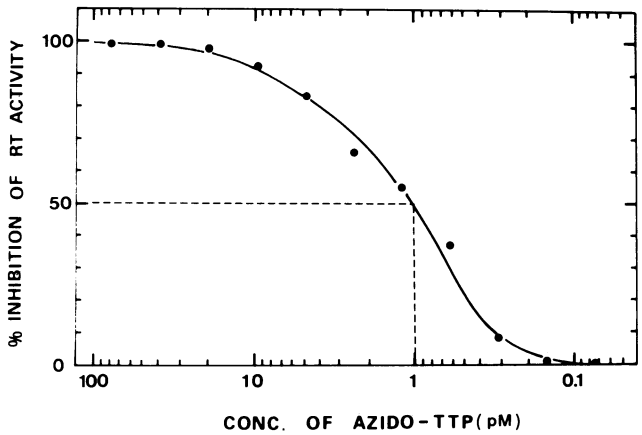

FIG. 5. Effect of azido-TTP on the RT activity of HTLV-III. [^{3}H]TTP (22 pM) was added to each well. The dotted line represents the concentration of azido-TTP that inhibits 50% of the initial RT activity.

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