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Potent, Broad-Spectrum Inhibition of Human Immunodeficiency Virus Type 1 by the CCR5 Monoclonal Antibody PRO 140

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CCR5 serves as a requisite fusion coreceptor for clinically relevant strains of human immunodeficiency virus type 1 (HIV-1) and provides a promising target for antiviral therapy. However, no study to date has examined whether monoclonal antibodies, small molecules, or other nonchemokine agents possess broad-spectrum activity against the major genetic subtypes of HIV-1. PRO 140 (PA14) is an anti-CCR5 monoclonal antibody that potently inhibits HIV-1 entry at concentrations that do not affect CCR5's chemokine receptor activity. In this study, PRO 140 was tested against a panel of primary HIV-1 isolates selected for their genotypic and geographic diversity. In quantitative assays of viral infectivity, PRO 140 was compared with RANTES, a natural CCR5 ligand that can inhibit HIV-1 entry by receptor downregulation as well as receptor blockade. Despite their divergent mechanisms of action and binding epitopes on CCR5, low nanomolar concentrations of both PRO 140 and RANTES inhibited infection of primary peripheral blood mononuclear cells (PBMC) by all CCR5-using (R5) viruses tested. This is consistent with there being a highly restricted pattern of CCR5 usage by R5 viruses. In addition, a panel of 25 subtype C South African R5 viruses were broadly inhibited by PRO 140, RANTES, and TAK-779, although ~30-fold-higher concentrations of the last compound were required. Interestingly, significant inhibition of a dualtropic subtype C virus was also observed. Whereas PRO 140 potently inhibited HIV-1 replication in both PBMC and primary macrophages, RANTES exhibited limited antiviral activity in macrophage cultures. Thus CCR5-targeting agents such as PRO 140 can demonstrate potent and genetic-subtype-independent anti-HIV-1 activity.

Entry of human immunodeficiency virus type 1 (HIV-1) into susceptible host cells requires that they express CD4 and a fusion coreceptor such as the chemokine receptors CCR5 and CXCR4 (reviewed in reference 10). CCR5 is the predominant coreceptor used by viruses present during the early stages of HIV-1 infection, and half or more of all infected individuals progress to AIDS harboring only R5 viruses, i.e., those that use CCR5 exclusively (19, 39). In the remaining individuals, viruses acquire the ability to use CXCR4 exclusively or in addition to CCR5 (X4 and R5X4 viruses). Little is known regarding the factors that contribute to the selective bias against transmission and emergence of CXCR4-using viruses, but the broadening of coreceptor usage during natural infection is not correlated in any obvious way with CCR5 availability. Indeed, CCR5 expression on T cells in the periphery reportedly increases throughout the course of HIV-1 infection (18), perhaps reflecting chronic stimulation of the immune system, but little is known regarding the temporal patterns of CCR5 expression in other anatomical compartments.

Molecular-epidemiology studies clearly demonstrate that CCR5 plays a critical role in HIV-1 transmission and pathogenesis in vivo. Individuals who possess two copies of a nonfunctional CCR5 allele (Δ32 allele) are strongly (17, 31, 45), but not absolutely (8, 11, 50, 63), protected against infection by HIV-1. Individuals with one Δ32 and one normal CCR5 gene on average express lower levels of CCR5 on their T cells (73). Heterozygosity for the Δ32 allele does not protect against HIV-1 infection but does confer an improved prognosis in the form of significantly increased AIDS-free and overall survival periods (4, 17, 34, 47). Moreover, CCR5 heterozygotes are overrepresented among long-term nonprogressors, i.e., those individuals who do not progress to AIDS after 10 or more years of infection (17, 34, 61). Polymorphisms in the regulatory regions of the CCR5 gene also influence HIV-1 transmission and disease progression (36, 41, 42, 49).

Because it is an essential fusion coreceptor for clinically relevant strains of HIV-1 yet is apparently dispensable for human health, CCR5 provides an attractive target for new antiretroviral therapies (46). Moreover, CCR5 belongs to a family of seven transmembrane-spanning receptors that have historically provided excellent targets for pharmaceutical interventions (62).

A number of CCR5-targeting antibodies, chemokines, chemokine analogs, and small molecules are capable of inhibiting HIV-1 replication in vitro (3, 7, 14, 30, 44, 51, 60, 74). Of the CC-chemokines that bind CCR5, RANTES possesses significantly greater breadth of antiviral activity than MIP-1α and MIP-1β, although all CC-chemokines show interisolate variation in potency (69). The antiviral activity of the CC-chemokines better correlates with their ability to downregulate rather

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than to bind CCR5 on $CD4^+$ T cells, and sustained downregulation of CCR5 has been suggested to be a principal mechanism of action for the chemokine analog aminooxypentane (AOP)-RANTES (40). Similar isolate-dependent variations in potency have been reported for chemokine analog AOP-RANTES (64) and inhibitory CCR5 antibodies such as 2D7 (32, 33).

Thus it is unclear at present whether CCR5 antibodies or small-molecule CCR5 antagonists can broadly inhibit diverse HIV-1 isolates. The ability of nonagonists (i.e., agents that do not downregulate CCR5) to broadly inhibit CCR5-mediated entry may ultimately depend on whether wild-type HIV-1 isolates utilize a restricted or a dispersed set of epitopes on CCR5. In addition, there are discordant reports on the effects of CC-chemokines on HIV-1 replication in macrophages, and factors that may influence the inhibitory activity include the source of donor cells, isolation methods, culture conditions, and proteoglycan levels (2, 3, 20, 52, 53, 59, 60, 72, 77). While some chemokine derivatives are more potent than natural chemokines in inhibiting HIV-1 replication in macrophages (3, 60, 77), little is known regarding the activity of nonchemokine agents.

PRO 140 (previously described as PA14) is a murine monoclonal antibody that binds a complex epitope spanning multiple extracellular domains on CCR5 (51). It potently inhibits CCR5-mediated HIV-1 entry at concentrations that do not prevent CC-chemokine signaling, although PRO 140 acts as a weak CCR5 antagonist at higher concentrations. Because PRO 140 does not induce signaling or downregulation of CCR5, its antiviral effect is probably exerted through a mechanism involving receptor blockade.

In this study, we have examined PRO 140's breadth of activity against a panel of primary HIV-1 isolates selected for their geographic, genotypic, and phenotypic diversity. In studies carried out with both peripheral blood mononuclear cell (PBMC) and primary macrophage cultures, PRO 140 was compared with RANTES, the natural ligand for CCR5, and/or TAK-779, a small-molecule CCR5 antagonist (6). Our major conclusion is that PRO 140 potently and broadly prevents R5 viruses from infecting their principal target cells, namely, $CD4^+$ T cells and macrophages. PRO 140 therefore merits evaluation as an agent to prevent or treat HIV-1 infection in vivo.

MATERIALS AND METHODS

Viruses. All virus isolates and clones were grown and titered exclusively in stimulated PBMCs as described previously (65). HIV-$1_{\text{Case C 1/85}}$ and HIV-$1_{\text{Case C 7/86}}$ viruses were isolated from the same individual on the successive dates indicated (15). Virus isolates from South Africa (see Table 3) were from three different cohorts. Isolates from acutely infected commercial sex workers were labeled Du (Durban, KwaZulu/Natal); isolates from hospitalized AIDS patients were labeled according to their presenting symptoms: tuberculosis (TB), pneumocystis pneumonia (PCP), and cryptococcal meningitis (CM). Isolates from pediatric AIDS cases were labeled COT. The viruses were isolated by coculture with stimulated PBMCs and assayed for coreceptor usage using U87.CD4 cells expressing either CCR5 or CXCR4 and GHOST cells expressing minor coreceptors (67).

Inhibitors. PRO 140 (PA14) is a mouse immunoglobulin G1 antibody whose isolation, preparation, and purification have been described (51). The antibody was prepared at Progenics Pharmaceuticals as a 5-mg/ml solution in phosphate-buffered saline and maintained at −70°C prior to use. TAK-779 was obtained as described elsewhere (22). RANTES was purchased from PeproTech, Inc. (Rocky Hill, N.J.) and handled in accordance with the manufacturer's instructions.

Inhibition of HIV-1 replication in PBMC cultures. PBMCs were isolated from healthy blood donors with Ficoll-Hypaque and then stimulated with 5 or 0.5 μg of phytohemagglutinin/ml or with surface-immobilized anti-CD3 antibody OKT3 in RPMI 1640 medium containing 10% fetal calf serum, 100 U of interleukin-2/ml, glutamine, and antibiotics (PBMC culture medium) as previously described (68). After 72 h, equal numbers of PBMCs stimulated by one of these three methods were combined for use in infection assays as follows. PBMCs (2×10^5) in 100 μl of PBMC culture medium were combined with 50-μl aliquots of serially diluted PRO 140 or RANTES for 1 h at 37°C. The virus inoculum was adjusted to 400 to 1,000 times the 50% tissue culture infectious dose per ml in PBMC culture medium, and a 50-μl aliquot was added to each culture. The inhibitory doses refer to the concentrations of the agents present at this point in the culture, when virus, cells, and inhibitors were all present at their final concentrations. Thereafter the inhibition assay was performed as described previously (69), with the extent of HIV-1 replication being determined by measuring the p24 antigen content of the culture supernatants. The inhibition data were analyzed by linear regression to calculate the concentrations of PRO 140 or RANTES that afforded 50 and 90% reductions in p24 antigen production. These were designated the 50 and 90% inhibitory concentrations (IC_{50} and IC_{90}), respectively.

Inhibition of HIV-1 replication in macrophage cultures. Monocytes were isolated at >90% purity from freshly separated PBMCs using positive selection with anti-CD14-coated magnetic beads (Miltenyi Biotech, Auburn, Calif.) according to the manufacturer's instructions. Twenty-four-well culture plates were pretreated for 2 h with 300 μl of poly-L-lysine (50 μg/ml in phosphate-buffered saline). Excess poly-L-lysine was then removed, and 6×10^5 monocytes were seeded per well. Monocytes were cultured in RPMI 1640 containing 10% fetal calf serum, 5% heat-inactivated normal human serum, 5% macrophage-conditioned medium (harvested from macrophage cultures on day 7 of differentiation), glutamine, and antibiotics. Cells were allowed to differentiate for 7 days and then were incubated for 1 h in fresh medium containing PRO 140 or RANTES. In studies examining the ability of proteoglycans to potentiate the antiviral activity of RANTES and PRO 140, heparan sulfate or chondroitin sulfate was also added at this time. Cultures were then infected with 1,000 50% tissue culture infective doses of virus and maintained in a total volume of 1 ml per well. The extent of HIV-1 replication was determined on day 7 postinfection by measuring p24 production as described above.

Env amino acid sequences. Env sequences obtained from GenBank were as follows (virus isolate/accession number): 92US657/U04908, DJ259/L22940, JR-FL/U63632, 92TH001/U39256, CM235/L03698, 92RW026/U08658, 92US714/U08450, JR-CSF/U45960, DJ258/L22939, BZ162/L22084, SF162/M65024, and HxB2/K03455.

RESULTS

Subtype-independent inhibition of HIV-1 replication. PRO 140 and RANTES were directly compared in infectivity assays carried out using a diverse panel of PBMC-grown primary HIV-1 isolates. Figure 1 depicts representative PRO 140 and RANTES inhibition curves for selected HIV-1 isolates. Tables 1 and 2 summarize the median IC_{90} values observed in two to five replicate assays performed on each of 17 R5 viruses representing subtypes A to C, E, and F. Overall, PRO 140 and RANTES were virtually equipotent against this virus panel (Table 1). Both agents effectively (>90%) inhibited all nine subtype B viruses. The median IC_{90} values were 17 nM for PRO 140 and 13 nM for RANTES. The results are in good agreement with the PRO 140 concentration (~25 nM) required to inhibit entry of HIV-$1_{\text{JR-FL}}$ Env-complemented virus in luciferase-based entry assays (51). The greatest differences in viral sensitivities were observed for HIV-$1_{92\text{US}657}$, which was 14-fold more sensitive to inhibition by PRO 140, and HIV-$1_{\text{SF}162}$, which was 3.4-fold more sensitive to RANTES. HIV-$1_{\text{SF}162}$, the virus least susceptible to PRO 140, is known to be relatively resistant to inhibition by other CCR5-targeting agents (32, 33, 69) and was included in the panel for this reason.

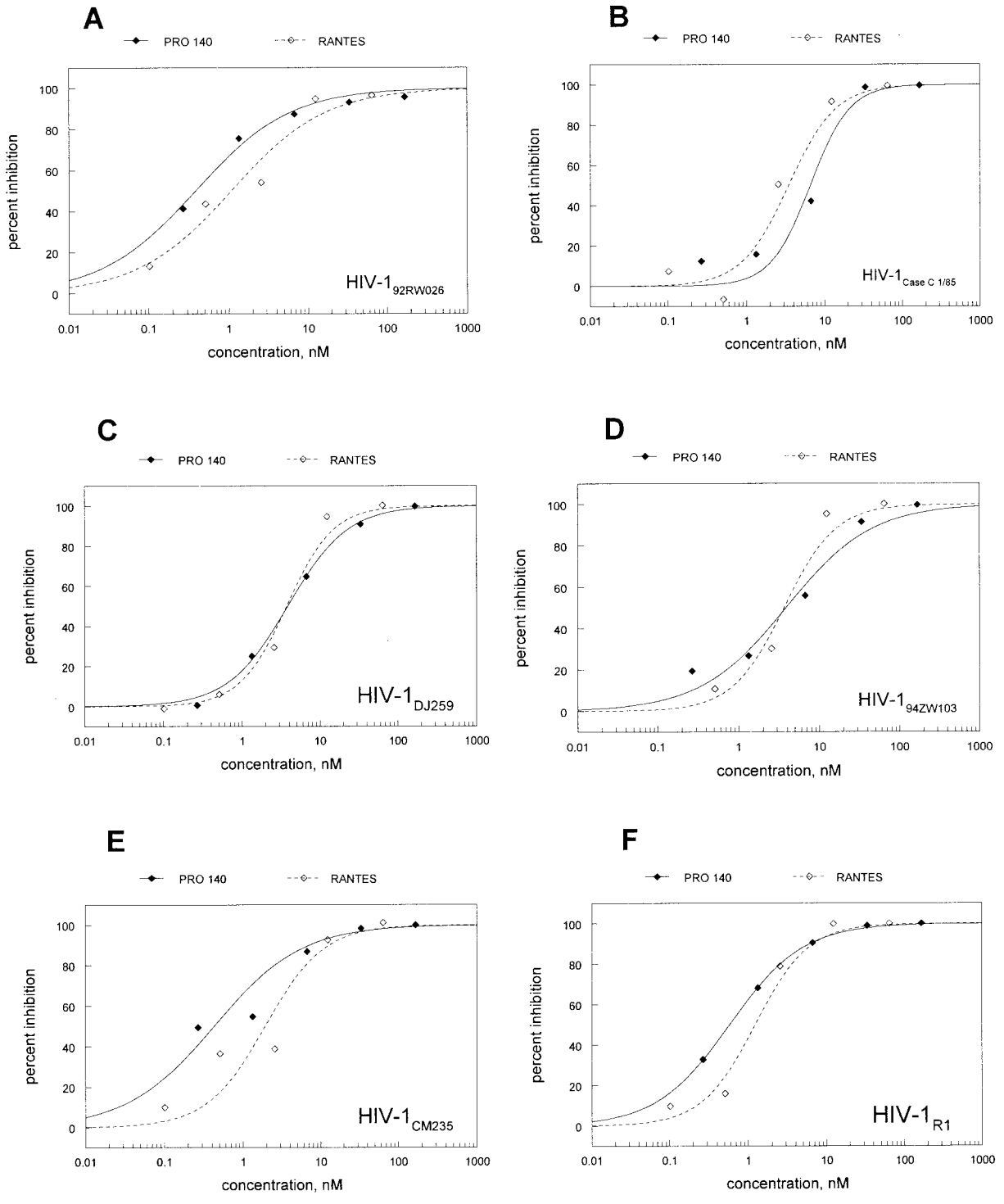

FIG. 1. Inhibition of HIV-1 replication in PBMC cultures. The extent of R5 virus replication was assessed by measuring the p24 antigen content of 7-day culture supernatants. The inhibition curves depict data from a representative assay. (A) subtype A isolate 92RW025; (B) subtype B isolate Case C 1/85; (C) subtype C isolate DJ259; (D) subtype C isolate 94ZW103; (E) subtype E isolate CM235; (F) subtype F isolate R1.

A similar breadth of potency was observed when PRO 140 and RANTES were tested against a panel of eight additional R5 viruses consisting of two each from subtypes A, C, E, and F. As indicated in Table 2, both PRO 140 and RANTES potently inhibited all eight viruses. There were no obvious genetic-subtype-dependent differences in viral sensitivities to either agent. For the non-B virus panel as a whole, the median IC_{90} values were 15 nM for PRO 140 and 12 nM for RANTES. The relative potencies of PRO 140 and RANTES (as determined by the ratio of their IC_{90} values) never varied more than

TABLE 1. Inhibition of subtype B R5 viruses by PRO 140 and RANTES

Virus	Median IC_{90} (nM) for:		PRO 140/RANTES IC_{90} ratio
	PRO 140	RANTES	
JR-FL	11	12	0.92
SF162	100	29	3.4
JR-CSF	19	9.7	2.0
92US714	17	52	0.33
93US075	12	6.8	1.8
92US657	3.4	46	0.074
Case C 1/85	28	37	0.76
WWD	23	13	1.8
RDM	14	12	1.2
Median	17 (2.5μg/ml)	13 (100ng/ml)	1.2

fourfold from the median value of 1.3. Such differences are within the limit of assay error.

Several syncytium-inducing subtype B viruses were included in these analyses. The panel included four R5X4 viruses (HIV-$1_{92US076}$, HIV-$1_{2076\ clone\ 3}$, HIV-$1_{Case\ C\ 7/86}$, and HIV-1_{DH123}) and one X4 virus (HIV-1_{HC4}). When used at concentrations ranging to 25 and 0.5 μg/ml, respectively, neither PRO 140 nor RANTES measurably inhibited or enhanced the replication of these CXCR4-using subtype B viruses in PBMC cultures (data not shown).

Inhibition of South African subtype C viruses. In addition to the 2 subtype C viruses described in Table 2, 27 subtype C viruses from South African patients with acute or advanced infection were tested for sensitivity to PRO 140, RANTES, and TAK-779. Each compound was tested at two concentrations: 33 and 167 nM for PRO 140, 13 and 64 nM for RANTES, and 1,000 and 5,000 nM for TAK-779. Each agent mediated >90% inhibition of 24 of 25 R5 test isolates when used at the higher concentration and had considerable potency at the lower concentration (Table 3). One R5 virus (HIV-1_{COT6}) from a 2-year-old child with tuberculosis appeared to be partially resistant to each of the CCR5-targeting agents. Notably, all eight viruses from acutely infected individuals were fully sensitive to the inhibitors.

Interestingly, one of two R5X4 viruses was >90% inhibited by PRO 140 and RANTES. The second subtype C R5X4 virus was not effectively inhibited by any of the CCR5-targeting agents under these conditions (Table 3). Both of the R5X4 subtype C viruses (HIV-1_{Du179} and HIV-1_{CM9}) replicate efficiently on U87.CD4-CXCR4 cells and form syncytia in MT-2 cell cultures. Other viruses (HIV-1_{PCP1} and HIV-1_{TB5}) that were capable of sustaining only low-level replication (75 to 600 pg of p24/ml in day 8 supernatants) on U87.CD4-CXCR4 cells were classified as R5 viruses (data not shown).

TABLE 2. Inhibition of R5 viruses from subtypes A, C, E, and F by PRO 140 and RANTES

Virus	Genetic subtype	Median IC_{90} (nM) for:		PRO 140/RANTES IC_{90} ratio
		PRO 140	RANTES	
92RW026	A	15	12	1.3
DJ258	A	28	11	2.5
DJ259	C	6.5	12	0.54
94ZW103	C	17	11	1.5
CM235	E	14	12	1.2
92TH001	E	13	11	1.2
BZ162	F	87	19	4.6
R1	F	5.2	9.6	0.54
Median		15 (2.2 μg/ml)	12 (94 ng/ml)	1.3

Inhibition of HIV-1 replication in macrophages. PRO 140 and RANTES were also tested for their ability to block HIV-1 replication in cultures of freshly isolated macrophages. Figure 2 depicts comparative inhibition data obtained for HIV-1_{JR-FL} and HIV-1_{SF162} in macrophage and PBMC cultures. In total, two subtype A and four subtype B isolates were analyzed in three to five replicate assays. PRO 140 effectively inhibited all six test isolates (Table 4). The overall median IC_{90} value was 110 nM, sixfold higher than the median IC_{90} value (18 nM) observed for the same six isolates in PBMC cultures (Tables 1 and 2). One isolate, HIV-1_{SF162}, was fourfold more sensitive to PRO 140 when assayed on macrophages than when assayed on PBMCs (Fig. 2 and Table 1). Of note is that HIV-1_{SF162} is unusually insensitive to PRO 140 and RANTES in PBMC cultures.

In contrast to PRO 140, RANTES was a poor inhibitor of HIV-1 replication in macrophages (Fig. 2 and Table 4). When used at concentrations ranging to 64 nM (0.5 μg/ml), RANTES failed to mediate 90% inhibition of any test isolate, and only three of six viruses were inhibited by 50%. However, RANTES potently blocked replication of the same viruses in PBMC cultures, indicating that the variable was the target cell, not the virus or inhibitory agent (Tables 1 and 2).

Since exogenous and cell surface proteoglycans have been reported to modulate the anti-HIV-1 activity of CC-chemokines (2, 13, 53, 72), additional experiments examined the antiviral effects of heparan sulfate and chondroitin sulfate used alone and in combination with RANTES and PRO 140. Used alone at concentrations ranging to 0.5 μg/ml, the proteoglycans demonstrated weak, sporadic antiviral effects. Thus, IC_{50} values of ~0.45 μg/ml were observed for heparan sulfate against HIV-1_{JR-FL} and HIV-1_{SF162}, but the other four isolates were not significantly inhibited by heparan sulfate. Chondroitin sulfate was tested against isolates HIV-1_{JR-FL} and HIV-1_{JR-CSF} and had no measurable activity against either. When used in combination with PRO 140 and RANTES, 0.5 μg of heparan sulfate/ml exerted a modest additive antiviral effect: PRO 140's median IC_{90} was reduced by half and RANTES now exhibited a measurable IC_{90} (46 nM or 0.36 μg/ml) against one of the six viruses (HIV-1_{JR-FL}). The antiviral activities of PRO 140 and RANTES were unaffected by chondroitin sulfate. Hence the addition of exogenous proteoglycans did not substantially potentiate the antiviral activity of either RANTES or PRO 140 in macrophage cultures.

Comparison of Env sequences and viral sensitivity to PRO 140 and RANTES. Amino acid sequences of the various test isolates were compared in an effort to identify the molecular basis for the variation in viral sensitivity to PRO 140 and RANTES. Results for the V3 loop are listed in Table 5. Amino acid position 319 near the crown of the V3 loop has been implicated in viral sensitivity to AOP-RANTES, with 319A viruses demonstrating the greatest sensitivity (64). A weak, opposite correlation was seen for PRO 140 in PBMC cultures, with 319A viruses showing somewhat diminished sensitivity

TABLE 3. Inhibition of subtype C viruses

Virus	Phenotype	% Inhibition of viral replication[a] by:					
		PRO 140 at:		TAK-779 at:		RANTES at:	
		167 nM	33 nM	5,000 nM	1,000 nM	64 nM	13 nM
Du104	R5	100.0	99.2	100.0	100.0	100.0	97.5
Du151	R5	99.3	99.3	97.4	91.4	99.1	98.1
Du156	R5	100.0	97.7	100.0	100.0	100.0	93.6
Du174	R5	100.0	100.0	99.9	99.1	99.9	100.0
Du204	R5	100.0	100.0	99.9	97.6	100.0	97.9
Du301	R5	100.0	100.0	100.0	100.0	98.2	100.0
Du368	R5	98.2	91.8	94.0	92.0	97.3	90.5
Du422	R5	100.0	100.0	99.2	100.0	99.2	99.1
CM1	R5	92.5	**40.9**	**86.8**	95.5	91.7	95.2
CM4	R5	100.0	**69.9**	99.4	99.6	100.0	99.5
CM7	R5	99.0	92.9	95.1	97.4	99.2	98.0
PCP1	R5	100.0	99.7	94.4	**10.1**	100.0	**88.9**
TB1	R5	100.0	97.6	100.0	97.6	100.0	100.0
TB2	R5	100.0	99.8	100.0	99.3	100.0	100.0
TB3	R5	94.2	100.0	100.0	100.0	100.0	99.8
TB5	R5	100.0	**89.4**	100.0	**73.0**	100.0	93.8
TB6	R5	100.0	100.0	100.0	100.0	100.0	100.0
TB7	R5	100.0	100.0	100.0	100.0	100.0	99.7
TB8	R5	100.0	96.6	100.0	100.0	100.0	96.1
TB9	R5	100.0	100.0	98.7	91.4	98.8	99.7
TB10	R5	100.0	100.0	100.0	100.0	100.0	100.0
COT1	R5	100.0	100.0	100.0	100.0	100.0	100.0
COT2	R5	100.0	98.6	100.0	98.5	100.0	100.0
COT6	R5	**82.4**	**81.2**	100.0	**32.9**	**88.2**	100.0
COT9	R5	99.4	90.5	100.0	**87.3**	100.0	98.6
Du179	R5X4	92.1	**47.2**	**80.4**	**84.6**	99.8	**58.8**
CM9	R5X4	**0.0**	**0.0**	**0.0**	**8.0**	**4.1**	**48.2**

[a] Inhibition levels of <90% are in boldface. Numbers of R5X4 (out of 2) and R5 (out of 25) viruses inhibited by >90% are, respectively, as follows: PRO 140 at 167 nM, 1 and 24; PRO 140 at 33 nM, 0 and 21; TAK-779 at 5,000 nM, 0 and 24; TAK-779 at 1,000 nM, 0 and 21; RANTES at 64 nM, 1 and 24; RANTES at 13 nM, 0 and 24.

(median IC_{90} = 22 nM) compared to 319T and 319R viruses (median IC_{90} = 13 nM). The differences between 319A and other viruses did not reach statistical significance ($P = 0.13$ in a two-sided Student t test). A similar trend was observed for PRO 140's activity in macrophage cultures (median IC_{90} = 110 nM for 319A viruses and 62 nM for other viruses; $P = 0.44$). RANTES activity in PBMC cultures was unaffected by the amino acid at position 319 (median IC_{90} = 22 nM for 319A viruses and 18 nM for other viruses; $P = 0.73$). RANTES was not sufficiently active in macrophage cultures to make a similar comparison. As indicated in Table 5, there was no obvious correlation between viral sensitivity and either the length or net charge of the V3 loop. No obvious correlation could be established upon sequence analysis of other regions of Env, including those that have been implicated in binding CCR5 (57, 58).

DISCUSSION

The principal conclusion of this study is that PRO 140, a weak CCR5 antagonist, potently blocks the replication of a broad range of primary HIV-1 isolates on both primary T cells and macrophages, which represent the two chief targets for HIV-1 infection in vivo. Interestingly, PRO 140 was markedly more active than RANTES in blocking HIV-1 in macrophage cultures. This study is the first to demonstrate such broad-spectrum inhibition in the absence of CCR5 downregulation and suggests that genotypically diverse R5 viruses utilize a highly restricted set of epitopes on CCR5. In another novel finding, PRO 140 and RANTES were shown to inhibit certain R5X4 viruses. These findings have important and positive implications for CCR5-targeted therapy of HIV-1 infection.

In infectivity assays employing PBMCs and HIV-1 viruses representing subtypes A to C, E, and F, PRO 140 inhibited 17 of 17 R5 viruses with a median IC_{90} value of ~15 nM (~2 μg/ml). PRO 140 and small-molecule CCR5 antagonist TAK-779 were also tested against a larger panel of subtype C viruses, which presently account for half of all new infections worldwide (23). Both PRO 140 and TAK-779 were broadly inhibitory, although ~30-fold-higher concentrations of TAK-779 were required to achieve the same level of inhibition. PRO 140's inhibitory activity is therefore independent of the genetic subtype of the virus.

In PBMC cultures, PRO 140's breadth of antiviral activity was similar to that of RANTES. Moreover, viruses tended to have similar relative sensitivities to PRO 140 and RANTES, with the observed differences largely falling within the range of experimental error. Despite the occasional exception, RANTES antiviral activity correlated more closely with that of PRO 140 than that of either MIP-1α or MIP-1β, which are not universally active against R5 viruses (69). It was unexpected that PRO 140, a weak CCR5 antagonist, would possess breadth and potency of antiviral activity as great as or greater than those of RANTES, an agonist capable of downregulating CCR5. However, RANTES can exert both inhibitory and en-

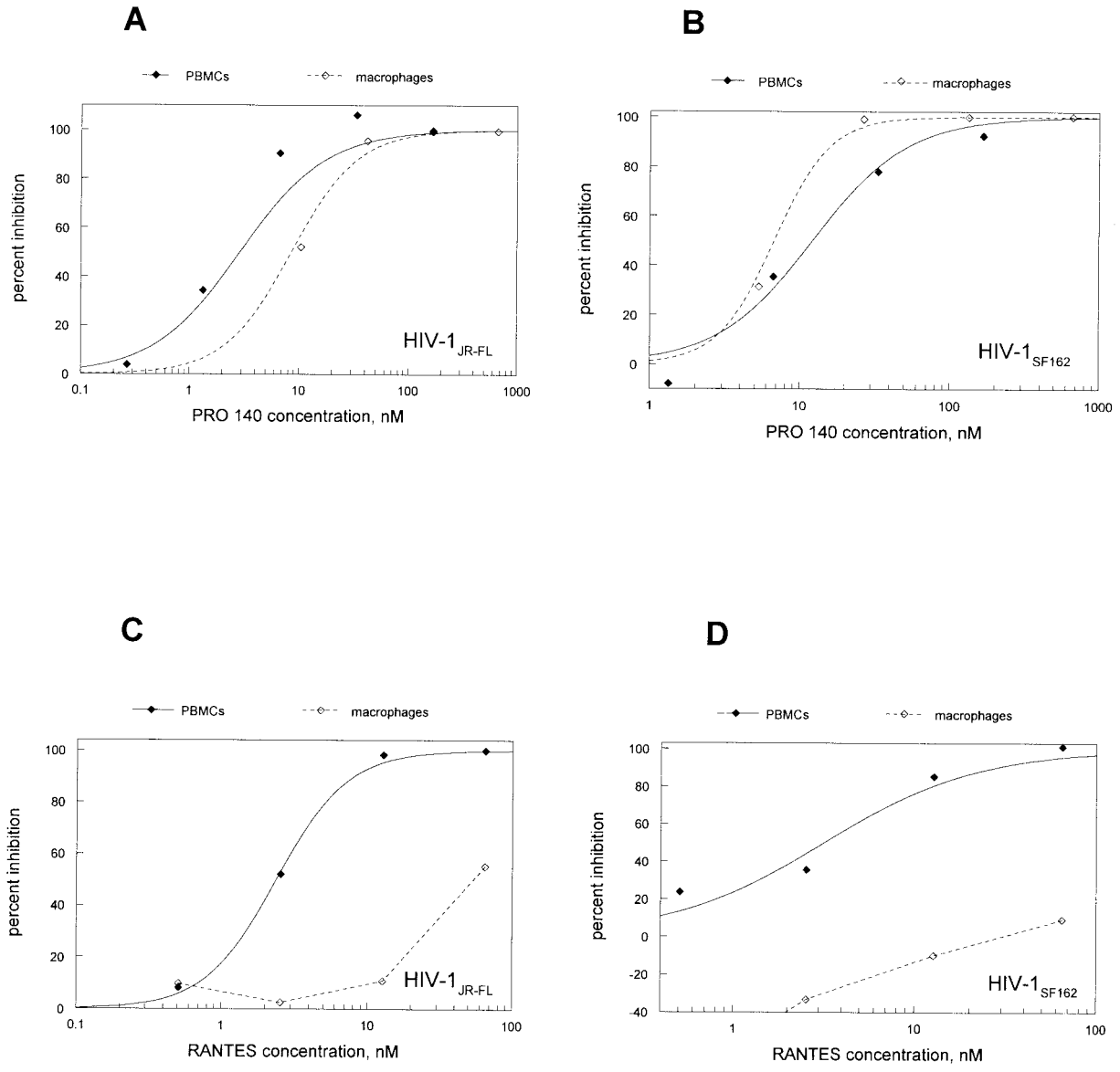

FIG. 2. Inhibition of HIV-1 replication in PBMC and macrophage cultures. The extent of R5 virus replication was assessed by measuring the p24 antigen content of 7-day culture supernatants. The inhibition curves depict data from a representative assay. HIV-1_{JR-FL} and HIV-1_{SF162} are subtype B viruses.

hancing effects on HIV-1 replication. The enhancing effects, which involve signal transduction events mediated through glycosaminoglycans (66), may diminish RANTES's overall inhibitory activity. There is no evidence, or reason to expect, that PRO 140 is capable of interacting with glycosaminoglycans or acting through this mechanism. In addition, PRO 140's epitope on CCR5 may more closely overlap the site used by HIV-1.

Although PRO 140 and RANTES possessed comparably

TABLE 4. Inhibition of HIV-1 replication in macrophage cultures

Virus	Genetic subtype	PRO 140		RANTES	
		IC_{90} (nM)	IC_{50} (nM)	IC_{90} (nM)	IC_{50} (nM)
92RW026	A	120	14	>64	>64
DJ258	A	120	15	>64	>64
JR-FL	B	100	16	>64	58
SF162	B	25	16	>64	22
JR-CSF	B	23	<5.3	>64	58
92US714	B	160	42	>64	>64
Median		110 (16 μg/ml)	16 (2.4 μg/ml)	>64 (>500 ng/ml)	>61 (>480 ng/ml)

TABLE 5. V3 loop sequences of test isolates

Virus	Subtype	Coreceptor usage	IC_{90} (nM)[a] for:		V3		
			PRO 140	RANTES	Length (aa)[c]	Net charge	Sequence[b]
92US657	B	R5	3.4	46	35	+6	CTRPNNNTRKGIHI–GPGRAFYTTGEVIGNIRQAHC
DJ259	C	R5	6.5	6.5	35	+2	CTRPNNNTRESIRI–GPGQTFY**A**TGDIIGDIRQAHC
JR-FL	B	R5	11	12	35	+5	CTRPNNNTRKSIHI–GPGRAFYTTGEIIGDIRQAHC
92TH001	E	R5	13	11	35	+3	CTRPSNNTRTSINI–GPGQVFYRTGDIIGDIRKAYC
CM235	E	R5	14	12	35	+3	CTRPSNNTRTSIPI–GPGQAFYRTGDIIGDIRKAYC
92RW026	A	R5	15	12	35	+5	CTRPNNNTRRSIRI–GPGQAFY**A**TGDIIGNIRQAHC
92US714	B	R5	17	52	35	+5	CIRPNNNTRRSIHM–GPGRAFY**A**TGDIIGDIRQAHC
JR-CSF	B	R5	19	9.7	35	+5	CTRPSNNTRKSIHI–GPGRAFYTTGEIIGDIRQAHC
DJ258	A	R5	28	11	35	+4	CSRPGNNTRKSVRI–GPGQTFY**A**TGDIIGDIRQAHC
BZ162	F	R5	87	19	35	+6	CTRPNNNTRKSIHI–GPGRALY**A**TGDIIGDIRKAHC
SF162	B	R5	100	29	35	+4	CTRPNNNTRKSITI–GPGRAFY**A**TGDIIGDIRQAHC
Du179	C	R5X4	~100	~50	34	+6	CTRPGNNTRKSIRI–GPGQAFYTNH-IIGDIRQAHC
CM9	C	R5X4	≫100	≫50	35	+6	CARPGNNTIKRIRI–GPRYAFY**A**KETIIGDIRQAHC
HxB2	B	X4	Not done	Not done	36	+10	CTRPNNNTRKRIRIQRGPGRAFVTIGKI-GNMRQAHC

[a] IC_{90} for inhibition of viral replication in PBMC cultures.
[b] Position 319 (boldface) has been implicated in sensitivity to AOP-RANTES.
[c] aa, amino acids.

broad-based antiviral activity in PBMC cultures, only PRO 140 was effective in macrophage cultures. Thus, PRO 140 was nearly equipotent at inhibiting HIV-1 replication in primary T cells and macrophages, the two principal cellular targets for virus infection in vivo. In contrast, when tested against the same virus isolates, RANTES was far less effective in blocking macrophage infection. Cell type-dependent differences in the antiviral activities of SDF-1α and CXCR4 antagonists have also been reported (43).

From the initial observation that CCR5 functions as a fusion coreceptor for what were then known as macrophagetropic HIV-1 isolates, it was recognized that CC-chemokines were relatively weak inhibitors of macrophage infection (3, 20, 48). Subsequently, there have been conflicting reports on the anti-HIV-1 activity of CC-chemokines in macrophage cultures; the discrepant findings have been attributed to variations in cell donors, isolation conditions, culture conditions, CC-chemokine preparations, and proteoglycan levels (2, 13, 55, 71, 72). In the present study, the activity of RANTES was not potentiated by exogenous proteoglycans, so the explanation of RANTES's impotence lies elsewhere. Cellular activation by CC-chemokines undoubtedly adds an additional level of complexity (28, 35, 66), particularly since antagonistic CC-chemokine analogs are often more potent antivirals than the unmodified agonists in macrophage cultures (3, 60, 77). Like certain CC-chemokine analogs, PRO 140 is unaffected by the factors that limit RANTES's ability to block HIV-1 replication in macrophages.

Regarding the dichotomy in the activities of PRO 140 and RANTES in macrophage cultures, it may be important to note that CCR5 is expressed on the cell surface in multiple conformational states (37) characterized by different sulfation or other posttranslational modifications (16, 25), self-multimerization (9), association with CD4 or other cell surface proteins (75), or interaction with cytoplasmic G proteins. Since CCR5's activities as an HIV-1 coreceptor and chemokine receptor are clearly dissociable (5, 24, 29), HIV-1, RANTES, and PRO 140 may vary in the ability to recognize the various CCR5 conformations. Our inhibition data are consistent with the notion that RANTES, PRO 140, and HIV-1 recognize similar spectra of the CCR5 conformations present on PBMCs but that PRO 140 and HIV-1 recognize a broader range of macrophage CCR5 conformations. Ongoing studies in our laboratories are exploring whether HIV-1 and PRO 140 may recognize macrophage CCR5 more broadly or effectively than does RANTES.

Although there were measurable differences in PRO 140's potency against R5 viruses, the differences were relatively minor and in some cases anticipated. For example, $HIV\text{-}1_{SF162}$, the R5 virus that was least sensitive to PRO 140 in PBMC cultures, is also relatively insensitive to RANTES and completely insensitive to MIP-1α and MIP-1β (69). The presence of an alanine at position 319 of the V3 loop has been implicated in conferring increased viral susceptibility to a RANTES derivative in PBMC cultures (64), but we could not confirm this result for either PRO 140 or RANTES. For PRO 140, a weak, opposite trend was observed in both PBMC and macrophage cultures, with A319 viruses demonstrating somewhat decreased susceptibility to PRO 140. The mechanisms whereby viruses become refractory to inhibition by CCR5-targeting agents are being explored in our laboratories in ongoing studies that seek to generate PRO 140- and RANTES-resistant viruses in vitro.

Interestingly, $HIV\text{-}1_{SF162}$ was unusually sensitive to PRO 140 in macrophage cultures, and thus it is unclear whether or not this virus would display unusual sensitivity to PRO 140 in the context of natural infection. Notably, replication of all R5 viruses on PBMCs and macrophages was effectively (>90%) inhibited by PRO 140 at concentrations that are readily achievable in vivo, suggesting that interisolate variations in viral sensitivity could be largely obviated via adequate dosing in therapeutic settings.

Our findings support the notion that wild-type R5 viruses use a highly restricted set of epitopes that overlap the PRO 140 binding site, which includes elements of the N terminus and the second extracellular loop (ECL2) of CCR5 (51). The restricted pattern of CCR5 usage was first suggested by alanine scanning mutagenesis studies, which demonstrated that CCR5's fusogenic activity mapped to a limited number of charged, polar, and hydrophobic amino acids within the N-terminal and ECL2

regions (21, 25, 26, 56). It may also be that PRO 140 can sterically block HIV-1's access to regions outside the PRO 140 epitope. This possibility is not excluded by the observation that RANTES is able to bind to CCR5 and induce signaling in the presence of HIV-1-inhibitory concentrations of PRO 140 (51). Clearly, a native, oligomeric HIV-1 envelope glycoprotein on the surface of a virion may be more susceptible to steric inhibition than an 8-kDa chemokine protein.

Despite the shared sensitivity of R5 viruses to CCR5-targeting agents, there may be subtler intersubtype differences in CCR5 usage. For reasons that are not understood at present, CCR5 is the predominant coreceptor used by subtype C viruses at all stages of infection, whereas CXCR4 usage, although not unknown, is relatively uncommon (1, 12, 54, 70). Our subtype C panel included two R5X4 viruses, one of which was potently inhibited by PRO 140 and RANTES. To our knowledge, this is the first reported inhibition of R5X4 virus replication on normal PBMCs by a CCR5-targeting agent. HIV-1_{Du179}, the susceptible R5X4 virus, met rather strict criteria for CXCR4 usage, including robust replication on U87.CD4-CXCR4 cells and the ability to form syncytia in MT-2 cultures. Interestingly, this virus possesses a somewhat atypical GPGQAFYTNHII motif at the crown of the V3 loop (Table 5), but further studies are needed to understand the correlates of R5X4 virus susceptibility to CCR5-targeting agents. In contrast, replication of prototypic subtype B R5X4 viruses on PBMCs was insensitive to inhibition by these agents, which are nonetheless capable of blocking CCR5-mediated entry of such viruses into engineered cell lines expressing CD4 and CCR5 but not CXCR4 (51).

Thus, although subtype C viruses can clearly adapt to use CXCR4 efficiently, the adaptation may occur less readily; and the nominally dualtropic viruses may retain a bias for CCR5 usage, at least during the early stages of adaptation. Moreover, the ability of a dualtropic isolate to use CXCR4 on a transfected cell does not necessarily mean that it can use CXCR4 on a primary cell (27, 78). In contrast, prototypic subtype B R5X4 isolates generally show more-balanced coreceptor usage or even demonstrate a bias in favor of CXCR4, as suggested by the observation that SDF-1α, the natural ligand for CXCR4, can suppress the replication of some subtype B R5X4 viruses in PBMC (69). One possible implication of these observations is that CCR5-targeting agents might be particularly effective against the increasingly prevalent subtype C viruses. These concepts can be tested by examining additional R5X4 viruses and by examining the development of PRO 140-resistant viruses in in vitro or in vivo models of HIV-1 infection.

There was no evidence for enhancement of R5X4 or X4 virus replication on PMBCs in the present study, which employed RANTES concentrations (<0.5 μg/ml) lower than those required for enhancement (28, 35, 66). That is, CXCR4-using viruses were either inhibited or unaffected by PRO 140 and RANTES. Recently, CC-chemokines and other anti-CCR5 antibodies have been reported to enhance syncytium formation between macrophages and cells expressing recombinant envelope glycoproteins derived from X4 viruses (38), perhaps by decreasing CCR5-CD4 interactions and thereby increasing CXCR4-CD4 interactions. On the other hand, in studies employing macrophages derived from individuals who do not express CCR5 (Δ32 homozygotes), lack of CCR5-CD4 interactions did not enhance the entry of CXCR4-using viruses (76), arguing against a role for coreceptor competition for CD4 in viral entry. The effect of PRO 140 on the replication of CXCR4-using viruses in macrophage cultures is being explored in ongoing studies.

In summary, PRO 140 potently protected the major cellular targets of HIV-1 from in vitro infection by a diverse panel of R5 viruses. The data support the notion that HIV-1 uses a restricted set of epitopes on CCR5 and that these epitopes are similarly presented on T cells and macrophages. In addition, the demonstration of potent cross-subtype activity further supports the development of CCR5-targeting molecules as a new class of antiretroviral agents. PRO 140 therefore is a promising, prototypic coreceptor-targeting agent that warrants further testing in preclinical models of HIV-1 infection.

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