ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1996, p. 271–273 Vol. 40, No. 1
0066-4804/96/$04.00+0

Mechanism of Selective Inhibition of Human Immunodeficiency Virus by Ingenol Triacetate

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Received 2 June 1995/Returned for modification 15 August 1995/Accepted 14 October 1995

Ingenol 3,5,20-triacetate (ITA), one of the ingenol derivatives, is a selective inhibitor of human immunodeficiency virus (HIV) replication in vitro. ITA inhibited the replication of HIV strains in MT-4 cells at concentrations of 0.051 to 0.65 μM. This concentration was approximately 10^3-fold lower than its cytotoxic threshold. The mechanism of action of ITA is primarily attributed to the inhibition of viral adsorption to the host cells, but it is distinct from the mechanism of inhibition by other adsorption inhibitors.

Kansui has been used as a herbal remedy for edema, ascites, and cancer in China (22). Ingenol derivatives extracted from Kansui, the dried roots of *Euhorbia kansui* Liou (*Euhorbiaceae*), have various biological activities such as activation of protein kinase C (PKC) (10), antitumor activity (22), tumor promotion activity (20), and up-regulation of the macrophage Fc receptor (12). In the study described in this report we examined the anti-human immunodeficiency virus (anti-HIV) activities of ingenol derivatives and found that ingenol 3,5,20-triacetate (ITA) (13) is an inhibitor of HIV replication in vitro.

ITA was prepared by partial synthesis from ingenol (13, 19). The purity of ITA was checked by high-performance liquid chromatography. Zidovudine (AZT) and aurintricarboxylic acid (ATA) were purchased from Sigma Chemical Co. and Aldrich Chemical Co. (Brussels, Belgium), respectively. The HIV type 1 (HIV-1) protease inhibitor A-75925 was synthesized by Yamanouchi Pharmaceutical Co. Ltd. (Tokyo, Japan).

MT-4, MOLT-4, and chronically HIV-1-infected MOLT-4 cells (MOLT-4/HTLV-III_B) were used in the antiviral assays. They were grown and maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin G per ml, and 100 μg of streptomycin per ml. Eight strains of HIV-1 (HTLV-III_B, HIV-1_{HE}, A012B, A012D, A018A, A018C, HTLV-$III_{B\text{-}R}$, and HIV-$1_{HE/NEV}$) and one strain of HIV-2 (LAV-2_{EHO}) were used in the antiviral assays. HIV-1_{HE} is a clinical isolate (15). A012B and A018A are AZT susceptible, while A012D and A018C are AZT-resistant clinical isolates (11). HTLV-$III_{B\text{-}R}$ and HIV-$1_{HE/NEV}$ are nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant mutants established by serial passages in cell culture in the presence of escalating concentrations of MKC-442 (4) and nevirapine (18), respectively. The titers of virus stocks were determined in MT-4 cells, and virus stocks were stored at −80°C until use.

Except for their activities against strains A012B, A012D, A018A, and A018C, the anti-HIV activities of the compounds was based on the inhibition of virus-induced cytopathogenicity in MT-4 cells. The number of viable cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described previously (16). Determination of the activity against the replication of A012B, A012D, A018A, and A018C was based on the inhibition of p24 antigen production in MT-4 cells (1). The cytotoxicities of the compounds were evaluated in parallel with their antiviral activities. Cytotoxicity was based on the viabilities of mock-infected MT-4 cells as determined by the MTT method.

When ITA was examined for its inhibitory effect on HIV-1 (HTLV-III_B) replication in MT-4 cells, it suppressed HIV-1-induced cytopathogenicity with a 50% effective concentration (EC_{50}) of 0.1 μM (Table 1). The concentration of ITA which affected the viabilities of mock-infected MT-4 cells (CC_{50}) was 211 μM. Thus, the selectivity index, based on the ratio of the CC_{50} to the EC_{50}, was 2,110. When the anti-HIV-1 activity of ATA was examined under the same assay conditions and compared with that of ITA, ATA was found to be a weaker inhibitor than ITA (Table 1). Furthermore, ITA equally inhibited the replication of three clinical isolates of HIV-1 (HIV-1_{HE}, A012B, and A018A) and one HIV-2 isolate (LAV-2_{EHO}). The EC_{50}s for these viruses ranged within 0.7 to 7 times the EC_{50} for HTLV-III_B. ITA was equally active against AZT-resistant mutants (A012D and A018C) and the corresponding AZT-susceptible strains (A012B and A018A) (Table 1). As expected, AZT was 60- and 54-fold less inhibitory to A012D and A018C, respectively, than to their corresponding clinical isolates. ITA also proved to be as inhibitory against NNRTI-resistant mutants (HTLV-$III_{B\text{-}R}$ and HIV-$1_{HE/NEV}$) as it was against their parental strains (HTLV-III_B and HIV-1_{HE}) (Table 1).

The drug-susceptible phase in the HIV-1 replicative cycle was determined by the time-of-addition experiment in which the test compounds were added to MT-4 cells at different times after virus infection (14). The amount of p24 antigen in culture supernatants was determined at 48 h after virus infection. In the absence of inhibitors, the p24 antigen levels in culture supernatants increased within a few days after virus infection (data not shown). However, when ITA was present in the culture medium immediately after virus infection, the com-

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TABLE 1. Inhibition of HIV-1 and HIV-2 replication in MT-4 cells by ITA, ATA, and AZT[a]

Virus	ITA		ATA		AZT	
	EC_{50} (μM)[b]	CC_{50} (μM)[c]	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
$HTLV\text{-}III_B$	0.10 ± 0.07	>211	0.69 ± 0.2	126 ± 14	0.0049 ± 0.002	3.2 ± 0.2
$HTLV\text{-}III_{B\text{-}R}$	0.051 ± 0.01		0.73 ± 0.1		0.0014 ± 0.0006	
$HIV\text{-}1_{HE}$	0.099 ± 0.002		0.72 ± 0.1		0.0037 ± 0.003	
$HIV\text{-}1_{HE/NEV}$	0.13 ± 0.007		0.64 ± 0.2		0.0020 ± 0.001	
A012B	0.073 ± 0.01		4.3 ± 0.07		0.0011 ± 0.0002	
A012D	0.017 ± 0.004		0.89 ± 0.3		0.066 ± 0.03	
A018A	0.65 ± 0.2		21.1 ± 0.07		0.0024 ± 0.0009	
A018C	0.096 ± 0.06		4.5 ± 0.2		0.13 ± 0.03	
$LAV\text{-}2_{EHO}$	0.074 ± 0.02		0.012 ± 0.002		0.0018 ± 0.0002	

[a] All data represent means ± standard deviations for at least three separate experiments.
[b] The EC_{50} is based on the inhibition of virus-induced cytopathogenicity or the reduction of p24 antigen in culture supernatants.
[c] The CC_{50} is based on the reduction of viability in mock-infected cells.

pound could sufficiently reduce the p24 antigen level in the culture supernatants (Fig. 1). Like ATA (a virus adsorption inhibitor), ITA lost its inhibitory effect on HIV-1 replication when it was added at 2 h or later (Fig. 1). By contrast, the reverse transcriptase inhibitor AZT and the protease inhibitor A-75925 could inhibit HIV-1 replication even when they were added at 7 and 24 h after virus infection, respectively. These results suggest that the mechanism of action of ITA is similar to that of ATA. In addition, ITA did not inhibit either HIV-1 reverse transcriptase or protease activity in cell-free assay systems at concentrations up to 200 μM (data not shown).

The syncytium formation assay was carried out to check whether ITA suppressed the fusion process between the viral envelope and the host cell membrane. In this assay, MOLT-4 cells were cultured with equal numbers of MOLT-4/$HTLV\text{-}III_B$ cells, and the number of giant cells (syncytia) was recorded microscopically after 24 h of cocultivation. In the presence of ITA, syncytium formation was completely inhibited at a concentration of 2 μM (data not shown). We next examined whether ITA directly inhibited the binding of gp120 to CD4 in a cell-free system by using a gp120 antigen-capture enzyme-linked immunosorbent assay (ELISA) kit (Intracel Co.). The test compounds and gp120 (1 ng per well) were added into a microtiter tray coated with CD4 and incubated at room temperature. After a 60-min incubation, the amount of gp120 that bound to CD4 was determined according to the manufacturer's instruction. ITA did not block the binding of gp120 to CD4 even at the highest concentration examined (10 μM), whereas ATA inhibited the binding of gp120 to CD4 by 87% at 10 μM and 62% at 2 μM. Thus, unlike ATA or dextran sulfate, (2, 3, 17, 21), ITA did not prevent the binding of gp120 to CD4, as determined by a cell-free ELISA. These results indicate that although both ITA and ATA inhibit the virus adsorption process, the mode of inhibition by ITA is apparently different from that by ATA.

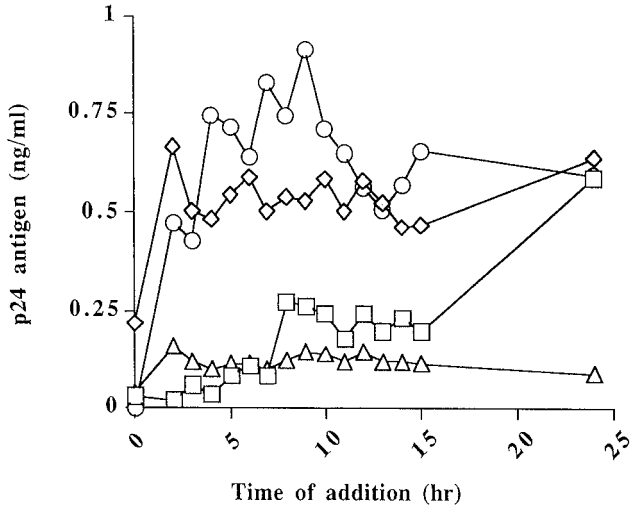

FIG. 1. Time-of-addition experiment. MT-4 cells were infected with HIV-1 ($HTLV\text{-}III_B$), and 5 μM ITA (◇), 118 μM ATA (○), 0.1 μM AZT (□), and 50 μM A-75925 (△) were added at different times after virus infection. The amounts of p24 antigen in culture supernatants were measured at 48 h.

To further elucidate the mechanism of inhibition, ITA was examined for its effect on CD4 expression in MT-4 cells. The effects of the compounds on surface CD4 expression were determined quantitatively by immunofluorescence with anti-OKT-4A monoclonal antibody (Ortho Diagnostics) and FACScan analysis (Becton-Dickinson, Ermbodegem, Belgium) (18). When the number of $CD4^+$ cells was assessed, the numbers of positive cells decreased with increasing concentrations of ITA and incubation times (Fig. 2). More than 90% of total cells became CD4 negative after a 24-h incubation with the compounds at 10 or 50 μM. This down-regulation of CD4 expression was also observed at a lower concentration (0.08 μM). However, ITA did not affect the expression of CD26, which is a related molecule of virus-cell fusion (5), at a concentration of 50 μM (data not shown).

Ingenol and its derivatives are known to have several biological activities. In particular, they have a high degree of potential to activate PKC (10). The activation of PKC leads to the down-regulation of cell surface CD4, which has already been shown with phorbol 12-myristate 13-acetate (PMA) (6, 8). Although ITA is a weaker activator of PKC than PMA, activation of PKC by ITA certainly occurs and leads to the down-regulation of CD4 molecules (data not shown). In fact, the correlation coefficient between EC_{50}s of the ingenol derivatives for HIV-1 replication and the 50% inhibitory concentrations for CD4 expression was 0.82 (data not shown). Taken together, the mechanism of action of ITA may be attributed in part to the inhibition of viral adsorption to host cells through the down-regulation of CD4. An effect on host cells is also consistent with our studies to determine whether ITA induces drug-resistant mutants during long-term treatment of infected cells. At present, HIV-1 mutants resistant (or less susceptible) to ITA have not been isolated (data not shown).

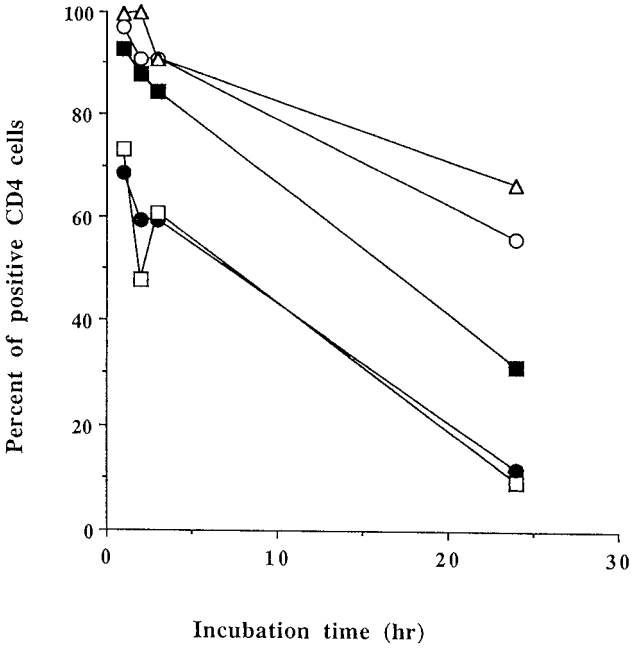

FIG. 2. Down-regulation of CD4 by ITA. MT-4 cells were incubated with ITA at concentrations of 50 μM (□), 10 μM (●), 2 μM (■), 0.4 μM (○), and 0.08 μM (△). The number of $CD4^+$ cells was examined by indirect immunofluorescence and FACScan analyses.

Another important issue is that ITA belongs to the family of phorbol esters. The phorbol ester PMA is known to be a potent transcriptional activator of HIV-1 expression in chronically infected cells through the activation of PKC (6). Therefore, it is possible that ITA also up-regulates viral expression. Indeed, we found that some ingenol derivatives activated the expression of HIV-1 in the OM-10.1 cell line (data not shown), which is a chronically infected clone derived from HL-60 cells and which harbors a single HIV-1 provirus (7). However, it should be noted that there was some dissociation between their anti-HIV-1 activities and PKC activation (data not shown). Ideally, the derivative should have potent anti-HIV-1 activity but little or no effect on viral expression. Prostratin, a non-tumor-promoting phorbol, has recently been shown to selectively inhibit HIV-1 replication in vitro (9). Further studies on the structure-activity relationship are needed to make the claim that the ingenol derivatives, including ITA, would be candidate drugs for the treatment of AIDS.

We thank E. Sato and H. Sato for excellent technical assistance. The strain of HIV-2 ($LAV\text{-}2_{EHO}$) was provided by L. Montanier (Pasteur Institute, Paris, France), while HIV-1 ($HTLV\text{-}III_B$) was originally obtained from R. C. Gallo (National Cancer Institute, Bethesda, Md.). The four clinical HIV-1 isolates (A012B, A012D, A018D, and A018C) were obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. (D. D. Richman)

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