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MINIREVIEW

Inhibition of HIV Infection by Bicyclams, Highly Potent and Specific CXCR4 Antagonists

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This paper is available online at http://www.molpharm.org

ABSTRACT

The bicyclams represent a new entity of low-molecular weight molecules that inhibit human immunodeficiency virus (HIV) infection through a specific blockade of CXCR4 (fusin), the receptor for the CXC chemokine SDF-1 (soluble-derived factor), which is also used as coreceptor by T-lymphotropic HIV strains to enter their target cells. The bicyclam AMD3100 or 1,1′-[1,4-phenylenebis(methylene)]-bis-1,4,8,11-tetraazacyclotetradecane octahydrochloride dihydrate, is able to block the CXCR4 receptor and to inhibit HIV replication at nanomolar concentrations while not being toxic to the host cells at 100,000-fold higher concentrations. It is the most specific and most potent CXCR4 antagonist that has been described to date.

After the identification of CD4 as the primary receptor for human immunodeficiency virus (HIV) entry into the cells of the immune system, it soon became evident that CD4 alone was not sufficient to establish a productive infection, but it took another 10 years, until 1996, for the G-protein-coupled 7-transmembrane chemokine receptors CXCR4 and CCR5 to be finally identified as the coreceptors for HIV-1 entry (Cammack, 1999). CCR5 is the most important coreceptor for the macrophage (M)-tropic (now also designated as R5) strains that are commonly transmitted between individuals, and CXCR4 is the most relevant coreceptor for the T-cell-line-tropic (now also referred to as X4) isolates that emerge after several years of HIV-1 infection (Zhang and Moore, 1999). The HIV-1 coreceptors CXCR4 and CCR5 present exciting new therapeutic targets for the development of new antiretroviral agents (Cammack, 1999; Proudfoot et al., 1999; Zhang and Moore, 1999).

Here, I will focus on the potential of the so-called bicyclams in the treatment and prevention of HIV-1 infections caused by the T-tropic or X4 virus strains that are using the CXCR4 coreceptor to enter their target cells. CXCR4 is the natural receptor for the CXC-chemokine SDF-1α (stromal cell-derived factor 1α), whereas CCR5 is recognized by a number of CC-chemokines [i.e., RANTES (regulated upon activation normal T cell expressed and secreted), MIP-1α, and MIP-1β]; accordingly, RANTES, MIP-1α, and MIP-1β block the entry of M-tropic (R5), whereas SDF-1 blocks the entry of T-tropic (X4) virus strains into the cells (Luster, 1998).

The Four Key Players

The viral component involved in the interaction of HIV-1 with CXCR4 is the V3 loop (third variable loop) of the viral envelope glycoprotein gp120 (Fig. 1). This gp120 segment, together with CXCR4 (Fig. 2) (Berson et al., 1996), the bicyclams (Fig. 3), and SDF-1α (Dealwis et al., 1998), represent the four key players in the inhibitory effects of the bicyclams on the CXCR4-mediated cell entry of T-tropic HIV-1 strains and the CXCR4-mediated signaling by SDF-1α. It is remarkable that SDF-1α, the natural ligand for CXCR4, is highly basic (overall charge, +8), just like the V3 loop of gp120 (Fig. 1), whereas the bicyclam derivatives (Fig. 3) contain a cluster of eight amines, and the corresponding net charge of the extracellular regions of CXCR4 is −9 (Fig. 2).

The Bicyclams

The bicyclams, originally discovered as an impurity in the large-scale preparation of the macrocyclic polyamine cyclam [1,4,8,11-tetraazacyclotetradecane], can be described as two such ring structures tethered by either an aliphatic linker (i.e., propylene, as in JM2763, now referred to as AMD2763) or an aromatic linker (i.e., 1,4-phenylenebis(methylene), as in JM3100, now referred to as AMD3100) (Fig. 3) (De Clercq,

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ABBREVIATIONS: RANTES, regulated upon activation normal T cell expressed and secreted; SIV, simian immunodeficiency virus; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; AZT, azidothymidine (zidovudine).

1995). The bicyclams are highly potent and selective inhibitors of HIV-1 and HIV-2 replication. Whereas JM2763 was found to inhibit HIV replication in various human T-cells at a concentration of 0.14 to 1.4 μM (De Clercq et al., 1992), AMD3100 (*alias* JM3100) proved inhibitory to HIV replication at roughly a 100-fold lower concentration, that is within the nanomolar concentration range (De Clercq et al., 1994). As AMD3100 did not prove toxic to the host cells at concentrations up to 500 μM, its selectivity index, or ratio of 50% cytotoxic concentration (CC_{50}) to 50% antivirally effective concentration (EC_{50}), could be estimated at >100,000. An initially puzzling observation was the lack of activity of AMD3100 against simian immunodeficiency virus (SIV; strains MAC-251, AGM-3, and MND-GB1) (De Clercq et al., 1994). The reason for this discrepancy has now become clear: SIV uses CCR5 (or other coreceptors), but not CXCR4, to enter human cells, and AMD3100 is unable to block virus entry through CCR5 [in fact, the mandrill strain (MND-GB1) of SIV uses CXCR4 to enter CEM cells (which are CCR5$^-$), and, accordingly, its replication in CEM cells is blocked by AMD3100, as well as SDF-1α] (Schols and De Clercq, 1998).

Mode of Action

From time-of-addition experiments, whereby the compounds are added at different times after infection (De Clercq et al., 1994), it was concluded that the bicyclams (i.e., AMD2763 and AMD3100) interact with a stage of the virus replicative cycle that is intermediate between the virus adsorption step (where dextran sulfate interacts) and the reverse transcription step [where zidovudine (AZT), didanosine and the non-nucleoside reverse transcriptase inhibitors (such as TIBO) interact] (De Clercq et al., 1992, 1994). Hence, it was surmised that the bicyclams must act at the level of the HIV fusion-uncoating process (De Clercq, 1992). Further experiments ascertained that the bicyclams do not interfere with the binding of the virus to its main receptor (CD4), and thus must block viral entry after the virus has become cell-bound (De Vreese et al., 1996b).

To gain further insight in the mode of anti-HIV action of the bicyclams, resistance to the prototype bicyclams (AMD2763 and AMD3100) was developed by repeated passages of the HIV-1 clone NL4-3 in the presence of the compounds (De Vreese et al., 1996b; Esté et al., 1996). It took more than 60 passages (300 days) in MT-4 cells for the virus

Amino acid sequences of the third variable (V3) loop of HIV-1 strains

V3 loop sequence	virus	CCR5-tropic	CXCR4-tropic
C T R N N N T R K S I H I . . G P G R A F Y T T G E I I G D I R Q A H C	consensus		
C T R N N N T R K S I *R* I *Q R* G P G R A F *V* T *I* G *K* I I G *N M* R Q A H C	NL4.3	no	yes
C T R N N N T R *R R L* S I . . G P G R A F Y *A R R N* I I G D I R Q A H C	89.6	yes	yes
C T R N N N T R K *G* I H I . . G P G R A *I* Y T A *E K* I I G D I R Q A H C	HE	yes	yes
C T R N N N T R K S I H I . . G P G R A *L* Y T T G E I I G D I R Q A H C	BaL	yes	no

Fig. 1. V3 loop sequence encompassing the amino acid fragment 263–299 of the viral envelope gp120 glycoprotein.

Fig. 2. CXCR4, receptor for the CXC-chemokine SDF-1α (stromal cell-derived factor) and coreceptor for the T-tropic (X4) HIV-1 strains. Figure taken from Berson et al. (1996).

to become 300- to 400-fold resistant to AMD3100 (Esté et al., 1996). The resistant virus had several mutations scattered over the whole gp120 glycoprotein, but primarily clustered in the V3 loop: i.e., R272T, S274R, Q278H, I288V, N293H, and A297T. The substitutions of the highly conserved amino acids in close proximity to the disulfide bridges of the V3 and V4 loops (A297T and P385L, respectively) may be of particular importance. But most, if not all, of the mutations, including those outside the V3 loop, may have contributed to the resistant phenotype, as indicated by recombination experiments with overlapping parts of the envelope gene (De Vreese et al., 1996a). Based on the nature and location of the amino acid substitutions, it has been postulated, therefore, that the overall, three-dimensional, conformation of gp120, rather than the individual amino acid substitutions, was the prime determinant of the resistance/sensitivity profile of HIV strains to bicyclams (De Vreese et al., 1997).

The Target of Action

A particularly striking observation, that made us look into a specific coreceptor antagonism of the bicyclams, was the finding that AMD3100, like SDF-1α and unlike RANTES, was active against a variety of T-tropic HIV strains (i.e., III$_B$,

Fig. 3. Bicyclams, consisting of two cyclam (1,4,8,11-tetraazacyclotetra-decane) moieties, tethered via an aliphatic bridge [i.e., propylene, as in JM2763 (now referred to as AMD2763)] or an aromatic bridge [i.e., phenylenebis(methylene), as in JM3100 (now referred to as AMD3100)].

RF, NL4-R, ROD, which are all using CXCR4 as coreceptor), but inactive against a series of M-tropic virus strains (i.e., BaL, SF-162, ADA, JR-FL, which are all using CCR5 as coreceptor) (Schols et al., 1997b). Because of the specific and potent inhibitory effect of AMD3100 on T-tropic viruses, it was then verified whether AMD3100 interacts with CXCR4. As shown by Schols et al. (1997b), AMD3100 at 1 μg/ml completely inhibited the binding of the monoclonal antibody (mAb) 12G5, a specific marker for the human CXCR4 protein. Under the same conditions, SDF-1α (2 μg/ml) also inhibited, but less efficiently than AMD3100, the binding of the mAb to CXCR4 (Schols et al., 1997b). Even when washed away before addition of the mAb, AMD3100 inhibited the binding of the CXCR4 mAb as efficiently as when the compound was present during the whole incubation period with the mAb. Adding AMD3100 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 alone. This points to a very strong and direct interaction of AMD3100 with the CXCR4 receptor and excludes the possibility that the receptor may have become internalized under the influence of AMD3100 (Schols et al., 1997a).

CXCR4 Antagonism

In contrast to AMD3100, which showed a concentration-dependent inhibition of the binding of mAb 12G5 to CXCR4 at a concentration range of 0.2 to 2.5 μg/ml (Schols et al., 1997a), various other compounds, which are known to interact with the viral entry into the cells, such as the polyanions dextran sulfate and zintevir (AR177, a 17-mer oligodeoxynucleotide with two G-quartet motifs) at 25 μg/ml, proved totally ineffective in blocking mAb 12G5 to CXCR4 (Schols et al., 1997a).

AMD3100 at 100 ng/ml completely blocked signal transduction from CXCR4 in both SUP-T1 and THP-1 cells, as monitored by the $[Ca^{2+}]_i$ response to SDF-1α; at 10 ng/ml, AMD3100 effected a partial reduction in the Ca^{2+} flux (Schols et al., 1997b). In contrast, AMD3100 at 100 ng/ml failed to inhibit Ca^{2+} flux induced by RANTES (Schols et al., 1997b), MIP-1α and MCP-3 in THP-1 cells (Schols et al., 1997b). Similarly, AMD3100 had no influence on the Ca^{2+} flux induced by RANTES in 293T cells, whereas it completely inhibited SDF-1α-induced signaling from CXCR4 in 293T cells at a concentration of 0.1 nM (Donzella et al., 1998). AMD3100 (12 nM) did not induce a Ca^{2+} flux, nor did it affect the response of other G-protein-coupled receptors to ligands such as carbachol (3 mM) or somatostatin (3 mM) (Donzella et al., 1998). Although inhibitory to the binding of monoclonal antibodies reactive with CXCR4 (12G5), AMD3100 did not affect the binding of mAb 2D7 reactive with CCR5 (Donzella et al., 1998). Over a concentration range of 0.1 to 1000 ng/ml, a nice correlation was found for the AMD3100 concentrations required to inhibit HIV-1 (NL4-3) replication, CXCR4 mAb binding, and SDF-1α-induced Ca^{2+} flux, all measured in SUP-T1 cells (Fig. 4), suggesting a close relationship between these three parameters (Schols et al., 1997a,b).

The specificity of AMD3100 in blocking T-tropic (or dual-tropic) HIV-1 entry into the cells was attested furthermore by using env-complemented virus strains in U87 MG-CD4 cells expressing either CXCR4 or CCR5 (Donzella et al., 1998). Entry into the CXCR4-expressing cells mediated by the envelopes of the TCLA strain HxB2 or the dual-tropic SF162-
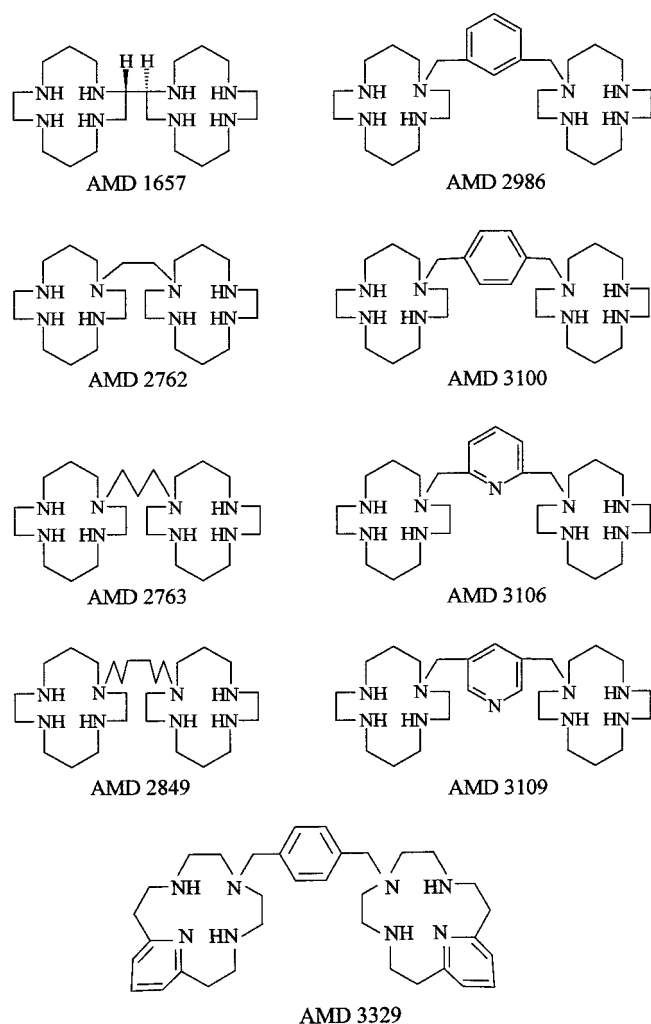
DBL was strongly inhibited by AMD3100 (IC_{90}, 0.01–0.1 nM) (Donzella et al., 1998). However, the entry of M-tropic (ADA and JR-FL) or dual-tropic (SF162-DBL) viruses into the CCR5-expressing cells was insensitive to AMD3100 at the highest concentrations tested (1 μM) (Donzella et al., 1998). Thus, AMD3100 can be considered a specific probe for CXCR4, and to this end it has been used to ascertain that primary macrophages can be infected by HIV through a functional CXCR4 (Simmons et al., 1998). Given the high specificity of AMD3100 for CXCR4, AMD3100 has proved useful in discerning between those HIV-1 strains that use both CXCR4 and CCR5 on macrophages and T-cell lines (dual-tropic R5X4), and those HIV-1 strains that use CXCR4 on both macrophages and T-cell lines (dual-tropic X4) (Yi et al., 1999).

The inhibitory effects of AMD3100 on the T-tropic HIV-1 NL4-3 strain have been demonstrated in a wide variety of cells expressing CXCR4, including peripheral blood mononuclear cells (PBMCs) (Table 1) [D. Schols and E. De Clerq, unpublished data (1999)], and vice versa, various T-tropic and dual-tropic, but not M-tropic, HIV-1 strains have proven sensitive to AMD3100 in PBMCs (Table 1) [D. Schols and E. De Clerq, unpublished data (1999)]. T-tropic HIV-1 strains can be made resistant to AMD3100 (i.e., NL4-3[AMD3100res] and HE[AMD3100res]) or SDF-1α (i.e., NL4-3[SDFres]), upon repeated passages of the virus in the presence of the compound. Whereas NL4-3[AMD3100res] showed complete cross-resistance to SDF-1α, NL4-3[SDFres] had only slightly reduced sensitivity to AMD3100 (Schols et al., 1998). Resistance to AMD3100 or SDF-1α did not lead to a switch in coreceptor use (Schols et al., 1998).

Mode of Interaction with CXCR4

The tropism of the HIV-1 strains for CXCR4 or CCR5 may seem related to the viral gp120 V3 loop sequence, containing eight arginine/lysine residues, thus eight positive charges in the CXCR4-tropic NL4-3 strain, and only five arginine/lysine residues, thus five positive (actually four, because one of the lysine residues is replaced by glutamic acid) charges in the CCR5-tropic BaL strain (Fig. 1). CCR5 tropism seems to be possible only when two consecutive amino acids (glutamine and arginine at positions 278 and 279) in gp120 are absent. Resistance development to SDF-1α (NL4-3) and AMD3100 (HE) is accompanied by a reduction in the overall positive charge (i.e., substitution of glutamic acid for asparagine at position 270, or substitution of glycine for arginine at position 283, respectively).

Some determinants for sensitivity of the coreceptor CXCR4 to AMD3100 have been characterized (Labrosse et al., 1998). AMD3100 completely blocked HIV-1 infection mediated by a mutant CXCR4 bearing a deletion in most of the amino-terminal extracellular domain. In contrast, relative resistance to AMD3100 was conferred by different single amino acid substitutions in the second extracellular loop (ECL2) or in the adjacent membrane-spanning domain (TM4). Only substitutions of a neutral amino acid residue for aspartic acid and of a nonaromatic residue for phenylalanine were associated with resistance to AMD3100 (Fig. 5) (Labrosse et al., 1998). The interaction of the aspartic acids of ECL2 and TM4 with AMD3100 is consistent with the multipositive charge of the bicyclams, which may block HIV-1 entry by preventing the electrostatic interactions between CXCR4 and the HIV-1
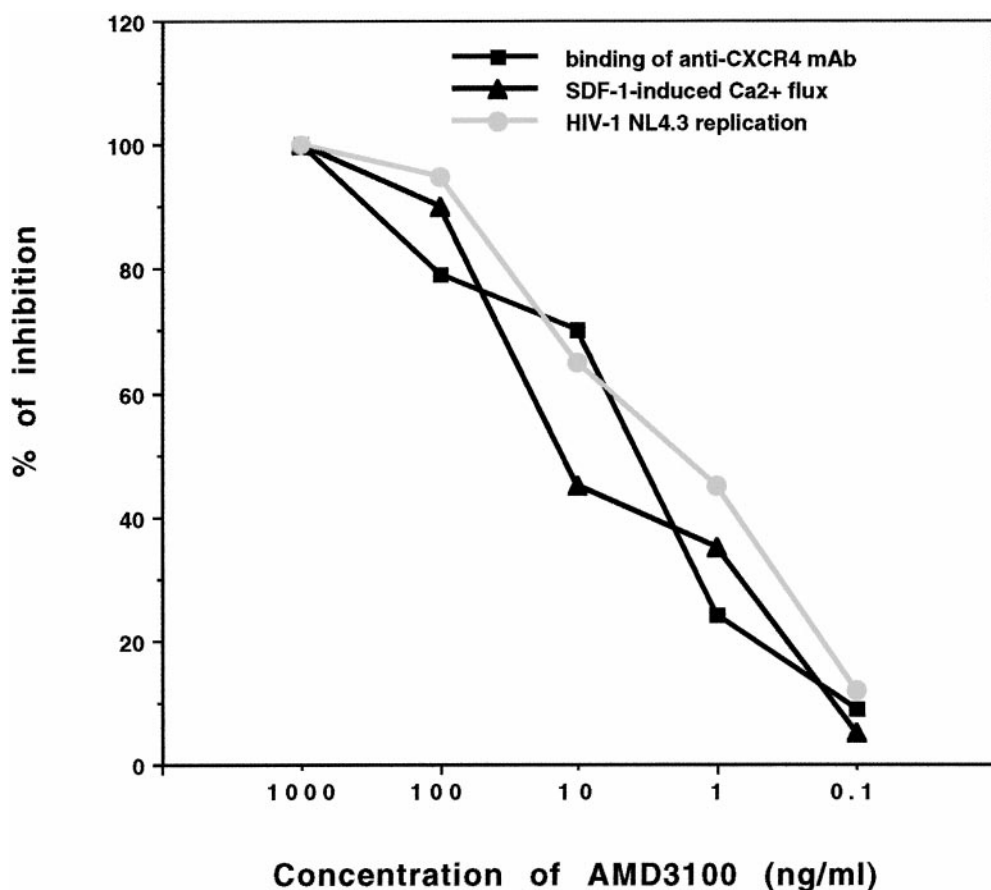
Fig. 4. Correlation between the inhibitory effects of AMD3100 on HIV-1 NL4-3 replication, binding of CXCR4 mAb, and SDF-1-induced Ca^{2+} flux, all performed in SUP-T1 cells.

envelope glycoprotein gp120. The aromatic linker [i.e., phenylenebis(methylene) in AMD3100] between the cyclam rings might engage in hydrophobic interactions with the Phe-X-Phe motifs of ECL2 or TM4 (Labrosse et al., 1998).

Structure-Activity Findings

The structure-activity relationship of the bicyclams has been assessed (Bridger et al., 1995, 1996, 1999). Structural features required for anti-HIV activity include specific macrocyclic ring size, metal chelating ability, plane torsion and plane angles, and distance between the metal-binding centers (Joao et al., 1995). Of all the bis-azamacrocyclic analogues reported to date, the *para*-phenylenebis(methylene)-linked dimer of the py[*iso*-14]aneN$_4$ (AMD3329) (Fig. 3) displayed the highest antiviral activity. Its EC$_{50}$ against HIV-1 and HIV-2 replication was 0.8 and 1.6 nM, respectively, that is about 3- to 5-fold lower than the EC$_{50}$ of AMD3100. AMD3329 also inhibited the binding of specific CXCR4 mAb, and the Ca^{2+} flux induced by SDF-1α, more potently than AMD3100. Furthermore, AMD3329 interfered with virus-induced syncytium formation at an EC$_{50}$ of 12 nM (Bridger et al., 1999).

For a number of different bicyclam derivatives, a close correlation was found between the anti-HIV potency of the

TABLE 1
Inhibitory effects of SDF-1α, RANTES, and AMD3100 on the T-tropic HIV-1 NL4-3 strain in different cell lines and on different HIV-1 strains in PBMCs

Virus yield was monitored by viral p24 Ag ELISA in the cell-free supernatant at 5 to 12 days after HIV-1 infection. All data represent mean values for at least three to eight separate experiments.

Cell Line (Coreceptors Expressed)	IC$_{50}$		
	SDF-1α	RANTES	AMD3100
	ng/ml		
HIV-1 NL4-3 strain in different cell lines			
MT-4 (CXCR4, CCR5)	130	>1,000	6
CEMX 174 (CXCR4, CCR3, BOB)	900	>1,000	21
PM1 (CXCR4, CCR1, CCR3, CCR4, CCR5)	450	>1,000	10
PBMC (CXCR4, CCR5, CCR3)	100	>1,000	3
U87, CXCR4 (CXCR4, Bonzo)	730	>1,000	2
HOS.CXCR4 (CXCR4)	610	>1,000	4
HOS.pBabe (CXCR4)	95	>1,000	1
Different HIV-1 strains in PBMCs			
T-tropic NL4-3	100	>1,000	3
T-tropic NL4-3[AMD3100res]	>1,000	>1,000	2,100
T-tropic NL4-3[SDFres]	>1,000	>1,000	8
Dual-tropic 89.6	450	>1,000	45
T-tropic HE	>1,000	240	200
T-tropic HE[AMD3100res]	>1,000	900	>25,000
M-tropic BaL	>1,000	25	>25,000

IC$_{50}$, 50% inhibitory concentration based on virus yield reduction.

bicyclams and their ability to inhibit the binding of CXCR4 mAb (12G5), on the one hand, and their inhibitory effect on SDF-1α-induced intracellular Ca^{2+} mobilization (in SUP-T1 cells), on the other hand (Esté et al., 1999a). For a series of metal-AMD3100 complexes, again a close correlation was found between anti-HIV activity, inhibition of CXCR4 mAb binding, and inhibition of SDF-1α-induced Ca^{2+} flux, the order of decreasing activity being Zn > Ni > Cu > Co > Pd (Esté et al., 1999a).

Shift from T- to M-tropic HIV Strains

When PBMCs were infected with a mixture of 99% T-tropic (NL4-3) and 1% M-tropic (BaL) and then exposed for four passages (28 days) to AMD3100, the only virus recovered from the cultures was the M-tropic (BaL) strain (Esté et al., 1999b). When AMD3100 was added to PBMCs infected with clinical HIV isolates, displaying the syncytium-inducing phenotype, i.e., strains CST, AOM, and FCP (Esté et al., 1999b), these strains reverted to the nonsyncytium-inducing phenotype, and, concomitantly, the CST, AOM, and FCP strains that originally used CXCR4 to enter the cells (as monitored in CXCR4-expressing U87-CD4 cells) switched to CCR5 coreceptor use (as monitored in CCR5-expressing U87-CD4 cells) after they had been exposed to AMD3100. These findings indicate that selective blockade of CXCR4 by AMD3100 may prevent the switch from the less pathogenic M-tropic (R5) to the more pathogenic T-tropic (X4) HIV strains. As in vivo, this process heralds the progression to AIDS, AMD3100 should be examined further in vivo for its potential to block the switch from M- to T-tropism and prevent or arrest progression of the disease.

The Bicyclam AMD3100 Compared with Other Compounds

In conclusion, the bicyclams, and in particular AMD3100, offer great potential for the prevention and suppression of T-tropic HIV infections. They are highly potent and specific antagonists of CXCR4, the coreceptor used by T-tropic HIV strains to enter their target cells. The bicyclam AMD3100 is more potent an inhibitor of HIV replication than the natural CXCR4 ligand SDF-1, and in addition, AMD3100 also seems to be more potent than several other, arginine-rich, peptidic molecules that have been reported to interact with CXCR4, such as T22 ([Tyr[5,12], Lys[7]]-polyphemusin II) (Murakami et al., 1997; Tamamura et al., 1998), T134, a shortened version of T22 (Arakaki et al., 1999), ALX40-4C (*N*-α-acetyl-nona-D-arginine) (Doranz et al., 1997) and CGP64222, an inhibitor of the Tat/TAR interaction (Daelemans et al., 2000) (although all these molecules have not been compared directly with one
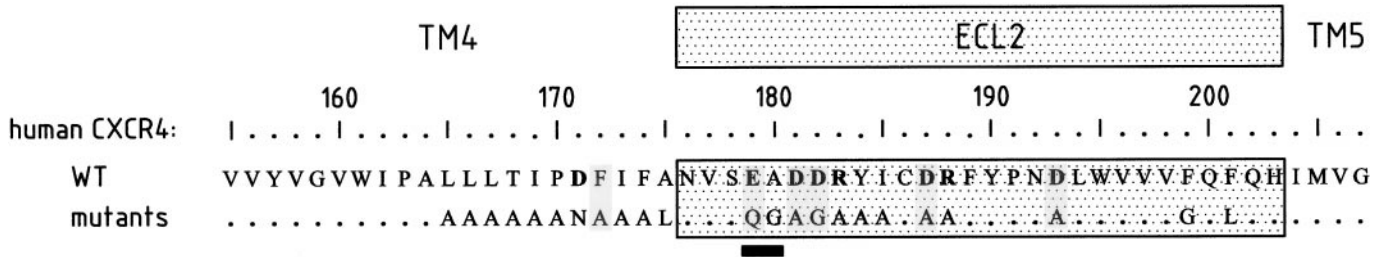

Fig. 5. Partial amino acid sequence of (human) wild-type and mutant CXCR4. Mutations affecting sensitivity to AMD3100 are highlighted. All mutations in CXCR4 correspond to single amino acid substitutions, except for EA to QG in ECL2 (underlined). Charged amino acids are shown in boldface type.

another in the same assay systems). AMD3100 also appears to be a far more potent inhibitor of T-tropic HIV strains than NSC 651016, a distamycin analog that has been recently reported to target some chemokine receptors (although not exclusively CXCR4) (Howard et al., 1998).

Also, AMD3100 has been covalently linked (via an ester linkage) to AZT, with the aim to create bipharmacophoric drugs, associating in a single molecule a CXCR4 antagonist (or fusion inhibitor) and (the precursor of) a reverse transcriptase inhibitor (Dessolin et al., 1999). It remains to be established whether the anti-HIV activity accomplished by such bicyclam-AZT conjugates is due to the bicyclam or AZT, or both.

Clinical Perspectives

AMD3100 has proved efficacious alone and in combination with AZT or didanosine in achieving a marked reduction in viral load in the SCID-hu Thy/Liv mouse model (Datema et al., 1996) without causing obvious toxicity. The in vivo efficacy of AMD3100 against T-tropic HIV strains has been recently confirmed in the SCID-hu PBMC mouse model [D. Schols and E. De Clerq, unpublished data (1999)]. AMD3100 and bicyclams in general are also effective against feline immunodeficiency virus (Egberink et al., 1999), which is not surprising because feline immunodeficiency virus strains, like T-tropic HIV strains, use CXCR4 for cell fusion and viral entry.

Recently published data (Blanco et al., 2000) indicate that AMD3100 not only inhibits HIV replication but also blocks cell-surface-expressed HIV-1-envelope-induced apoptosis of uninfected cells. The glycoprotein complex gp120/gp41 seems to be the major determinant for the induction of apoptosis (i.e., in $CD8^+$ T-cells and neurons) that involves CXCR4 signaling and appears to be restricted to X4 isolates. The fact that AMD3100 is able to block these apoptotic events adds further to the potential of AMD3100 in the treatment of (X4) HIV-1-infected individuals.

Clinical studies with AMD3100 have been recently initiated (Hendrix et al., 1999): in a phase I clinical trial in normal healthy volunteers, AMD3100 was well tolerated following single 15-min i.v. injections of doses of 10 μg and 20 μg/kg. A median peak serum concentration of 118 ng/ml (that is almost 100-fold higher than the minimum antivirally effective concentration in cell culture) was obtained with a dose of 20 μg/kg. AMD3100 has recently entered phase II clinical trials in HIV-infected individuals, which will be monitored for the coreceptor (CXCR4 and/or CCR5) use of their HIV isolates.

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