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Biomed & Pharmacother (1994) 48, 55-61

Dossier "AIDS"

Inhibitory effect of the oral immune response modifier, bestatin, on cell-mediated and cell-free HIV infection *in vitro*

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Summary – The antiviral effect of the immunomodulating anti-cancer agent, bestatin, was examined *in vitro* by exposing MT-4 lymphocytes to HIV in the presence of 10-fold dilutions of drug (range 100 µg–100 pg/ml). The reduction in infectivity was measured by p24 ELISA and compared to the effect of established anti-HIV drugs–azidothymidine (AZT) and dextran sulfate. The results indicate that low doses of bestatin (1 µg/ml) can completely inhibit viral infection resulting either from inoculation with free virus or coculture with infected lymphocytes. Unlike AZT or dextran sulfate, bestatin prevents HIV infection without interfering with the rate of cell growth. No appreciable decrease in HIV production was observed when chronically infected virus-producing T cell lines *ie*, H9, MOLT-4, HPB-ALL, 8E5 and MT-2 were treated with bestatin. Bestatin appears to act in the early stages of viral penetration, possibly through inhibition of lymphocyte-associated aminopeptidases.

bestatin / HIV / T lymphocytes

Introduction

Bestatin, a low molecular weight dipeptide (2S, 3R)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leuc ine, is an orally active biological response modifier derived from a microbial source (*Streptomyces olivoreticuli*) and is known as an inhibitor of exopeptidases such as leucine aminopeptidase and aminopeptidase B, found mostly on the membrane of human lymphocytes and monocytes [1, 2]. We recently reported [3, 4] that coumarin-derived compounds, also major inhibitors of aminopeptidases [5] and serine proteases [6], display anti-HIV activity *in vitro* possibly through inhibition of hydrolases on the surface of lymphocytes.

Bestatin was discovered by Umezawa in 1976 [1] and has been successfully used since for the treatment of certain forms of leukemias and solid tumors [7, 8]. Mathé *et al* [9, 10] reported immunorestorative action of bestatin in immunocompromised cancer patients and in individuals infected with human immunodeficiency virus (HIV). However, recent clinical trials conducted in Denmark [11] concluded that no significant difference was observed between immunological parameters of HIV-positive patients treated with bestatin and patients on placebo. Because of the discrepancy between these clinical studies and the absence of any reported data on antiretroviral activity of aminopeptidase inhibitors, we investigated whether bestatin has a direct effect on HIV infection *in vitro*.

Materials and Methods

Infection assay using free virus

To determine whether bestatin may affect HIV infectivity in the virus-target cell system, filtered (pore size, 0.22 µm) culture medium from HIV-producing H9 line (infected with IIIB strain of HIV-1) titrated to 100 ng/ml of p24 *gag* protein or an equivalent of 10^9 viral particles per ml was used as a viral inoculum. Since only one out of a hundred released particles is infectious, 100 ng of p24 would in fact correspond to 10^7 infectious virions per ml. The rationale and methodology for these calculations have been described earlier [12-14]. Target MT-4 lymphocytes (10^5 cells/ml) were exposed to 10-fold dilutions of drug (from 100 µg/ml to 100 pg/ml) immediately followed by the addition of virus, and were cultured in 96-well plates (200 µl of culture medium/well) for 1 hour. Each dose was tested in triplicate

in four separate experiments. Inoculated cells were then washed and subcultured for 4 days at 37°C in 5% CO_2 until signs of infection, *ie*, syncytial cells in untreated control wells were observed under microscope. Productivity of infection was then tested by p24 ELISA. The antiviral effect of bestatin (M_r 345) was compared to the effect of established anti-HIV drugs *ie*, dextran sulfate (M_r 8000; range 100 μg-100 pg per ml) and AZT (M_r 267; range 10^{-3}-10^{-9} M). All drugs were purchased from Sigma, St Louis, MO.

Infection assay in cell-cell system

HIV-1 producing unstimulated HPB-ALL T lymphocytes were incubated at a ratio of 1:10 with uninfected MT-4 lymphocytes (10^4:10^5 cells/ml) in the continuous presence of $\log_{10}$ dilutions of bestatin or two control drugs, AZT and dextran sulfate. Upon appearance of syncytial cells at 2 days post-infection, the coculture medium was tested for viral production by measuring the release of HIV-1 core protein p24 by ELISA. Spontaneous release of virus in control wells containing HIV-infected HPB-ALL alone was subtracted from experimental values.

Effect of drugs on HIV synthesis from persistently infected T cell lines

HIV-1 producing H9, MOLT-4, HPB-ALL, 8E5, and MT-2 T lymphocytes (10^6 cells/ml) were grown in 96-well Multiscreen filtration plates with gravity-permeable bottom (Millipore, Bedford, MA) in the presence of various concentrations of drugs for 3 days. In order to fit into the detection range of p24 ELISA (c. 10 ng–10 pg of *gag* antigen per ml) the samples of culture medium were appropriately diluted and added to a 96-well ELISA plate. All cultures were maintained in RPMI 1640 culture medium with 10% FBS, L-glutamine, penicillin and streptomycin in 5% CO_2 atmosphere.

Determination of viral production by ELISA

The dose-response to drugs was quantitated by measuring the amount of cell-free p24 antigen in culture supernatants of infected cells according to the manufacturer's (HIV core p24 *gag* antigen ELISA kit, Coulter, Hialeah, FL). The amount of released p24 was calculated by comparing to the optical density of supplied p24 standards. The lower limit of reliable sensitivity of this kit was set at 10 pg/ml or p24.

Toxicity assay

MT-4 cells and HIV-infected H9, MOLT-4, HPB-ALL, and MT-2 lymphocytes were grown in the presence or absence of $\log_{10}$ dilutions of bestatin, AZT, or dextran sulfate. The cells were pulsed for 4 h with 0.5 μCi/ml of [^{3}H] thymidine (Amersham, Arlington Heights, IL). The incorporation of the labeled DNA precursor was determined by scintillation spectroscopy and compared to the cpm values of the control cells cultured without drugs.

Results

To determine whether bestatin may affect the infectivity of HIV, we evaluated its effect in infection assays in which target MT-4 cells were exposed either to cell-free HIV (fig 1a) or HIV-infected HPB-ALL T lymphocytes (fig 1b). The inhibitory effect of bestatin was compared to the antiviral action of previously reported compounds with anti-HIV activity-dextran sulfate [15] and AZT [16]. All three drugs demonstrated an inhibitory capacity. Viral infection was completely abrogated at a microgram concentration of bestatin. Although infectious doses used in two types of assays were titrated to achieve the same level of viral production in MT-4 (c. 40 ng/ml of p24 in untreated controls), it appears that AZT and dextran sulfate exhibit lower antiviral activity against cell-mediated infection. Unlike bestatin which remained active at a dose of 1 μg/ml, there was approximately one $\log_{10}$ drop in drug potency when AZT and dextran sulfate were tested in cell-cell systems using HPB-ALL cells as the HIV donor. The end-point antiviral concentrations attained by these drugs are shown in figures 1a and 1b.

To determine whether observed inhibition in viral production was not related to the growth suppression of host cells, we examined the effect of drugs on incorporation of isotope-labeled thymidine. As opposed to AZT, bestatin-caused reduction in p24 production from HIV-exposed MT-4 lymphocytes was not correlated to the inhibition of cell growth. Surprisingly, the treatment with dextran sulfate was associated with an increase in [^{3}H] TdR uptake (fig 2). Similar effects were observed when HIV-infected T cell lines (H9, MOLT-4, HPB-ALL, 8E5, and MT-2) were treated with these drugs and then tested for thymidine uptake (data not shown).

We then tested the effect of bestatin on viral synthesis in established T cell lines which spontaneously produced various levels of p24 (range 40 ng-0.2 ng of p24 per ml). Five cell lines routinely maintained in our laboratory, H9, MOLT-4, HPB-ALL, 8E5, and MT-2, were washed and then grown in triplicate in a 96-well plate in the presence of various concentrations of antiviral compounds for 3 days. Following initial titration, appropriately diluted culture supernatants were added into a 96-well ELISA plate and tested for p24 production. The representative dose-response to bestatin and AZT treatment is illustrated in figures 3a and 3b. Although a limited decrease in viral production was observed for

some of the cell lines, no reduction in p24 release comparable to the effect shown in figure 1 was observed at any time.

Discussion

Bestatin displays anti-HIV as demonstrated by reduction in productive viral infection in MT-4 lymphocytes. Complete inhibition of viral infection resulting from exposure to free HIV or infected cells was achieved by doses of bestatin starting from as low as 1 μg/ml). The highest (100 μg/ml) concentration of bestatin employed in our study did not show any appreciable inhibition of [^{3}H]thymidine uptake by MT-4, suggesting that antiviral activity was specific and not related to the suppression of cell proliferation. We have also carried out a set of experiments using one primary HIV-1 isolate and PHA-stimulated peripheral lymphocytes as target cells. The concentration of bestatin required to inhibit de *novo* HIV infection in this situation was the same as with the laboratory strain of HIV. Since we have not yet tested a larger number of primary isolates, these results were not included in this study.

Earlier *in vitro* screenings demonstrated that proliferation of some but not all leukemic cell lines was inhibited only in the presence of extremely high doses of bestatin (4.5 mg/ml). No negative effect was observed after exposure to 450 μg of bestatin [17]. Usual pharmacological doses of bestatin prescribed for oral administration do not exceed 30–60 mg per day [7, 8]. This dosage is at least 10-fold lower than for AZT–routinely administered at doses equal to 500 mg/day or higher and causes serious adverse effects such as myelosuppression and inability to tolerate the drug. The antiviral effect of AZT was associated in a dose-dependent manner with significant growth-suppressive activity and cell death was observed at a millimolar concentration or an equivalent of 267 μg/ml. Interestingly, antivirally active concentrations of dextran sulfate appeared to have an opposite effect to AZT, *ie*, increased proliferation of target MT-4 cells. Al-

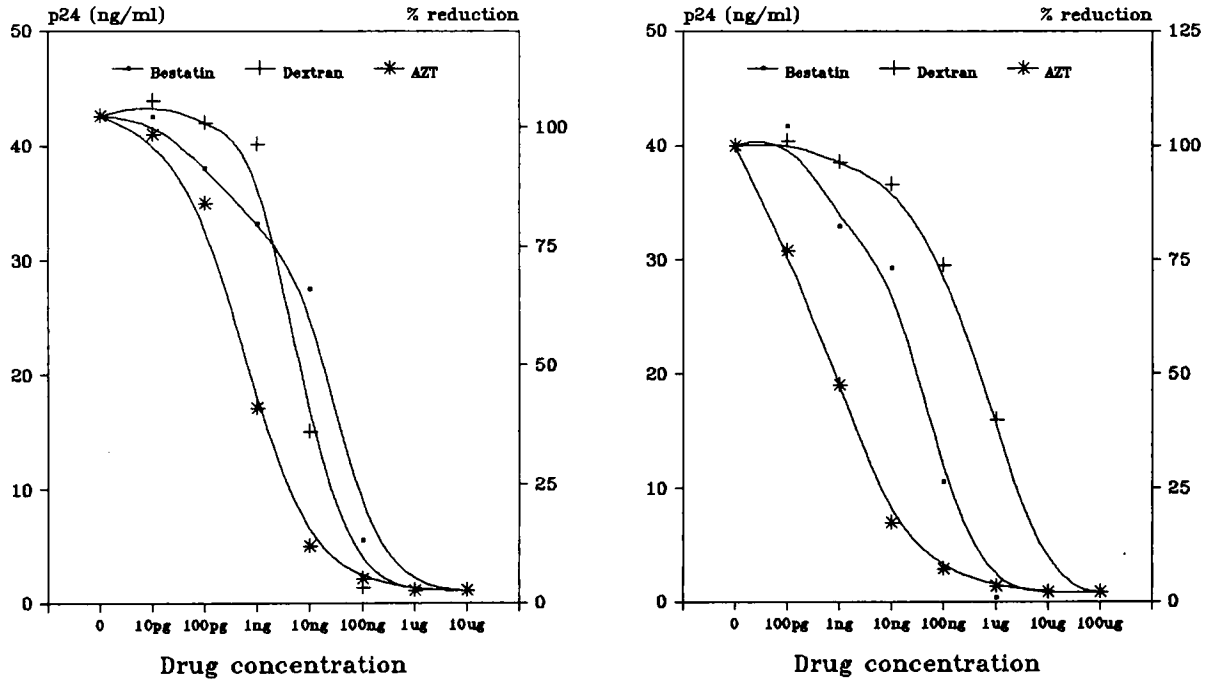

Fig 1. Effect of bestatin, dextran sulfate, and AZT in blocking de *novo* HIV infection in MT-4 lymphocytes (note that concentrations for AZT are in $\log_{10}$ molar increments starting from 10^{-3} M or 267 μg/ml). The experiments using free virus (a) or infected HPB-ALL lymphocytes co-cultured with MT-4 (b) were repeated at least four times and typical results reflecting the decrease of p24 release at 4 (a) and 2 (b) days post-infection are shown. The complete inhibition of infection in either types of antiviral assays is observed at 1 μg/ml for bestatin. In experiments with free HIV, starting concentrations of drugs are 10-fold lower than in chart (b). Decrease in drug potency is observed for AZT and dextran sulfate when tested against cell-to-cell HIV spread.

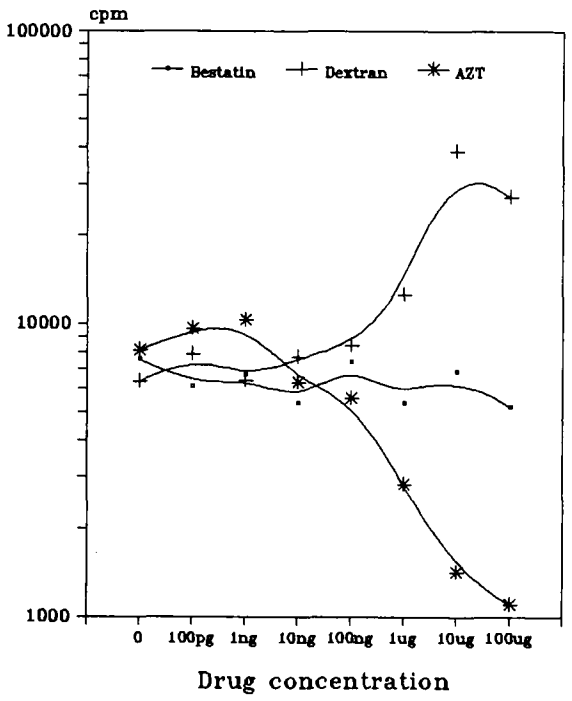

Fig 2. Effect of bestatin, dextran sulfate, and AZT on proliferation of MT-4 lymphocytes as measured by [^{3}H]TdR incorporation assay. Results indicate that reduction in p24 production from HIV-infected lymphocytes is not correlated to cell growth pattern as there is no observable dose-effect for bestatin. Antivirally active doses of AZT and dextran sulate caused inhibition and activation of proliferation respectively.

though dextran sulfate has been known for two decades to be an activator of polyclonal B-cell proliferation [18], it has also been reported as mitogenic to normal T lymphocytes [19]. To our knowledge, induction of proliferation of human T lymphocytic lines by this polysaccharide anticoagulant has never been reported before. It is conceivable that this phenomenon can be related to the results of the recent clinical trial showing the increase of plasma viremia in patients treated with dextran sulfate [20]. It cannot be ruled out that such an unfavorable effect results from the reactivation of resting T lymphocytes harboring HIV and subsequent upregulation of retroviral expression. It appears that *in vitro* treatment with AZT and dextran sulfate causes two opposite extremes in the cell replication process, suppression and activation of proliferation. Presumably, neither of these effects can be of a neutral nature without provoking undesirable side-effects in the human body. In contrast, pharmacological doses of bestatin do not cause any detectable effect on proliferation of MT-4 cell lines. Moreover, bestatin has been reported to have a beneficial effect by enhancing the growth of bone marrow stem cells [21, 22] and elevating the number of T cell precursors which are committed to differentiate into CD4+ cells [23].

In an infection assay with free virus, antiviral drugs were present during the brief period of inoculation and productivity of infection was tested 4 days later by ELISA. This type of assay was primarily aimed at demonstrating the effect of the drug on earlier stages of the viral life cycle, *ie*, adsorption and penetration. On the other hand, in already infected virus-producing cell lines, viral integration is already completed and, thus, the expression of proviral genome and assembly of viral particles are independent from the initial events. The experiment aimed at revealing the effect of bestatin on post-translational expression of HIV in steady producing T cell lines, were intended to identify different mechanism(s) of action of the drug–undetectable under conditions of the infectivity assay described above. In these experiments, however, we were unable to identify an effect which could have been interpreted as independent from the negative effect on cell growth. Thus, it appears that bestatin may act preferentially in the initial steps of viral replication, suggesting that this drug would be useful in blocking de *novo* infection resulting from exposure to free virions or cell-associated HIV. Unfortunately, as clinical experience with AZT has already shown, this effect may be of limited benefit in a situation in which viral infection is already established and progressed beyond an irreversible point.

The polysaccharide anticoagulant, dextran sulfate, is known to inhibit the adherence of HIV to the surface of target cells [15] and its antivirally active concentrations are comparable to bestatin. Although the mechanism of dextran sulfate action remains a subject of controversy, it has been proposed that this compound may act by inhibiting the binding of the viral gp120 envelope glycoprotein to the CD4 molecule which serves as a viral receptor on the surface of permissive cells [24]. Mathé *et al* proposed that the target of bestatin is also the CD4 [7, 9, 10]. This possibility has been supported by Sakurada *et al* [17], who established that the cell-aggregating action of bestatin was counteracted by pre-treatment with the CD4 antibody but not by antibodies against LFA1, CD11a, CD11b, CD11c, or CD18. Although the elucidation of the role of CD4 may be worthy of exploration, it is unlikely that such a mechanism

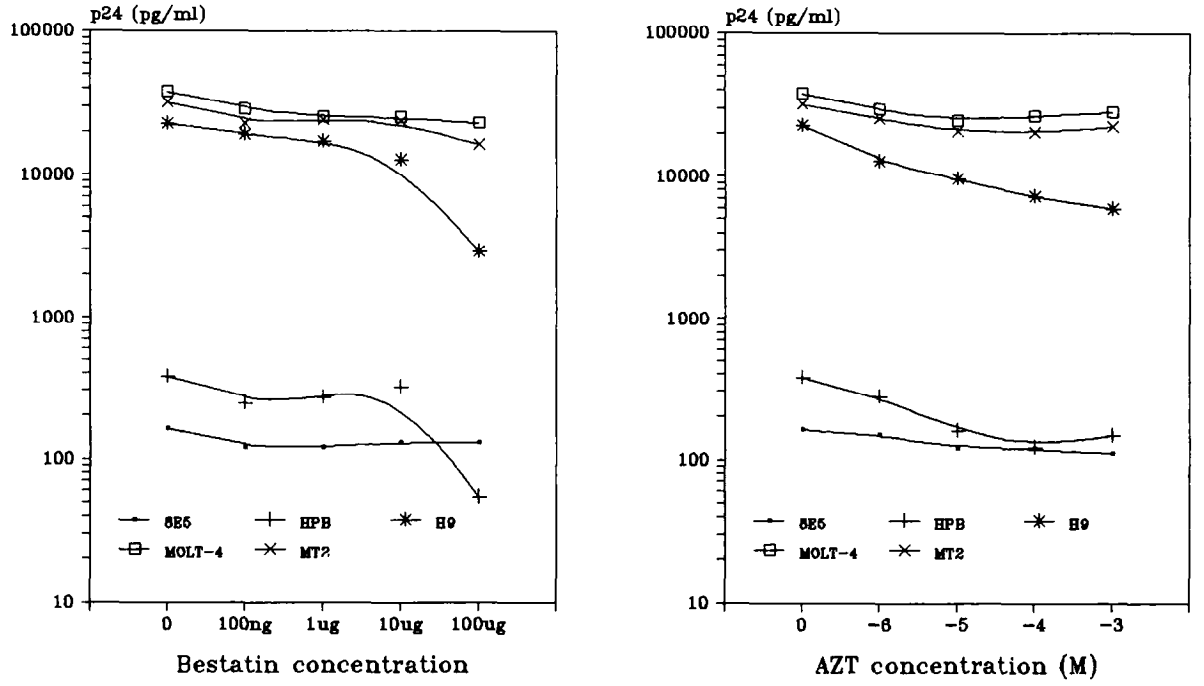

Fig 3. Effect on viral replication in HIV-infected H9, MOLT-4, HPB-ALL, 8E5, and MT-2 lymphocytes as measured by p24 ELISA following 3 days of continuous exposure to bestatin (a) and AZT (b). Although bestatin appears to be slightly more potent than AZT the effect was not as dramatic when compared to the results of infection assays shown in figure 1.

of action can provide an exclusive explanation for the observed biological effect. Both drugs, dextran sulfate and bestatin, were capable of preventing HIV infection when CD4-negative placental trophoblasts were used as target cells [25, Bourinbaiar *et al*, in preparation).

Current opinion on the antiviral effect of hydrolase inhibitors is based on experimental evidence indicating that binding capacity and infectivity of viruses depends on the cleavage of envelope glycoproteins upon entry into the target cell [26]. This cleavage is believed to assure functional activation of retroviral fusion proteins and presumably occurs either before entry of the virus into the cell or in the endosomal compartment [27]. A wide range of membrane-bound host enzymes, including serine [6, 26-31], aspartic [32], and cysteine proteases [33] were implicated in this process. Recent discovery of the involvement of furin, a subtilisin-type human endoprotease, in the cleavage of HIV-1 envelope gp160 glycoprotein and inhibition of HIV infection by peptidyl-chloromethylketones may indicate the target to intervention [4, 34]. However, as opposed to serine proteases which belong to the endopeptidase class of hydrolases, aminopeptidases act primarily as exopeptidases, catalyzing the hydrolysis of N-terminal amino acid residues from the oligopeptides, and have never been reported to be involved in the HIV infection process. Thus, the mechanism of anti-HIV action of bestatin may be different from that reported for serine proteases. Alternatively, it is possible that inhibition of HIV infection by bestatin conforms to a general bioisosteric mechanism. Both types of lymphocyte ectoenzymes, trypsin-like proteases and aminopeptidases, can be inhibited by ketone-containing peptides [36] as well as by coumarin-based suicide substrates [5]-compounds which have been shown by us to display anti-HIV activity [3, 4]. It is likely that bestatin acts by regulating peptidases on the surface and/or cytosol of host lymphocytes [2, 7, 8], but at present only a few of them have been characterized [2, 35-40], *eg*, aminopeptidase N. We are now working in this direction in order to identify which one could be the most crucial for viral infectivity. It has recently been reported that aminopeptidase

N on the surface of epithelial cells serve as the receptor for coronaviruses–responsible for respiratory infections [41-42]. Prevention of HIV infection by bestatin, a specific inhibitor of aminopeptidases [43-45], supports the hypothesis regarding the existence of alternative HIV receptor(s) [28, 30] and modes of viral spread [46], rationalizing the paradox of the permissiveness of CD4-negative cells, *eg*, epithelial cells to HIV [47]. Bestatin is known as a potent orally active immunomodulator and has been successfully used for adjuvant immunotherapy of malignant diseases [7, 8] as well as an enhancer of host resistance to opportunistic infections [48]. Bestatin has been reported to improve the survival rate in immunosuppressed mice infected with *Candida albicans* – a frequently found fungal infection in patients with HIV [49]. In view of the demonstrated effect against HIV *in vitro*, bestatin should be considered for re-examination in extended clinical trials and its therapeutic value must be verified by detailed analysis of immunological as well as virological parameters.

Acknowledgments

We thank CF Coleman and EC Fruhstorfer for editorial assistance. This work was supported in part by grants from the NIH T32 AI07382, RO1 AI31343, RO1 DA04791, RO1 HD26613, and a grant from Schubert Foundation.

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