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Bicyclams, a class of potent anti-HIV agents, are targeted at the HIV coreceptor Fusin/CXCR-4

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Abstract

Bicyclams are a novel class of antiviral compounds which are highly potent and selective inhibitors of the replication of HIV-1 and HIV-2. The prototype compound, AMD3100, has an IC_{50} of 1–10 ng/ml, which is a least 100 000 fold lower than the cytotoxic concentration. AMD3100 does not inhibit virus binding to the CD4 receptor and based on time-of-addition experiments, has been assumed to interact with the HIV fusion-uncoating process. Resistance of HIV-1 strains to AMD3100 is associated with the accumulation of several mutations in the viral envelope glycoprotein gp120. Here, we demonstrate that AMD3100 interacts with fusin (CXCR-4), the coreceptor used by T-tropic viruses to infect the target cells. The replication of NL4-3 wild type virus and NL4-3 dextran sulfate-resistant virus was inhibited by the CXC-chemokine, stromal cell-derived factor 1 (SDF-1), the natural ligand for CXCR-4. In contrast, the replication of the HIV-1 NL4-3 AMD3100-resistant virus was no longer inhibited by SDF-1. The bicyclams are the first low-molecular-weight anti-HIV agents shown to interact with the coreceptor for T-tropic viruses. © 1997 Elsevier Science B.V.

Keywords: Fusin/CXCR-4; Bicyclams; HIV; AMD3100; Stromal cell-derived factor-1

1. Introduction

The bicyclams are a novel class of highly potent antiviral compounds which selectively inhibit the replication of HIV-1 and HIV-2 (De Clercq et al., 1992, 1994). Time-of-addition experiments have indicated that the bicyclams block an early step in the viral replication cycle (De Clercq et al., 1992, 1994). Binding studies confined the inhibitory effect on an event following binding, but preceding reverse transcription. AMD3100, previously called JM3100 (De Clercq et al., 1994) or SID791 (De Vreese et al., 1996a,b), blocks syncytium formation at a concentration 10–100-fold higher than the concentration required to inhibit virus

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infection (De Clercq et al., 1992). The envelope gp120 glycoprotein has been considered as the major target molecule for this class of compounds because a number of mutations accumulate in the gp120, especially in the V3–V4 region of viruses that were rendered resistant to the bicyclams (De Vreese et al., 1996a,b). The V3 loop is important in the viral fusion process and because recently the gp120 of M-tropic viruses has been demonstrated to interact with CCR-5 (Trkola et al., 1996; Wu et al., 1996), experiments were performed to verify whether AMD3100 did not interact with CCR-5 or with fusin (Berson et al., 1996; Feng et al., 1996), recently named CXCR-4 (Mackay, 1996), the coreceptor for T-tropic HIV-1 and HIV-2 strains.

2. Materials and methods

2.1. Isolation and infection of lymphocytes

Buffy coat preparations from healthy donors were obtained from the Blood Bank in Leuven and kindly provided by Dr O. Souw. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Lymphoprep™ (d = 1.077 g/ml) (Nycomed, Oslo, Norway). The cells were stimulated with phytohaemagglutinin (PHA) at 1 μg/ml (Wellcome) for 3 days at 37°C. The activated cells (PHA blasts) were washed three times with phosphate buffered saline (PBS), pelleted and incubated with HIV (1 ng P24 per 1×10^6 cells) for 1 h at room temperature and non-adsorbed virus was removed by washing three times with PBS, as described by Cocchi et al. (1995). HIV-infected or mock-infected PHA blasts (5×10^5 cells per test) were then cultured in 500 μl complete medium (Life Technologies, Paisley, Scotland) in 24-well plates (Corning) in the presence of 25 IU/ml of IL-2 and varying concentrations of AMD3100. At day 3 and day 6 after infection, 200 μl of medium was removed and replaced with fresh culture medium with IL-2, but without the addition of new compound. Supernatant was collected at day 6 and at day 10 and HIV core antigen in the culture supernatant was analyzed by a sandwich enzyme-linked immunosorbent assay (ELISA). For HIV-1, the p24 ELISA kit from duPont (Wilmington, MA) and for SIV the SIV core Ag EIA kit from Coulter (Miami, FL) was used.

2.2. Viruses

The HIV-1 T-tropic viruses IIIB strain (Popovic et al., 1984) and RF strain (Popovic et al., 1984) and the HIV-1 M-tropic strains BaL (Gartner et al., 1986), SF-162 (Cheng-Mayer et al., 1991), ADA (Gendelman et al., 1988) and JR-FL (O'Brien et al., 1990) were all obtained through the MRC AIDS reagent project, Herts, UK. The HIV-1 T-tropic molecular clone NL4-3 (Adachi et al., 1986) was obtained from the National Institute of Allergy and Infectious Disease AIDS reagent program. The AMD3100-resistant NL4-3 virus and the DS-resistant NL4-3 virus were generated as described previously (De Vreese et al., 1996a,b; Esté et al., 1997b). The SIV MAC251 strain was originally isolated by Daniel et al. (1987) and obtained from Dr C. Bruck (Smith Kline-RIT, Rixensart, Belgium).

2.3. Compounds, monoclonal antibodies (mAbs) and flow cytometric analyses

The chemical compounds used were: dextran sulfate, a sulfated polysaccharide (Baba et al., 1990), an oligonucleotide AR177, also called T30177 or Zintevir (Ojwang et al., 1995) and the bicyclam derivatives, JM2763 and AMD3100, also previously called JM3100 (De Clercq et al., 1994) or SID791 (De Vreese et al., 1996a). The CXC-chemokine, stromal cell-derived factor 1, (SDF-1) was obtained from R and D Systems Europe, Oxon, UK. Compounds or chemokine were added to SUP-T1 cells at the indicated concentrations for 15 min on ice or at room temperature. Then the 12G5 mAb (Endres et al., 1996) (2 μg/ml) (kindly provided by Dr James A. Hoxie, University of Pennsylvania, PA) was added for 30 min at room temperature. The cells were washed twice in PBS and then incubated with fluorescein isothiocyanate-conjugated goat-anti-mouse antibody (GaM-IgG-FITC) (Caltag Laboratories) for 30 min at room temperature, washed twice in PBS

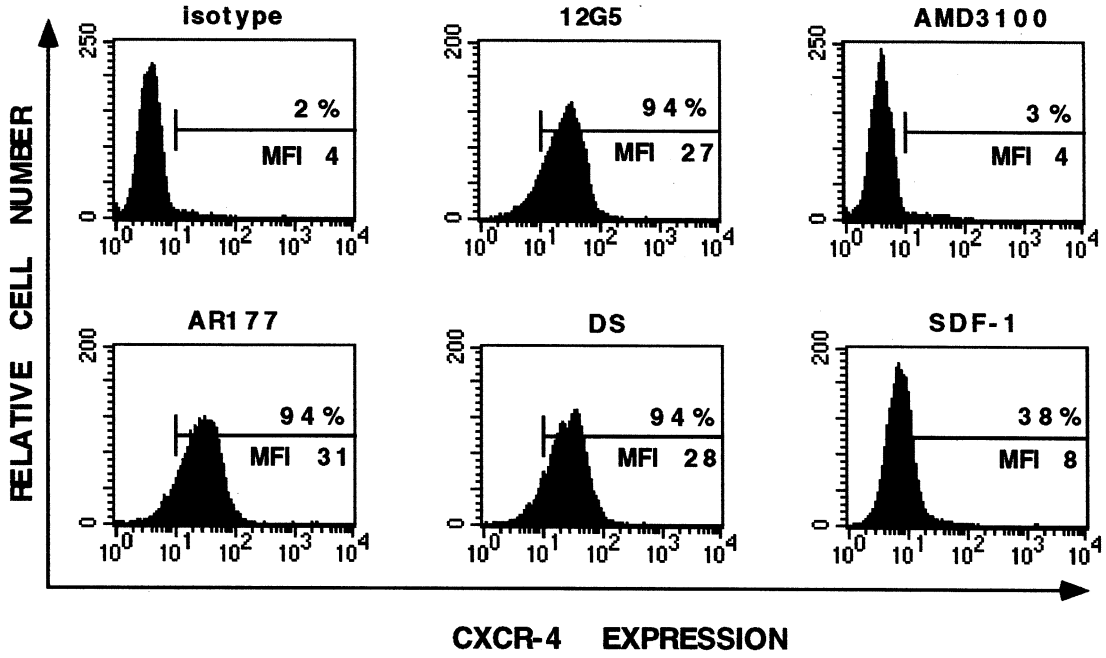

Fig. 1. Effects of AMD3100, dextran sulfate (DS) (M_w; 5000, Sigma) and AR177 (Zintevir) (all tested at 25 μg/ml) and SDF-1 (2 μg/ml) on binding of 12G5 mAb to CXCR-4 in SUP-T1 cells. The percentage of positive cells and the mean fluorescence intensity (MFI) values are indicated in each histogram. The region for positivity was defined using a control IgG_{2a} mAb (Becton Dickinson).

and analyzed by flow cytometry. The anti-CD4 mAb, Leu-3a and isotype-matched control mAbs were purchased from Becton Dickinson. Cells were analyzed by a FACScan™ (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer. Data were acquired and analyzed with CellQuest™ software (Becton Dickinson) on an Apple Macintosh computer.

3. Results

The monoclonal antibody (mAb), 12G5, reacts specifically with the human fusin protein and recognizes the protein on many T-cell lines such as the SUP-T1 cells (Endres et al., 1996). As shown in Fig. 1, AMD3100 at 25 μg/ml completely inhibited the binding of the 12G5 mAb to CXCR-4 on SUP-T1 cells. This is in contrast with two other potent HIV inhibitors, the sulfated polysaccharide dextran sulfate (DS) (Baba et al., 1990) and the oligonucleotide AR177, also called T30177 or Zintevir (Ojwang et al., 1995), which were both ineffective at 25 μg/ml in blocking the binding of 12G5 mAb to CXCR-4. The CXC chemokine, stromal cell-derived factor 1 (SDF-1) (Bleul et al., 1996; Oberlin et al., 1996), the natural ligand for CXCR-4 competed, as expected, with the binding of the mAb 12G5 to its receptor (Fig. 1).

In the following experiments, the compound AMD3100 was brought onto the cells in serial dilutions and washed away after a 15 min incubation period with the cells at room temperature before the 12G5 mAb was added (Fig. 2). As can be seen in Fig. 2, the compound strongly interacted with the CXCR-4 receptor and even when washed away, inhibited the binding of the mAb 12G5 as efficiently as when the compound was present during the whole incubation period with the mAb (compare with AMD3100 at 25 μg/ml in Fig. 1, and data not shown). This indicates that AMD3100 does not interfere with the mAb itself but binds directly to CXCR-4. From Fig. 2 it is also clear that even at a concentration of 0.2 μg/ml AMD3100 still interfered with the binding

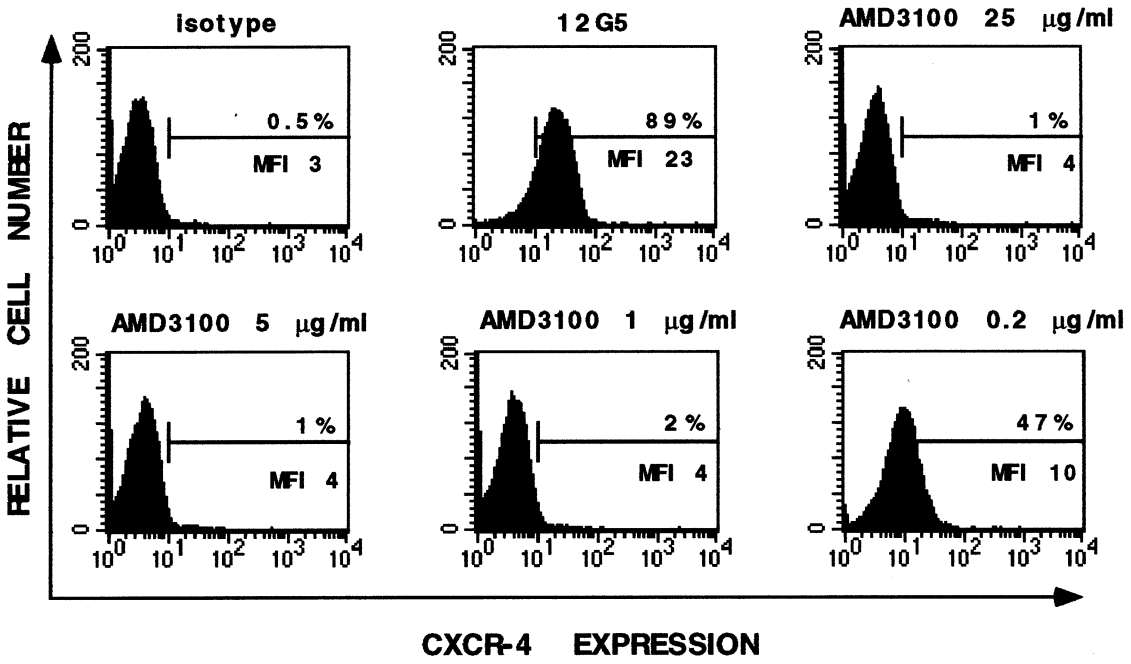

Fig. 2. Interaction of AMD3100 (at 25, 5, 1 and 0.2 μg/ml) with CXCR-4 in SUP-T1 cells is concentration-dependent. Staining was performed as for Fig. 1, except that after the 15 min preincubation at room temperature, the cells were washed twice in PBS to remove excess of compound.

of mAb 12G5 to CXCR-4. Adding AMD3100 together with the mAb, at room temperature or at 4°C, blocked the binding of the mAb as efficiently as adding the compound 15 min. before the mAb (data not shown). This also points to a very strong and direct interaction of AMD3100 with the CXCR-4 receptor and internalization of CXCR-4 can therefore be excluded.

The bicyclam JM2763, which is about 20 fold less potent than AMD3100 in inhibiting HIV-1 and HIV-2 replication (De Clercq et al., 1994), also proved about 20-fold less potent than AMD3100 in inhibiting the binding of 12G5 mAb to the SUP-T1 cells (Fig. 3). This demonstrates a direct correlation between the anti-HIV activity of the bicyclams and their interaction with CXCR-4.

If AMD3100 specifically interacts with CXCR-4, then the compound should be active only against viruses which use CXCR-4 as coreceptor to enter the target cells. This is indeed the case. As shown in Table 1, AMD3100 is very active against T-tropic viruses (IC_{50} between 0.003 and 0.01 μg/ml), but not active against M-tropic viruses (IC_{50}: >25 μg/ml). These virus strains preferentially use CCR-5, but some can also use CCR-2 and/or CCR-3 (but not CXCR-4) to enter the cells. In addition, AMD3100 is also not active against SIV, which uses CCR-5 as coreceptor (Table 1).

Because AMD3100 interacts specifically with CXCR-4, the receptor for SDF-1 and HIV is using CXCR-4 as its coreceptor to enter the cells, then SDF-1 should interfere with the infectivity of wild-type, but not AMD3100-resistant virus. Therefore, MT-4 cells were infected with 100 $CCID_{50}$ of the HIV-1 NL4-3 wild type (WT), NL4-3 AMD3100-resistant strain (De Vreese et al., 1996a) and NL4-3 DS-resistant strain (Esté et al., 1997b). SDF-1 was added to the cells at different concentrations starting from 2 μg/ml. At 4 days after infection the cells were analyzed for CD4 expression because productive infection of T-tropic viruses is generally accompanied by the disappearance of CD4 from the cell surface (Dalgleish et al., 1984). The uninfected MT-4 cells were 99% $CD4^+$ (Schols et al., 1989) whereas

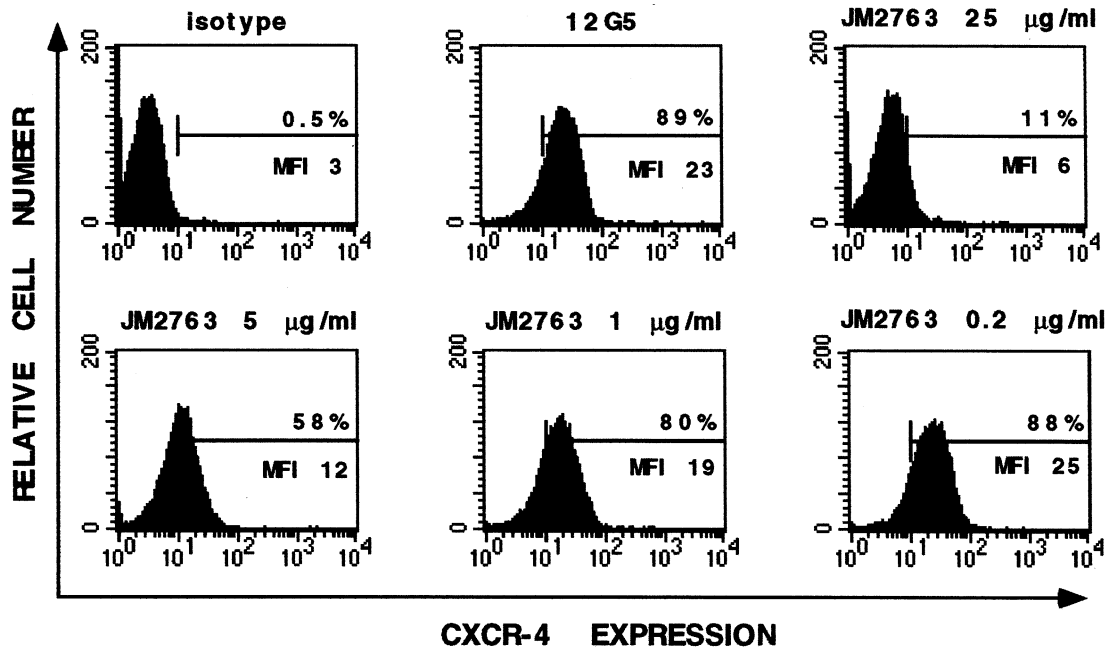

Fig. 3. Interaction of JM2763 (at 25, 5, 1 and 0.2 μg/ml) with CXCR-4 in SUP-T1 cells. The staining was performed as described in the legend to Fig. 2.

only 57%, 45% and 37% of the NL4-3 WT, AMD3100-resistant and the DS-resistant virus infected cells expressed CD4 (Fig. 4, upper panels). As can be seen in the lower panels of Fig. 4, SDF-1 was totally protective against the NL4-3 WT and also against the DS-resistant strain at 2 μg/ml (99% of the cells expressed CD4). However, SDF-1 (at 2 μg/ml) had no activity at all against the AMD3100-resistant strain (only 40% of the cells expressed CD4). The p24 viral Ag levels in the supernatant of the untreated cells infected with NL4-3 WT virus, NL4-3 DS-resistant virus and the NL4-3 AMD3100-resistant virus (Fig. 4) were determined and measured 416 840, 423 857 and 604 029 pg/ml, respectively. The p24 Ag level was below the detection limit of the ELISA (<5 pg/ml) in the supernatant of the cells treated with SDF-1 (at 2 μg/ml) and infected with the NL4-3 WT virus or the NL4-3 DS-resistant virus, but in the supernatant of the NL4-3 AMD3100-resistant virus with SDF-1 (at 2 μg/ml) a value of 672 416 pg/ml of p24 Ag was measured. SDF-1 had an identical IC_{50} (50% inhibitory concentration) of 100 ng/ml for the NL4-3WT and the DS-resistant strain, as determined with the MTT-method (Pauwels et al., 1988). Thus, the replication of AMD3100-resistant virus was not inhibited by SDF-1, whereas the replication of the WT virus and the DS-resistant virus were equally sensitive to this chemokine.

4. Discussion

Our data demonstrate that the bicyclam AMD3100 strongly interacts with CXCR-4, whereas DS and AR177, two other compounds that also interfere with virus entry into the cells (and interfere with virus binding), do not. AMD3100 is a unique compound in that it interferes with the chemokine receptor CXCR-4. AMD3100 does not appear to interact with CCR-5, or other CC chemokine receptors, as monitored by its inability to prevent the binding of biotinylated MIP-1α or biotinylated RANTES to SUP-T1 cells, MT-4 cells or freshly isolated PBMC (data not shown). Furthermore there is no inhibitory effect of AMD3100 on the replication of

SIV or HIV-1 M-tropic viruses in PBMC (Table 1), which all seem to use CCR-5, perhaps in addition to other coreceptors such as CCR-2 or CCR-3 (but certainly not CXCR-4), to enter the target cells (Alkhatib et al., 1996; Choe et al., 1996; Doranz et al., 1996; Feng et al., 1996). The specific interaction of AMD3100 with CXCR-4 explains why the bicyclams are not active against M-tropic HIV strains in human PBMC.

DS and AR177 directly interfere with binding of a mAb (9284) to the V3 loop (Schols et al., 1990; Esté et al., 1997a) but the bicyclams do not (De Clercq et al., 1992). However, when the cells are persistently infected with the AMD3100-resistant virus strain, the mAb 9284 does not longer bind to the cells. In contrast this mAb is able to bind to cells persistently infected with the WT strain (De Vreese et al., 1996a). Furthermore, mutations in the gp120 glycoprotein have been described for the AMD3100-, DS- and AR177-resistant virus strains, especially in the V3–V4 region (De Vreese et al., 1996a; Esté et al., 1997a,b), but unlike the DS- and AR177-resistant strains, the AMD3100-resistant strain has up to seven mutations clustered in the V3 loop. These alterations may induce profound changes in the V3 loop, the putative binding site of HIV with the coreceptors and also alter the affinity of the V3 loop for the mAb 9284. Our results demonstrate that mutations in the V3 loop are probably not caused by the direct interaction of AMD3100 with the viral envelope glycoprotein, but more likely emerge following the interaction of AMD3100 with the HIV coreceptor CXCR-4.

Table 1
Antiretroviral activity of AMD3100 is dependent on the coreceptor use

Strain	Coreceptor use	IC_{50} (μg/ml)
T-tropic		
IIIB	CXCR-4	0.01
RF	CXCR-4	0.005
NL4-3 WT	CXCR-4	0.003
NL4-3 AMD3100 res.	CXCR-4 (?)	0.6
M-tropic		
BaL	CCR-5	>25
SF-162	CCR-5	>25
ADA	CCR-2, CCR-3, CCR-5	>25
JR-FL	CCR-2, CCR-3, CCR-5	>25
SIV		
MAC251	CCR-5	>25

Effect of AMD3100 on infection by T-tropic and M-tropic HIV-1 strains and SIV in PHA-activated PBMC. Virus yield was monitored in the cell-free supernatant 7–10 days after infection by a p24 Ag capture ELISA for HIV-1 and the SIV core Ag EIA kit for SIV. Results of a representative experiment from three separate experiments from three different PBMC donors are shown.

It took 25 passages in cell culture before the NL4-3 strain became resistant to JM2763 (172-fold less sensitive) (IC_{50}: 127 μg/ml) and 60 passages before resistance to AMD3100 (300-fold less sensitive) (IC_{50}: 546 ng/ml) developed (De Vreese et al., 1996a). It is important to mention that we never obtained complete resistance against AMD3100 with the NL4-3 strain. However, for the HIV-1 T-tropic strain HE only 6 passages were required for JM2763, and 8 passages for AMD3100, to achieve complete resistance (IC_{50}: >250 μg/ml) (De Vreese et al., 1996a). Unlike the NL4-3 strain, the HE strain, may use other coreceptors besides fusin to infect the cells. In support of this assumption, the CC chemokine, RANTES, was found to be active against the HE strain in PHA-activated PBMC, whereas the NL4-3 strain was completely insensitive to RANTES (Schols et al., 1997).

The bicyclams are inhibitory to syncytium formation, although their IC_{50} in these assays are about 10–100 fold higher than their IC_{50} in the viral replication assays (De Clercq et al., 1994). This can also be explained by their interaction with fusin on the $CD4^+$ target cells or on the persistently HIV-infected cells. The AMD3100-resistant virus is cross-resistant to DS and AR177 (De Vreese et al., 1996a; Esté et al., 1997a), whereas, vice versa, the AR177- and DS-resistant strains are still sensitive to JM2763 and AMD3100 (De Vreese et al., 1996a; Esté et al., 1997a). This suggest that fusion inhibitors may be still active against virus that has become resistant to an adsorption inhibitor. The reverse does not hold, however, as resistance to fusion inhibitors may be accompanied by resistance to virus adsorption inhibitors as well.

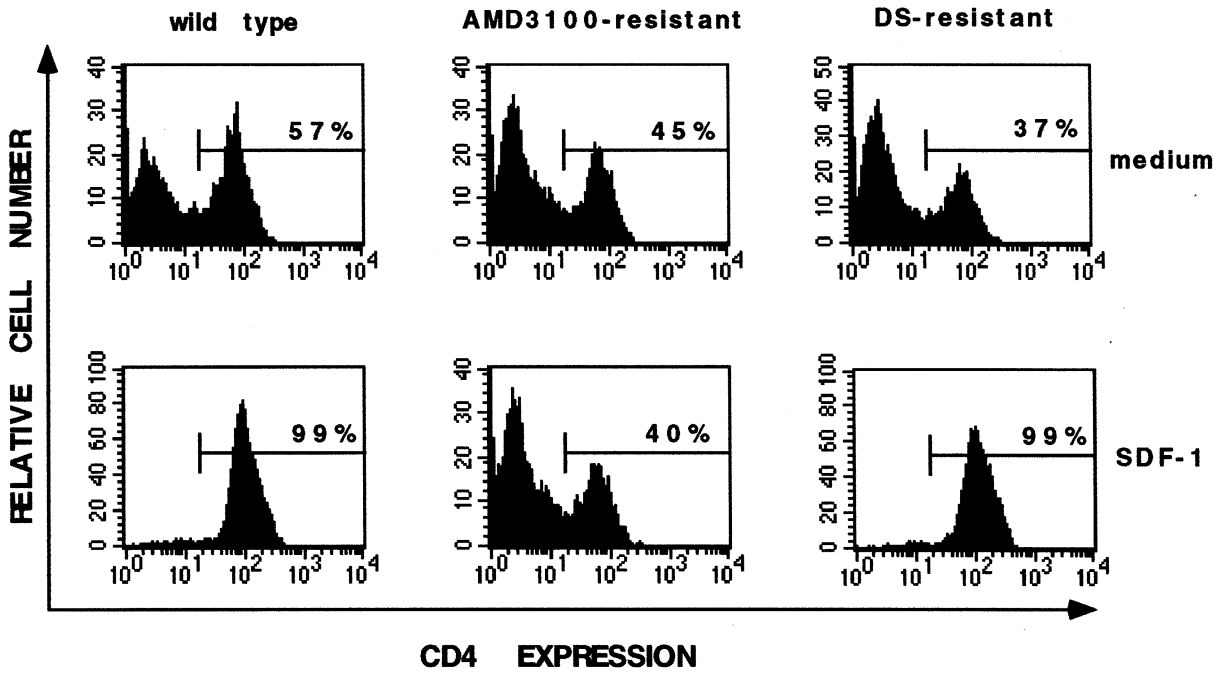

Fig. 4. Effect of SDF-1 (2 μg/ml) on NL4-3 WT, AMD3100-resistant and DS-resistant strain viral replication in MT-4 cells, monitored by CD4 expression. Cells were infected with the different virus strains at 100 $CCID_{50}$ (100 fold the 50% cell culture infective dose) and stained 4 days after infection with Leu-3a mAb directly labeled with phycoerythrin (Becton Dickinson). The percentage of positive cells are indicated in each histogram.

What remains to be resolved is why RANTES, MIP-1α and MIP-1β, that are active against M-tropic viruses in PM1 cells (a T-cell line) and PBMC (Cocchi et al., 1995) have no anti-HIV activity whatsoever in purified monocytes/macrophages (Deng et al., 1996; Dragic et al., 1996; Schmidtmayerova et al., 1996). In fact, some of these chemokines even enhance viral replication in these cells (Dragic et al., 1996; Schmidtmayerova et al., 1996). Although monocytes express CXCR-4 (McKnight et al., 1997), they cannot be infected with T-tropic viruses such as IIIB or NL4-3 for reasons which are not clear yet.

AMD3100 is equally active against a broad range of HIV-1 and HIV-2 strains, but not against simian immunodeficiency virus (SIV) strains in human PBMC (De Clercq et al., 1994). At this moment it is not clear what coreceptor SIV is using in human PBMC, but it does not seem to be CXCR-4 (Feng et al., 1996). There are some unpublished data mentioning that SIV is using CCR-5 as the coreceptor to enter the target cells (Trkola et al., 1996; Wu et al., 1996). This may explain why the bicyclams are not active against SIV in human PBMC.

SDF-1 has been shown to inhibit T-tropic (not M-tropic) viruses and primary HIV isolates (Feng et al., 1996; Wu et al., 1996). Also CXCR-4 is used by HIV-2 to enter the cells (Endres et al., 1996). The 12G5 mAb was found to inhibit HIV-1 and HIV-2 infection at 1–20 μg/ml, although the ability of this mAb to block infection of T-tropic isolates of HIV-1 is highly dependent on the viral isolate and the target cell (McKnight et al., 1997). This suggests that other cofactors may be involved or that some viruses may use a different epitope on CXCR-4 that is not blocked by the 12G5 mAb.

Some individuals repeatedly exposed to HIV infection have remained uninfected and found to be homozygous for a 32-base-pair deletion in the

CCR-5 receptor (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996). Perhaps also mutations in CXCR-4 and other coreceptors may be identified in individuals that are less susceptible to HIV infection and/or in individuals that have been infected but also do not proceed to AIDS, the so called long-term non-progressors (Schuitemaker et al., 1994), where there is a predominance of M-tropic viruses. AMD3100, because of its strong interaction with fusin, may become an important antiviral drug in vivo, because of its potential to block the transition of M-tropic to T-tropic viruses, which always precedes the decline in $CD4^+$ T-cells and the development of AIDS.

In conclusion, the bicyclams are the first among the low-molecular-weight anti-HIV agents to be found to interact with fusin (CXCR-4), the coreceptor for HIV. In particular, AMD3100 strongly binds to CXCR-4, thus inhibiting virus fusion and infectivity. AMD3100 holds great promise as a candidate anti-HIV drug. Clinical trials with the compound have been planned.

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