Sensitivity of primary R5 HIV-1 to inhibition by RANTES correlates with sensitivity to small-molecule R5 inhibitors

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Introduction

HIV-1 uses chemokine receptors in addition to the principal receptor CD4 for cellular entry. The two major coreceptors for HIV-1 are CCR5 and CXCR4 [1,2]. Generally, HIV-1 infection is established by macrophagotropic, CCR5-using (R5) variants. Approximately 50% of infected individuals progress to AIDS after the development of CXCR4-using (X4) HIV-1 variants [3], whereas the rest progress in the presence of R5 variants only [4].

The natural ligands for CCR5, the β-chemokines RANTES, MIP-1α and MIP-1β, down-modulate CCR5 expression and inhibit HIV-1 CCR5 usage [5–7]. Different reports have suggested a role for β-chemokines in AIDS pathogenesis and evolution of HIV-1 in vivo [8–11]. In a previous study, we reported a decreasing sensitivity to RANTES of HIV-1 variants obtained longitudinally in the course of infection in individuals who progressed to AIDS in the presence of R5 HIV-1 variants only [12]. In analogy, we have also reported a decreasing sensitivity to inhibition by CXCR4 antagonist AMD3100 of late-stage X4 HIV-1 variants as compared with X4 HIV-1 variants obtained earlier from the same individuals [13]. These observations imply an ongoing evolution of HIV-1 variants and may have implications for the therapeutic application of HIV-1 entry inhibitors.

HIV-1 entry inhibitors are a new class of HIV-1 inhibitors that target the attachment and subsequent fusion of the virus to the target cell. CCR5 and CXCR4 coreceptor antagonists, which specifically inhibit R5 or X4 HIV-1 variants, respectively, are being considered for therapeutic application [14]. At present, it is unclear how HIV-1 sensitivity profiles for synthetic R5 antagonists compare with those obtained with natural ligands. If sensitivity of primary HIV-1 variants to R5 inhibitors greatly differed between patients, this could have implications for the efficacy of application of CCR5 antagonists in antiretroviral therapy. Therefore, we studied a possible correlation between the sensitivity of primary R5 HIV-1 variants, isolated from different patients, to RANTES and two small-molecule R5 inhibitors, AD101 (SCH 350581), a compound chemically related to SCH-C [15], and TAK-779 [16].

Materials and methods

Subjects

Eighteen participants of the Amsterdam Cohort Study (ACS) on AIDS in Homosexual Men who harboured only R5 HIV-1 variants during their follow-up were selected. Seven of these individuals were classified as
long-term survivors, with an asymptomatic follow-up of at least 11 years in the absence of antiretroviral therapy, and eleven were classified as progressors with AIDS diagnoses after 25–136 months of follow-up [4]. Seven participants of the ACS who developed X4 HIV-1 variants during a progressive disease course were selected. None of the individuals studied received antiretroviral therapy at or before the moment of sampling.

Virus isolation, X4 phenotyping and characterization of coreceptor usage

Biological virus clones were obtained by co-cultivation of patients’ peripheral blood mononuclear cells (PBMCs) from 10 R5 inhibition assay

Reduction in p24 antigen production (IC50 and IC90) was determined by a four-parametric logistical analysis [17]. If the appropriate degree of inhibition was not achieved at the highest inhibitory concentration used, a value ‘greater than’ (>) was recorded and we assumed these to be equal to 250 ng/ml for RANTES, 25 M for AD101 and 100 nM for TAK-779 in figures and statistical tests, with the exception of analyses of correlations with TAK-779 sensitivity. In those cases, IC values of viruses insensitive to TAK-779 inhibition were omitted (‘greater than’ values were left out).

Spearman’s correlation coefficient was calculated for correlations and the Mann–Whitney U test was used to compare IC values of patient groups. Statistical analyses were performed using SPSS v10.0 (SPSS, Inc, Chicago, IL, USA).

**Results**

Potency of R5 inhibitors RANTES, AD101 and TAK-779

We simultaneously determined the in vitro sensitivity of 144 primary R5 HIV-1 variants, isolated from 25 participants of the Amsterdam Cohort of Homosexual Men, to inhibition by the natural CCR5 ligand RANTES and the small molecule R5 inhibitors AD101 and TAK-779. To avoid bias due to different numbers of variants from the different patients the average IC50 shown). In 96-well plates, 10^5 cells per well were inoculated with 40 TCID50 of a virus isolate, with an end volume of 200 µl per well, and cultured at 37°C. At day 7, supernatant was removed and cells were resuspended in fresh medium. The production of p24 was measured in supernatant samples taken 14 days after infection using an in-house p24 antigen capture enzyme-linked immunosorbent assay (ELISA). Antigen levels of p24 from cultures inoculated in the absence of inhibitors were designated as ‘maximum virus production’ and the ratios of p24 production in inhibitor-containing cultures were calculated relative to these maximum values. Supernatant from cells that were not incubated with either inhibitor or virus was used for background values of the p24 ELISA. All measurements were performed in triplicate. As a control, 12 virus variants with varying sensitivity for RANTES, AD101 and TAK-779 were tested for sensitivity to zidovudine (AZT), an inhibitor of reverse transcription. No correlation between sensitivity to AZT and R5 entry inhibitors was observed (data not shown), indicating that differences in CCR5 antagonist sensitivity were indeed determined at the level of viral entry.

**Determination of 50% and 90% inhibitory concentrations and statistical analyses**

The inhibitory concentrations causing 50% and 90% reduction in p24 antigen production (IC50 and IC90) 14 days after infection were determined by a four-parametric logistical analysis [17]. If the appropriate degree of inhibition was not achieved at the highest inhibitory concentration used, a value ‘greater than’ (>) was recorded and we assumed these to be equal to 250 ng/ml for RANTES, 25 M for AD101 and 100 nM for TAK-779 in figures and statistical tests, with the exception of analyses of correlations with TAK-779 sensitivity. In those cases, IC values of viruses insensitive to TAK-779 inhibition were omitted (‘greater than’ values were left out).

Spearman’s correlation coefficient was calculated for correlations and the Mann–Whitney U test was used to compare IC values of patient groups. Statistical analyses were performed using SPSS v10.0 (SPSS, Inc, Chicago, IL, USA).
and IC\textsubscript{90} values per patient were used. When an IC value ‘greater than’ the highest concentration used was determined, the IC was assumed to be equal to the highest concentration for use in figures and statistical tests, except when indicated otherwise.

RANTES and AD101 were both potent inhibitors of \textit{in vitro} replication of primary R5 HIV-1 (RANTES: n=23, median IC\textsubscript{50}=13.9 nM, interquartile range (IQR) 7.0–17.7 nM; AD101: n=23, median IC\textsubscript{50}=15.8 nM, IQR 10.0–20.7 nM), whereas TAK-779 barely inhibited the replication of the variants even at 100 nM, the highest concentration tested (n=21, median IC\textsubscript{50}=>100 nM, IQR 97.8 to >100 nM; Figure 1).

Correlation of sensitivity to R5 inhibitors

A significant correlation was found between the average IC\textsubscript{50} values for R5 variants per patient for RANTES and AD101 (n=21, r=0.642, P<0.05; Figure 2A, left panel). Exclusion of the single outlier from this analysis did not influence this result (data not shown). No significant correlations were found, with average IC\textsubscript{50} values per patient for TAK-779 and either of the other two R5 inhibitors, possibly because virus variants of most individuals were rather insensitive to TAK-779 inhibition.

When individual IC\textsubscript{50} values of each R5 virus variant were used to determine possible correlations, we again found a strong correlation between the sensitivity to RANTES and AD101 (n=96, r=0.610, P<0.001; Figure 2A, right panel; IC\textsubscript{50} n=99, r=0.447, P<0.001; data not shown) and also found that the sensitivity to TAK-779 correlated with the sensitivity to both RANTES (n=29, r=0.499, P<0.05; Figure 2B) and AD101 (n=30, r=0.543, P<0.05; Figure 2C). R5 variants that had IC\textsubscript{50} values of >100 nM TAK-779 were considered insensitive to TAK-779 and were excluded from correlation analysis.

Sensitivity to RANTES and AD101 of sequentially obtained R5 variants

We have previously reported that, in the course of progressive disease in the presence of only R5 variants, a decreasing sensitivity of R5 variants to RANTES may occur [12]. Here, we wanted to establish whether the observed correlation between sensitivity to RANTES and AD101 would also apply to longitudinally obtained R5 HIV-1 isolates with decreasing sensitivity to inhibition by RANTES. For comparison, we also tested two individuals’ sequentially obtained R5 HIV variants that had unchanged sensitivity to RANTES inhibition. In Figure 3, the inhibition curves for RANTES and AD101 of sequential R5 virus variants with either a previously established decreasing or unchanged RANTES sensitivity over time are shown. Each dot represents the average inhibition of one to five R5 virus variants obtained at a single time point from each patient.

From patients ACH38 and ACH142, who progressed to AIDS after 101 and 109 months of follow-up, respectively, the late virus variants, obtained 102 and 93 months after start of follow-up, respectively, were less sensitive to inhibition by RANTES than the early variants that were obtained 21 months after start of follow-up of both patients [mean IC\textsubscript{50} values per time point (±standard deviation): ACH38 early 2.9 (±0.8) nM vs late 9.2 (±1.2) nM and ACH142 early 9.0 nM vs late 25.7 (±4.2) nM]. In agreement with the observed correlation between sensitivity to RANTES and AD101, we also observed a lower sensitivity to AD101-mediated inhibition of late variants as compared with early variants from these patients [mean IC\textsubscript{50} values per time point: ACH38 early 1.0 (±0.3) nM vs late 14.7 (±2.1) nM and ACH142 early 2.5 nM vs late 11.0 (±0.2) nM].

However, in patients ACH78 and ACH434, who were classified as long-term survivors with asymptomatic follow-ups of 124 and 140 months, respectively, no difference in sensitivity to either RANTES or AD101 was observed between variants obtained early: 17 and 16 months after start of follow-up, respectively, and variants obtained late: 115 and 119 months after start of follow-up, respectively [RANTES mean IC\textsubscript{50} values per time point: ACH78 early 9.1 (±2.6) nM vs late 9.5 (±1.9) nM and ACH434 early 3.0 (±1.7) nM vs late 5.1 (±3.6) nM; AD101 mean IC\textsubscript{50} values per time point: ACH78 early 4.5 (±1.1) nM vs late 4.7 (±2.7) nM and...
ACH434 early 0.9 (±1.7) nM vs late 1.5 (±1.6) nM). These observations are in agreement with our previous observations [12] and underscore the variability in virus phenotype evolution between patients.

R5 sensitivity to RANTES and AD101 in individuals who develop X4 variants

It is still unclear why X4 variants evolve in some patients and not in others. To study if differences in CCR5 coreceptor usage, as reflected by variation in RANTES- and AD101-mediated inhibition of virus replication, may evolve before the first appearance of X4 variants, we studied seven individuals who developed X4 variants during follow-up. R5 variants obtained prior to the first appearance of X4 variants were compared with R5 variants obtained from individuals in whom X4 variants could never be detected for their sensitivity to RANTES- and AD101-mediated inhibition. There was no difference in the time point of virus isolation between the groups.

No significant difference was observed for RANTES-mediated inhibition of replication of R5 variants isolated from individuals who did or did not develop X4 variants later during follow-up (n=7, median IC_{50}=8.2 nM, IQR 2.5–13.3 vs n=17, median IC_{50}=5.7 nM, IQR 4.4–7.8; Figure 4A). However, we did observe a significant but slightly lower sensitivity to AD101-mediated inhibition of replication of R5 variants isolated from individuals who later developed X4 variants (n=6, median IC_{50}=8.7 nM, IQR 6.0–19.2) compared with R5 variants obtained from individuals who did not develop X4 variants (n=17, median IC_{50}=2.9 nM, IQR 1.8–5.0, P<0.05 Mann–Whitney U test; Figure 4B).

Figure 2. Correlation of sensitivity to R5 HIV-1 entry inhibitors

(A) Correlation of sensitivity to RANTES and AD101. Left panel: mean IC_{50} values of R5 HIV-1 variants per patient for RANTES and AD101 are depicted. Right panel: IC_{50} values of all primary R5 HIV-1 variants tested are depicted. (B) Correlation of sensitivity to TAK-779 and RANTES. (C) Correlation of sensitivity to TAK-779 and AD101. IC_{50} values of primary R5 HIV-1 variants that are sensitive to TAK-779 inhibition are depicted. Both axes are in log_{e} scale.
**Figure 3. Sensitivity to RANTES and AD101 of early and late R5 HIV-1 variants**

Sensitivity of early and late R5 HIV-1 variants to RANTES (top row) and AD101 (bottom row) from four individuals are shown. Percent replication relative to control infection was calculated. Average values and standard errors of one to five R5 virus variants obtained early after study entry and two or three R5 virus variants obtained late after study entry are shown. X-axes are in log scale. Experiments were performed in triplicate.

**Figure 4. Sensitivity to RANTES and AD101 of R5 HIV-1 variants obtained from individuals who did or did not show the appearance of X4 variants**

Boxplots of the mean IC_{50} values of R5 HIV-1 variants obtained from 17 individuals who did not show the appearance of X4 variants during follow-up (R5 only) and seven individuals who did show the appearance of X4 variants later in follow-up (pre-X4) for (A) RANTES and (B) AD101, are depicted. Boxes indicate the median values falling between the 25th and 75th percentiles, the bars that extent from the boxes indicate the 10th and 90th percentiles. *P<0.05, Mann-Whitney U test.
The slightly lower sensitivity to AD101-mediated inhibition of R5 variants isolated from individuals who did develop X4 variants later in follow-up compared with R5 variants isolated from individuals who did not develop X4 variants during follow-up did not influence the overall correlation between sensitivity to RANTES and AD101. Indeed, when also analysed separately, a correlation was found for the sensitivity to AD101- and RANTES-mediated inhibition of R5 virus variants of individuals who later developed X4 variants (data not shown).

Conclusions

Our previous results, which showed a decreasing sensitivity to RANTES-mediated inhibition in the course of disease progression in the presence of R5 variants only [12], prompted us to study whether this observation could be extrapolated to other CCR5 antagonists. Here, we demonstrate a correlation between the sensitivity of primary R5 HIV-1 variants to the natural CCR5 ligand RANTES and the small-molecule R5 entry inhibitors AD101 and, although less evidently, TAK-779. The only weak correlations with TAK-779-mediated inhibition may be due to the low inhibitory effect of TAK-779. Replication of most R5 HIV-1 variants was barely inhibited by TAK-779, even at the highest concentration used in our protocol (100 nM).

The sensitivity of individual virus variants to entry inhibitors may be influenced by several features such as (co)receptor affinity, coreceptor binding site and fusion kinetics. The requirement of lower levels of CCR5 expression for infection may explain the observed correlation between sensitivity to RANTES and AD101, as previously indicated [18]. Within the infected individual, virus variants may evolve towards a more efficient CCR5 usage, possibly due to selection pressure by the presence of RANTES or competition for available target cells.

Alanine substitution studies of the CCR5 transmembrane domains have revealed that the binding sites of TAK-779 and AD101 overlap and are located within a cavity near the extracellular surface formed by CCR5 transmembrane helices 1, 2, 3 and 7 [19,20]. Tsamis et al. [20] proposed that the binding of the small-molecule antagonists to the transmembrane domain of CCR5 might induce a conformational change in the gp120 V3-binding site of CCR5, possibly at the CCR5 N-terminus. The envelope protein of HIV-1 would then be unable to bind to this new conformation of CCR5. This proposed mechanism may help to explain our finding that R5 variants obtained from individuals who developed X4 variants had a lower sensitivity to AD101 inhibition than R5 variants isolated from individuals who did not show the emergence of X4 variants, while there was no difference in the sensitivity to RANTES inhibition. These virus variants may be dependent on other CCR5 domains and therefore may show a atypical sensitivity to specific entry inhibitors. A variable dependence on the CCR5 N-terminus of different R5 HIV-1 isolates has been reported in a study using CCR5 chimeric receptors [21].

In analogy with the correlation between the sensitivity to RANTES and AD101, we show that a decreasing sensitivity to RANTES over time coincides with a decreasing sensitivity to AD101 in two individuals with progressive disease, while no evident changes in sensitivity were observed over time in two individuals classified as long-term survivors. These results illustrate that, in the course of progressive infection, R5 HIV-1 variants may evolve towards a phenotype that coincides with a reduced sensitivity to CCR5 antagonists, even in the absence of therapy with R5 inhibitors. New, potentially more potent small-molecule R5 entry inhibitors are being developed. It is not very likely that the emergence of less sensitive R5 HIV-1 variants in the natural course of infection in the absence of X4 variants, will impact on the therapeutic benefit of these CCR5 inhibitors in patients, as reported plasma concentrations of AD101 exceed the in vitro IC₉₀ values of most R5 variants studied here (J Strizki, personal communications). Further characterization of viral and host determinants of entry inhibitor sensitivity may improve the design of optimal strategies for clinical application of entry inhibitors.

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