In vitro inhibition of HIV-1 by Met-SDF-1β alone or in combination with antiretroviral drugs

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Compounds that can block the CXCR4 chemokine receptor are a promising new class of antiretroviral agents. In these experiments we studied the effect of a modified form of the native stromal cell-derived factor-1 (SDF-1), Met-SDF-1β. The *in vitro* susceptibility of two different CXCR4-tropic HIV-1 strains was determined. Antiviral effect was assessed by the reduction of p24 antigen production in PHA-stimulated peripheral blood mononuclear cells with exposure to the modified SDF-1 molecule. The 50% inhibitory concentrations (IC_{50}) were derived from six separate experiments. The IC_{50} against the two HIV-1 isolates was in 1.0–2.8 μg/ml range for Met-SDF-1β. Met-SDF-1β showed synergy to additivity with either zidovudine or nelfinavir at IC_{75}, IC_{90} and IC_{95}. Additivity was seen when Met-SDF-1β was combined with efavirenz. No cellular toxicity was observed at the highest concentrations when these agents were used either singly or in combination. This compound is a promising new candidate in a receptor-based approach to HIV-1 infection in conjunction with currently available combination antiretroviral drug therapies.

Introduction

CD4 T lymphocytes and macrophages are the major targets of infection by HIV-1 [1–3]. The entry of virus into these cells is mediated by the interaction of the viral envelope protein gp120 with CD4 and chemokine 'co-receptors' [4,5]. The receptor for stromal cell-derived factor-1 (SDF) [6,7], CXC chemokine receptor 4 (CXCR4), has been identified as the co-receptor for T-cell-tropic strains of HIV-1 [4,8], and the CC chemokine receptor 5 (CCR5) [9,10] is associated with the binding and the entry of macrophage-tropic isolates of HIV-1 [11–13]. The existence of CD4-independent strains of HIV-1 that can enter by binding CXCR4 [14] or CCR5 [15–17] alone suggest that these receptors are actually the primary receptor for viral entry, rather than 'co-receptors'. There is also evidence of a tendency towards CXCR4 usage later in disease [18].

Early therapeutic attempts at blocking HIV-1 cellular entry focused on inhibition of HIV-1 attachment to cellular CD4 via competition by soluble CD4 [19]. This approach resulted in effective blocking of laboratory-adapted HIV-1 strains *in vitro*, but was less effective on primary HIV-1 isolates [20] and when tested in clinical trials [21]. The identification of the chemokine receptors as essential factors for HIV-1 entry has led to new therapeutic targets for viral inhibition. The chemokines themselves, or their variants, represent a potent and novel class of inhibitors [22–24]. HIV-1 strains that use CCR5 are blocked by RANTES, MIP-1α, MIP-1β and MCP-2; viral strains that use CXCR4 are blocked by the ligand SDF-1. These factors may work through two independent mechanisms: (i) competitive blockage of the interaction of gp120 with the chemokine receptors; and (ii) downregulation of chemokine receptor expression on target cells, thus limiting the possibility of viral entry.

Stromal cell-derived factor 1α and 1β (SDF-1α and SDF-1β: amino acids 1–67) differ by four amino acids at the C-terminus: both bind and signal through CXCR4 and prevent entry of T-tropic virus by blocking its interaction with gp120. In this study we have examined the activity of a modified form of the native SDF-1β in which an N-terminus methionine

residue was added (Met-SDF-1β) [25]. Met-SDF-1β was tested alone and in combination with other antiretroviral drugs.

Materials and Methods

Viruses

The two viruses used for these experiments were HIV-1 IIIB (a syncytium-inducing prototypic strain from RC Gallo) and 14a-Pre (a clinical isolate) [26]. 14a-Pre was derived from an HIV-1-seropositive individual before antiretroviral therapy and was wild-type at reverse transcriptase codons 41, 67, 70, 215 and 219. Virus stocks were prepared according to the method described by Johnson *et al.* [26] in peripheral blood mononuclear cells (PBMCs) obtained according to standard isolation techniques [AIDS Clinical Trials Group (ACTG) Virology Manual]. Cell-free supernatant fluids were harvested twice a week for HIV-1 p24 antigen production assay (NEN Research Products, Boston, Mass., USA), and cell-free supernatants containing breakthrough viruses were tested for syncytium-inducing (SI) capacity using the ACTG protocol for detection of SI isolates [27]. Viral titration was carried out in PBMCs and the viral titre, measured as $TCID_{50}$/ml, was calculated by the method of Reed and Muench [28].

Three CCR5-tropic viruses were used as a control and included Ba-L (a prototypic strain from RC Gallo), RM and MB; the latter two were described previously [29] and were derived from patients with primary HIV-1 syndromes (PHI). Cellular tropism of those viruses was tested on U87MG-CD4 lymphocytes (a gift from Dan Littman, New York University School of Medicine, N.Y., USA).

Compounds

Met-SDF-1β (lot no. 4488–208) was provided at a concentration of 0.32 mg/ml and had the sequence MKPV at the amino terminus [25] and was dissolved in Tris buffer (provided by Genetics Institute, Cambridge, Mass., USA).

Zidovudine was obtained from the Wellcome Research Laboratories (Beckenham, UK). It was dissolved in sterile PBS and stored at a concentration of 4 mM. Nelfinavir was obtained from Agouron Pharmaceuticals (La Jolla, Calif., USA), dissolved in DMSO, and stored at a concentration of 10 mM. Efavirenz was obtained from DuPont Pharmaceuticals (Wilmington, Del., USA), dissolved in PBS, and stored at a concentration of 10 mM.

Drug susceptibility test

The *in vitro* susceptibility of two different CXCR4 HIV-1 strains was determined in PBMCs. Fixed amounts of infectious virus (1000 $TCID_{50}$) with a multiplicity of infection of 0.01 $TCID_{50}$/cell were used to infect PHA-P-stimulated PBMCs that were either drug-free (control wells) or pretreated with four Met-SDF-1β concentrations each in duplicate wells. Met-SDF-1β was used at concentrations ranging from 0.35 to 2.80 μg/ml in experiments with 14a-Pre and from 0.875 to 7 μg/ml in experiments with IIIB. Met-SDF-1β was added to the culture, either simultaneously with virus, or 1 h prior to virus addition. Antiviral effect was assessed by reduction of p24 antigen production in PHA-stimulated PBMCs after harvesting of the cell-free supernatant fluids on days 4, 7 and 11. The IC_{50} values were derived from three separate experiments, and were expressed as mean ± standard error of the mean (SEM). The effects of Met-SDF-1β on cell viability were assessed on uninfected cultures maintained in parallel using the trypan blue dye exclusion method.

In a second set of experiments, we studied combinations of Met-SDF-1β with several other antiretroviral compounds, including zidovudine, nelfinavir and efavirenz. Diluted fixed-ratio combinations of the drugs were added to each well in duplicate. Combination drug assays were performed with the following concentrations of each drug: $0.5\times IC_{50}$, IC_{50}, $2\times IC_{50}$ and $4\times IC_{50}$. Each combination experiment was conducted three times.

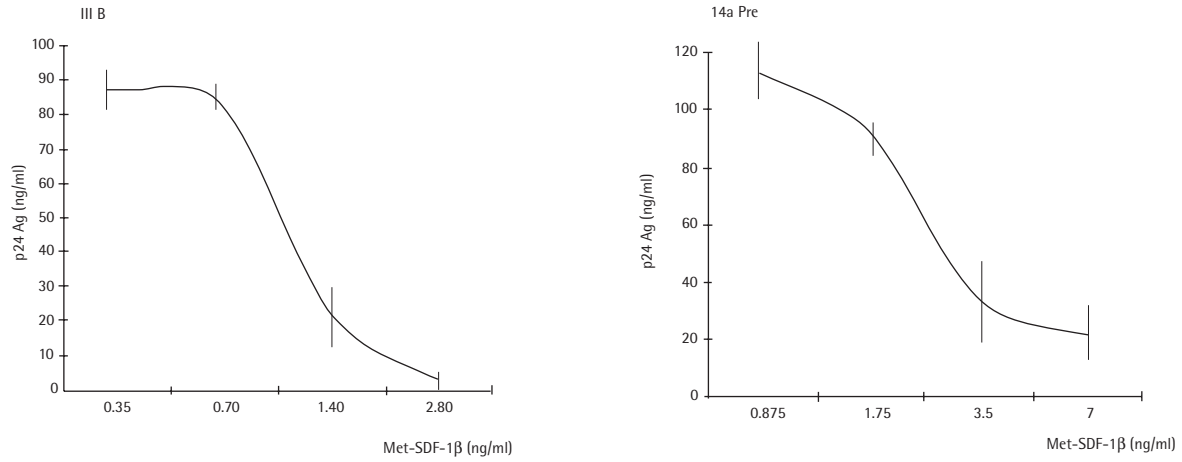
Mathematical analysis of single-agent inhibitory concentration

At different time-points the IC_{50} values for each viral isolate was determined by the dose–effect analysis [30,31] as described previously [26] using the Systat computer software program for Macintosh, version 5.1 (Evanston, Ill., USA).

Evaluation of the interaction between Met-SDF-1β and the different compounds

The multiple dose–effect equation of Chou and Talalay, based on the median-dose–effect principle and the isobologram technique, was used to analyse combined drug effects. The method involves plotting dose–response curves for each agent and for fixed-ratio combinations of the agents as previously described [32]. Drug interactions were determined by the median-dose–effect analysis with the combination index (CI) calculated at several efficacy doses (IC_{50-95}) [32]. Mean CI values were determined as well as SEM. In this study, we defined a combination as synergistic if the CI was <1, greater then the expected additive effect when two agents are combined, additive if the value was between 1 and 1.2, and antagonistic if the value is >1.2, less than the expected additive effect when two agents are combined.

Figure 1. Dose–response curves of Met-SDF-1β in HIV-1 infections



Results

Both virus isolates demonstrated susceptibility to Met-SDF-1β. Met-SDF-1β inhibited CXCR4-tropic HIV-1 isolates at concentrations lower than 3 μg/ml. Met-SDF-1β IC_{50} mean ± SEM was 1.040±0.180 μg/ml with IIIB and 2.850±1.030 μg/ml with 14a-Pre infections. Dose–response curves are shown in Figure 1. No differences were noted between pre-incubation and simultaneous administration of Met-SDF-1β, and a dose-related response was observed against both viruses. No cellular toxicity appeared at the highest concentrations used for the entire duration of the experiments.

Controls for the specificity of these compounds included CCR5-tropic viruses (Ba-L and the PHI isolates RM and MB). No activity was seen using a broad range of drug concentrations against these viruses (data not shown).

In the second part of the experiments Met-SDF-1β was evaluated in combination with other antiretrovirals. The CIs (mean ± SEM) for both CXCR4 viruses are shown in Table 1, assuming mutually exclusive effects. Met-SDF-1β showed synergy to additivity with either zidovudine or nelfinavir at IC_{75}, IC_{90} and IC_{95} against both viruses.

Against both isolates, Met-SDF-1β demonstrated an additive effect in combination with zidovudine, nelfinavir and efavirenz at IC_{50}, with CI values ranging from 1.021 to 1.202 (Table 1). Additivity (CIs 1–1.2) was also seen when Met-SDF-1β was combined with efavirenz at other concentrations (IC_{75}, IC_{90} and IC_{95}). No cellular toxicity was observed when these agents were used in combination, even at the highest concentrations used.

Zidovudine, nelfinavir and efavirenz were maintained as single-target controls in all experiments. IC_{50} values were as follows: zidovudine 0.0053±0.0028 μM, nelfinavir 0.0491±0.0215 μM and efavirenz 0.0027±0.0017 μM.

Discussion

The α-chemokine SDF and its derivatives, such as Met-SDF-1β, interfere with the fusion and entry of CXCR4-tropic HIV-1 strains into target cells by binding to the CXCR4 receptor [6,7]. Down-modulation of chemokine receptors is likely to be required for

Table 1. Combination index values

14a-Pre/PBMC	Combination Index values at			
	IC_{50}	IC_{75}	IC_{90}	IC_{95}
MetSDF-1β + zidovudine	1.021 ± 0.316	0.836 ± 0.174	0.690 ± 0.134	0.632 ± 0.114
MetSDF-1β + nelfinavir	1.202 ± 0.116	1.037 ± 0.074	0.921 ± 0.048	0.862 ± 0.039
MetSDF-1β + efavirenz	1.150 ± 0.034	1.257 ± 0.060	1.190 ± 0.070	1.153 ± 0.072

IIIB/PBMC	Combination Index values			
	IC_{50}	IC_{75}	IC_{90}	IC_{95}
MetSDF-1β + zidovudine	1.104 ± 0.186	0.560 ± 0.449	0.590 ± 0.273	0.755 ± 0.039
MetSDF-1β + nelfinavir	1.097 ± 0.891	0.942 ± 0.016	0.671 ± 0.343	0.610 ± 0.444
MetSDF-1β + efavirenz	1.077 ± 0.171	1.011 ± 0.061	1.055 ± 0.019	1.080 ± 0.068

efficient inhibition of HIV-1 [25,34], and Met-SDF-1β shown to cause prolonged down-modulation of its receptor CXCR4 [25]. The modified form of SDF-1, for example, Met-SDF-1β, more efficiently inhibited CXCR4-tropic HIV-1 strains than the wild-type SDF-1β (human SDF1β inhibited viral replication 1–3 $\log_{10}$ less than Met-SDF-1β). We have verified the tropism of the two isolates examined in this study on U87MG-CXCR4-transfected cells. The replication profile confirmed the lymphotropic nature of these isolates, in contrast to Ba-L, RM and MB, which replicated in CCR5-transfected cells.

The agent evaluated in this study, Met-SDF-1β, was tested as a single agent and was also examined in combination with other antiretroviral agents. Met-SDF-1β inhibits CXCR4 HIV-1 infections, either as a single agent or as part of a combination regimen. Synergistic effects were observed in dual treatments with zidovudine or nelfinavir at drug concentrations greater than IC_{75}. In our study, we found that the CIs between the two compounds of the combination decreased at higher inhibitory concentrations, with the exception of efavirenz, which demonstrated a stable additive effect at various inhibitory concentrations when combined with Met-SDF-1β. Of interest, preliminary studies suggest that Met-SDF-Fc, a new derivative of SDF-1α (Genetics Institute), may be active in inhibiting CXCR4 HIV-1 isolates at even lower concentrations (IC_{50} <0.5 μg/ml).

Other CXCR4-acting agents have been considered as antiviral agents. In contrast to Met-SDF-1β, which induces enhanced calcium flux through CXCR4 [25], other peptide [34,35] and small molecule [36,37] antagonists of CXCR4 have been found to inhibit HIV-1 replication without signalling through CXCR4. Overall, SDF-1 derivatives have shown a good therapeutic index as single agents and in combination regimens *in vitro*. This is particularly important when considering that disease progression is usually associated with a shift in chemokine receptor usage, from CCR5 to CXCR4 [18].

Current therapies approved for HIV-1 infection inhibit the two viral enzymes reverse transcriptase and protease. Although currently available combination regimens have shown a potent antiretroviral activity, many HIV-1-infected individuals develop virological failure over time because of incomplete viral suppression and the emergence of drug resistance. Thus, the development of new classes of compounds with a different mechanism of action is an important research objective in order to minimize the development of resistance.

Newer orally bioavailable inhibitors directed to X4 viruses are needed focusing the attention for the development of small molecules, such as AMD3100 [37–39]. Of interest, the HIV-1 (gp41) attachment/entry to the cell membrane as new site of attack has been recently reported with agents such as T-20 [40] and T1249 [41], and more recently by Kim *et al.* [42] and Harrison *et al.* [43]. Our study points out the likelihood that Met-SDF-1β will need to be used in combination with either: (a) existing antiretrovirals; or (b) other cell-membrane active agents.

To our knowledge this is the first experimental work dealing with *in vitro* combinations of entry/fusion blockers and enzymatic HIV-1 inhibitors. Our results suggest that Met-SDF-1β may be a candidate for development of a receptor-based approach to HIV-1 infection, most likely in combination with other potent antiretroviral drugs. However, it is only through carefully designed and conducted therapeutic trials that the clinical validity of these *in vitro* studies can be determined.

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