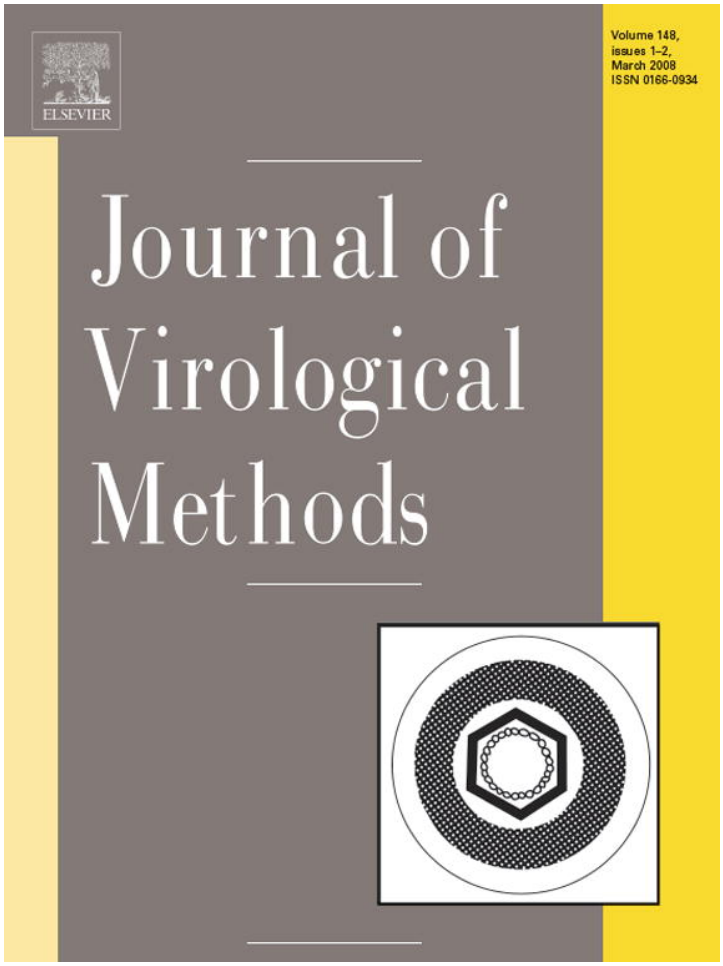

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Antiviral compounds show enhanced activity in HIV-1 single cycle pseudovirus assays as compared to classical PBMC assays

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Abstract

HIV-1 Env pseudotyped viruses (PV) are an attractive tool for studying the antiviral activities of compounds interfering with virus entry into a target cell. To investigate whether results obtained in PV assays are relevant biologically, the antiviral activity of 6 reference compounds was compared on 5 virus isolates of different clades using three assays: (1) replicating virus in peripheral blood mononuclear cells (PBMCs), (2) PV in CD4 and CCR5- or CXCR4 co-receptor expressing Ghost cells, and (3) PV in PBMCs. A significant linear relationship was found between both single-cycle PV assays ($P < 0.0001$, $R^2 = 0.75$). Moreover, both assays showed enhanced sensitivity to the antiretrovirals tested ($P = 0.013$ and 0.015, respectively) as compared to the PBMC assay with replication-competent virus. Most importantly, results from the latter assay could be predicted significantly from both PV assays, in which either Ghost target cells ($P < 0.0001$, $R^2 = 0.61$) or PBMCs ($P < 0.0001$, $R^2 = 0.55$) were used. The usefulness of the PV assay was demonstrated further by investigating the impact of the HIV-1 Env subtype on the antiviral activity of five new compounds derived from the entry inhibitor BMS806.

Keywords: HIV-1; Pseudovirus; Entry inhibitors; Antiviral activity

1. Introduction

Despite the tremendous benefits of highly active antiretroviral therapy (HAART) in controlling viral replication, HAART cannot eradicate the virus from infected patients. There are a limited number of antiretroviral compounds available and resistance is becoming a major problem in the treatment of HIV-1 infected patients. To overcome this, researchers are looking for drugs to tackle the virus at different stages of infection. HIV-1 entry inhibitors constitute an interesting class of compounds as these have the potential to block the earliest step of viral infection (Briz et al., 2006). Therefore, these compounds could also be used as topical microbicides for the prevention of new infections (Balzarini and Van Damme, 2007; Ketas et al., 2007). Despite the enormous variability found in the viral gp120 and gp41 envelope proteins (Env), several types of entry inhibitors are currently under development (Castagna and Biswas, 2005; Markovic, 2006) and some have already advanced to Phase I/II or even Phase III clinical trials (e.g. TNX355, Maraviroc) (Leonard and Roy, 2006; Schols, 2006; Westby et al., 2007).

Assays using replication-competent PBMC-derived viruses in combination with PBMCs are the most biologically relevant way to measure antiviral activity of anti-HIV drugs. However, as the preclinical evaluation of experimental antivirals in PBMCs is very cumbersome, this precludes the translation of PBMC assays into high-throughput systems. In addition, the diversity present within a heterogeneous virus population can complicate the interpretation of the results obtained. Moreover, donor cell dependency of viral replication hampers inter-lab comparison of data and therefore makes PBMC assays a less attractive approach. Therefore, other assays measuring antiviral activities of anti-HIV drugs use either molecular clones of viral isolates or single-cycle Env pseudotyped viral vectors. Using viral molecular clones might solve the problem of the viral diversity but

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these are not readily isolated, limiting the flexibility of the assay (Louder et al., 2005). A more attractive option is to use Env pseudotyped viral vectors, since amplifications of the complete HIV envelope is feasible starting from either plasma or from primary patient PBMCs. Cloning of the amplified Env gene into an expression vector can be routinely performed. After co-transfection of the Env gene plasmid with a second plasmid carrying the complementary HIV-1 information as well as a reporter gene, Env pseudoviruses are produced (Connor et al., 1996; Li et al., 2005; Seibert et al., 2006; Wei et al., 2002). Since Env pseudoviruses are only capable of a single round of infection and contain a reporter gene, precise quantification of infection is possible 48–72 h post-infection. Additionally, the pseudovirus system allows for high throughput screening and does not require an expensive Biosafety Level 3 Laboratory.

However, an important question is whether assays based on primary isolates or pseudoviruses produce similar results. To our knowledge, this question has never been addressed in a side-by-side setting. The activity of a well characterized set of anti-HIV compounds was therefore compared against both replication-competent virus isolates and derived single-cycle pseudoviruses in either PBMCs or a continuous cell line (Ghost). Furthermore, as an example of the utility of Env pseudoviruses for the evaluation of experimental HIV entry inhibitors, five BMS806 like compounds were evaluated against a panel of pseudoviruses belonging to different subtypes.

2. Material and methods

2.1. Replication-competent HIV-1 isolates and single-cycle HIV-1 pseudoviruses

Five replication-competent virus isolates were selected: two reference viruses: MN (subtype B, CXCR4) and SF162 (subtype B, CCR5) and three biologically cloned (Zhong et al., 1995) virus isolates: VI829-1 (subtype C, CCR5); VI525-1 (subtype A, CCR5) and CA10-3 (CRF01_AE, CXCR4). All isolates were grown and titrated on PHA/IL-2 stimulated peripheral blood mononuclear cells (PBMCs). For the evaluation of antiviral activities all viruses were used at a multiplicity of infection (MOI) of 10^{-4}.

For all replication-competent virus isolates, corresponding Env expressing constructs were prepared by DNA amplification of the complete Env gene starting from PBMC co-cultures and subsequent cloning into an expression vector (pSV7d or pcDNA4/TO (Invitrogen BV, Groningen, The Netherlands)). Env expressing constructs from another 4 replication-competent CCR5 using viruses: VI2809 and VI191 (both subtype A), VI886-1 (subtype B) and VI1888 (CRF01_AE) were also generated. Sequencing of the pseudovirus construct and phylogenetic analysis of the complete gp160 confirmed the association between the pseudovirus and the corresponding virus. The full length Env sequences of the PV constructs used have been deposited with GenBank (accession numbers EU191612–EU191618).

Pseudoviruses were generated in a 24-well plate by transfection of human embryonic kidney (HEK) 293T cells (obtained from ATCC) with pNL4-3.LucR^-E^- (NIH AIDS Research and Reference reagent program) and an Env expressing plasmid. In brief, 2×10^5 HEK293T cells were plated the day before transfection. Cells were co-transfected with 400 ng pNL4-3.LucR^-E^- and 1 μg of the Env expressing construct using the calcium phosphate method (ProFection® Mammalian Transfection Systems, Promega Benelux BV, Leiden, The Netherlands). After 24 h, the DMEM culture medium (Lonza, Verviers, Belgium) was replaced with DMEM culture medium containing 1 mM sodium butyrate (Sigma–Aldrich, Bornem, Belgium) and incubated for another 24 h. Two days post-transfection the pseudovirus was harvested and passed over a 45 μm pore sterile filter (Millex® HV, Millipore NV, Brussels, Belgium). Foetal bovine serum (FBS) was then added to a final concentration of 10% and the PV was frozen at −80 °C in 1 ml aliquots until needed. When larger volumes of pseudovirus were needed in order to concentrate the pseudovirus, transfections were done in the same manner, but 1×10^6 HEK293T cells were plated on the day before transfection. Transfections were done with 3.125 μg pNL4-3.LucR^-E^- and 9.375 μg of the Env expressing construct.

2.2. Target cells

Primary peripheral blood mononuclear cells (PBMCs) were obtained through density-gradient centrifugation (LymfoPrep, Axis-Shield, Oslo, Norway) of buffy coat from a HIV seronegative individual (further referred to as BC1). Aliquots of 25×10^6 cells/ml in 90% FBS and 10% DMSO were cryopreserved until use. To exclude differences in antiviral activity due to donor PBMC variability, all experiments (unless otherwise stated) were done with the same batch of cells derived from BC1. For each experiment, cells were thawed and washed (3X) with RPMI 1640 medium (Lonza, Verviers, Belgium) containing 10% FBS. Afterwards, the cells were cultured for 3 days in medium containing IL-2 (200 units/ml Gentaur, Brussels, Belgium) and 0,5 μg/ml phytohemagglutinin (PHA). Expression of the CD4 receptor and the CCR5 and CXCR4 co-receptors was determined in a 3-step immunostaining procedure using anti-human CD4, anti-human CCR5 and anti-human CXCR4 monoclonal antibodies (Becton Dickinson, Erembodegem, Belgium).

GFP-transduced Human Osteosarcoma cells (Ghost) expressing CD4 and CCR5 or CXCR4 were obtained from Dr. D. Littman (New York Medical Center, New York, USA).

2.3. Single cycle pseudovirus infection assay in Ghost cells

Fifty microliters of pseudovirus (yielding 50,000–100,000 relative luminescence units (RLU)) were pre-incubated for 15 min with 50 μl of a serial 10-fold dilution of anti-HIV compound (10,000–0.1 nM, except Efavirenz, used at 1000–0.01 nM). Afterwards, 100 μl of Ghost target cells (12,000 cells), expressing CD4 and either CCR5 or CXCR4, were added and cultures were incubated further at 37 °C in 5% CO_2. Seventy-two hours post-infection, 100 μl of the supernatant was discarded and replaced with an equal volume of Steadylite HTS (PerkinElmer Life Sciences, Zaventem, Bel-

gium) and Luciferase activity was determined in a TopCount™ (Canberra-Packard, Zellik, Belgium). All experiments were done in triplicate in at least two independent experiments.

2.4. Single cycle pseudovirus infection assay in PHA/IL-2 stimulated PBMCs

For experiments with PHA/IL-2 stimulated PBMCs, VI525-1, VI829-1 and CA10-3 pseudoviruses were first concentrated by ultracentrifugation. In brief, after harvesting, the PV was centrifuged for 15 min at 1200 × *g* (3000 rpm) to remove cell debris, followed by 1 h of ultracentrifugation of the supernatant at 50,000 × *g* (17,100 rpm) at 25 °C. The PV pellet was dissolved in 10% of the original volume of medium.

Experiments using PHA/IL-2 stimulated PBMCs were done once (unless otherwise stated) in duplicate. Briefly, 100 μl of PV was pre-incubated at room temperature for 15 min with 50 μl of a serial 10-fold dilution of each compound. Afterwards, 50 μl of PHA/IL-2 stimulated PBMCs (200,000 BC1 cells) were added. Seventy-two hours post-infection, 100 μl of the supernatant was discarded and replaced with an equal volume of Steadylite HTS (PerkinElmer Life Sciences, Zaventem, Belgium). Luciferase activity was determined in a TopCount™ (Canberra-Packard, Zellik, Belgium). This resulted in a signal to noise ratio of at least 15.

2.5. Infection experiments in PHA/IL-2 stimulated PBMCs using replication-competent virus

Fifty microliters of virus was pre-incubated at room temperature with 50 μl of a serial 10-fold dilution of compound for 15 min. Afterwards, 75,000 PHA/IL-2 stimulated PBMCs (BC1 cells) were added, resulting in 10^{-4} MOI, and cultures were incubated further at 37 °C in 5% CO_2. After 2 h, cultures were washed three times, and medium containing the same compound used during pre-incubation, at the same dilution, was added to the cells followed by further incubation for 7 days (37 °C, 5% CO_2). On day 7, the experiment was stopped and the HIV p24 antigen concentration in the supernatant was determined using a modified monoclonal p24 (HuMab-HIVp24) (Biomaric NV, Gent, Belgium) enzyme-linked immunosorbent assay (ELISA) (Beirnaert et al., 1998). For the evaluation of the co-receptor blockers AMD3100 and TAK779, an adapted set-up was used in which the cells were pre-incubated (15 min) with the compound before the virus was added. All experiments, unless otherwise stated, were done twice and in triplicate using PHA/IL-2 stimulated PBMCs (BC1).

2.6. Compounds

Six compounds of different antiviral classes were used: T-20 (fusion inhibitor), TAK779 (CCR5 antagonist), AMD3100 (CXCR4 antagonist), BMS-378806 (CD4-gp120 binding inhibitor; further referred to as BMS806), AZT (NRTI) and Efavirenz (NNRTI). All reagents except BMS806 were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The BMS806 and the five BMS806 related analogues (BA1–5), were provided by Tibotec (Mechelen, Belgium). All compounds were reconstituted in DMSO to obtain a stock concentration of 10 mM. In order to determine the EC50 values, serial 10-fold dilutions were made, with each dilution being tested in triplicate unless otherwise stated. All compounds were tested from a start concentration of 10,000 nM except for Efavirenz (EFV) where the highest final concentration was 1000 nM.

2.7. Determination of percentage inhibition and antiviral activity (EC50)

For calculations of the percentage inhibition of viral replication, the geometric means of all replicates were taken for each individual experiment. The percentage inhibition is calculated as $100 \times \{1 - \text{RLU (PV + compound)/RLU(PV - compound)}\}$. The 50% effective concentration (EC50) (nM) is the drug concentration where a 50% reduction of infectivity is obtained. For comparison between the different assays the geometric means of the individual EC50 values and the standard error of mean (SEM) were calculated.

2.8. Statistical analysis

Differences in sensitivity were analysed by a two-tailed Student's *T*-test. Linear regression analysis was performed on the $\log_{10}$ transformed EC50 data using JMP Version 5.0 software (SAS Institute Inc., Cary NC, 2002).

3. Results

3.1. Infectivity and titration of pseudovirus stocks in different target cells

Prior to determining the EC50 values of the compounds, the PV stocks were titrated on Ghost cells. The highest infectivity of the pseudoviral stocks, measured as Relative Luminescence Units (RLU) was obtained with MN, SF162 and VI829-1 (6–7 logs). For VI525-1 and CA10-3 an RLU of 6 and 5 logs, respectively, were obtained. Background signals were always below 400 RLU resulting in signal to noise ratios of 500 up to 25,000 (data not shown).

If PHA/IL-2 stimulated PBMCs were used as target cells, the RLU output was much lower and ranged from 10,000 to 60,000 for MN and SF162-derived PV, respectively. Moreover, RLU signals were even lower for PV derived from VI525-1 (500 RLU), VI829-1 (1400 RLU) and CA10-3 (3325 RLU). Background signals, obtained by mock PV (only pNL4-3.LucR^-E^-), were below 200 RLU which resulted in signal to noise ratios between 2.5 (VI525-1) and 15 (CA10-3). Comparable results were obtained using PBMCs from two other cell donors. Due to this low infectivity, SF162 and MN were used as undiluted pseudovirus stocks while the 3 other pseudoviruses (VI829-1, VI525-1 and CA10-3) had to be concentrated (as described in Section 2.1). As expected, the RLU measured in both Ghost and PBMCs was directly proportional with the amount of PV used (Fig. 1).

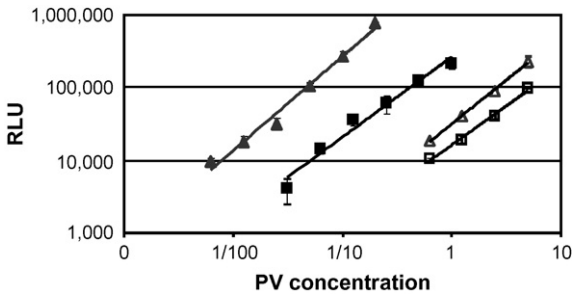

Fig. 1. Pseudovirus titration in PBMCs and Ghost cells. Pseudovirus particles (PV) were prepared by transfection of HEK293T cells with a pNL4-3.LucR^-E^- vector and a VI829-1 or CA10-3 Env expressing vector. Serial 10-fold dilutions of the supernatant were used to infect Ghost cells expressing CD4 and the appropriate co-receptor. When PBMCs were used as target cells, PV was concentrated prior to infection. All conditions were tested in triplicate and infection was assessed 72 h post-infection by measuring the Luciferase activity (RLU) using a TopCount^{TM}. Triangles and squares represent RLUs obtained for VI829-1 and CA10-3, respectively. Filled and open symbols represent infection in Ghost cells and PBMCs, respectively. Error bars show the mean RLU (±SEM) of the triplicates.

3.2. Phenotypical and functional characterization of donor PBMCs

Most experiments were carried out using PBMCs from a single donor (BC1) in order to avoid donor cell variability as a source of inter-experimental differences in EC50.

After PHA/IL-2 stimulation of PBMCs, no significant differences in expression levels of CD4 (55 and 52%), CCR5 (47 and 64%) or CXCR4 (93 and 97%) were found between fresh and cryopreserved cells, respectively.

Additionally, the functionality of cryopreserved cells was tested in comparison to fresh PBMCs from the same donor by an infection experiment using VI525-1 replicating virus at a multiplicity of infection (MOI) of 10^{-2}, 10^{-3} and 10^{-4}. No differences in p24 output, as measured by a modified in-house monoclonal p24 (HuMab-HIVp24) (Biomaric NV, Gent, Belgium) ELISA, were seen between the cryopreserved and fresh cells (data not shown).

3.3. Reproducibility of dose–response curves obtained using pseudovirus

The reproducibility of the antiviral activity of the fusion inhibitor T-20 was tested in both Ghost cells and PBMCs using PV derived from the subtype B HIV-1 strains SF162 (R5) and MN (X4). Highly reproducible dose–response curves were obtained with both PV isolates in Ghost cells (Fig. 2a). The antiviral activity of T-20 against SF162 PV resulted in a mean EC50 of 24 nM (range 15–42) while for MN PV the mean EC50 was 7 nM (range 4–12). Using fresh PHA/IL-2 stimulated PBMCs from 3 healthy donors slightly more variable dose–response curves were generated (Fig. 2b). The mean EC50 of T-20 was 17.7 nM (range 5.6–31.7) for SF162 PV, and 2.4 nM (range 1.5–5.0) for MN PV, respectively.

3.4. Evaluation of the antiviral activity of reference compounds in the PV/Ghost cell assay

The dose–response curves for all compounds were determined using a pseudovirus concentration resulting in 5×10^4 to 10^5 RLU. Each combination of PV and compound was tested in triplicate in at least two independent experiments and resulted in similar dose–response curves. Linear regression analysis was done to calculate the EC50 values. For comparison between the different assays, the geometric means of the individual EC50 values were calculated (Fig. 3). Set-up 1 in Fig. 3(a–e) depicts the EC50 values obtained for three R5 and two X4 pseudoviruses with 5 compounds, including the fusion inhibitor T-20, the gp120 binder BMS806, the CCR5 antagonist TAK779 or the CXCR4 antagonist AMD3100 as well as two reverse transcrip-

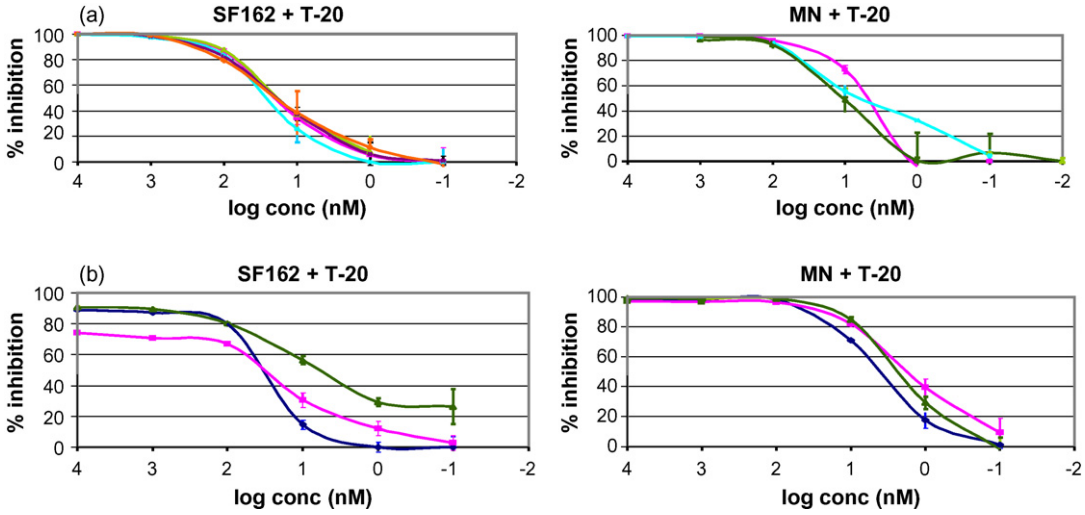

Fig. 2. (a) Reproducibility of dose–response curves in Ghost cells. (b) Reproducibility of dose–response curves in PHA stimulated PBMCs. Dose–response curves for T-20 were generated in both Ghost cells (a) and PHA/IL-2 stimulated PBMCs (b) using pseudovirus of SF162 (B, R5) and MN (B, X4). For infection of Ghost cells, a dilution of PV was used resulting in 50,000–100,000 RLU in the absence of compound. For the experiment in PHA stimulated PBMCs undiluted PV stocks were used (15,000 and 60,000 RLU in the absence of compound). To generate the dose–response curves in PBMCs, cells from 3 healthy donors were PHA/IL-2 stimulated before use. Each experiment was done in triplicate using six serial 10-fold dilutions of T-20. Error bars represent the standard errors of mean (SEM).

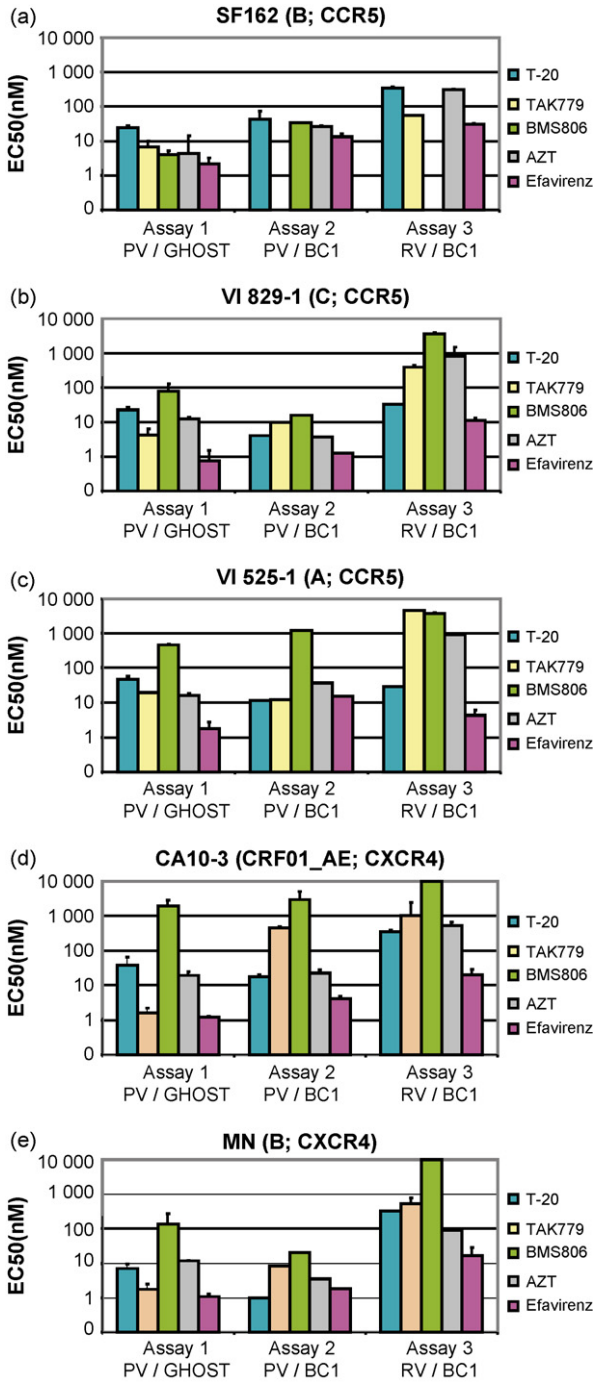

Fig. 3. Comparison of EC50 values obtained in the 3 used assays. Assay 1: pseudovirus in Ghost cells; assay 2: pseudovirus in PBMCs (BC1); assay 3: replication-competent virus in PBMCs (BC1). Each bar represents the 50% effective concentration (EC50) values ($\log_{10}$ transformed) for a particular compound against a particular virus isolate or PV in the 3 assays. Error bars represent the standard errors of mean (SEM), except for those experiments that could be done only once due to limitation of the available PBMCs. An asterix denotes that less than 50% inhibition was obtained, even at the highest concentration tested. Due to exhaustion of BC1 the EC50 for TAK779 and BMS806 could not be determined for SF162 in assay 2 and 3, respectively. TAK779 and AMD3100 showed no activity against the X4 and R5 using viruses, respectively. For clarity however these data were not included in the figure.

tase inhibitors: AZT (nucleoside-type or N-RTI) and Efavirenz (non-nucleoside type or NN-RTI).

Since the reverse transcriptase enzyme in all of the PV was derived from the pNL4-3 backbone, their susceptibility towards AZT and Efavirenz should be similar. The EC50 of AZT oscillates around 11 nM (range: 4–19) and that of Efavirenz around 1 nM (range: 0.7–2.1).

Taking the sensitivity range of AZT and Efavirenz as a reference, the EC50 values for the entry inhibitors T-20 (mean 23 nM; range: 7–46 nM) and TAK779 (mean 8; range 4–19 nM) or AMD3100 (mean 1.7 nM; range 1.6–1.8 nM) were also similar across the PV of various subtypes used.

In contrast, more pronounced differences in EC50 were obtained with BMS806 according to its known subtype dependency. The mean EC50 was 132 nM but it ranged from 4 nM for the SF162 PV (subtype B, R5) to 1979 nM for the CA10-3 PV (CRF01_AE, X4).

3.5. Evaluation of the antiviral activity of reference compounds in the PV/PBMC assay

To evaluate the activity of the compounds against PV infection of PHA/IL-2 stimulated BC1, the same set-up was applied as in Ghost cells, except that undiluted (SF162, MN) or concentrated PV was used and that each combination was only tested in duplicate in two independent experiments unless otherwise stated. The EC50 values are depicted in set-up 2 of Fig. 3(a–e).

For T-20 and TAK779 the EC50 values were in the same range irrespective of the subtype of the pseudovirus, yielding a mean EC50 of 8 nM (range 1–43 nM) and 11 nM (range 10–12 nM), respectively. For AMD3100, the mean EC50 value was 8 nM for MN PV (subtype B) and 451 nM for CA10-3 PV (CRF01_AE). The latter EC50 is remarkably high as compared to the EC50 obtained in the PV/Ghost assay. However, this cannot only be explained by differences in CXCR4 expression levels on the used target cells since no difference was found for MN.

Similar to the Ghost cell assay, significant differences in EC50 were found for BMS806. Low EC50 values were obtained for SF162 PV (33 nM), MN PV (20 nM) and VI829-1 PV (16 nM), while for VI525-1 and CA10-3 PV much higher EC50 values were obtained (1191 and 2974 nM, respectively). As expected, the EC50 values of the NRTI AZT (mean 12 nM; range 4–36 nM) as well as of the NNRTI Efavirenz (mean 7 nM; range 2–15 nM) were similar for all pseudoviruses tested.

3.6. Evaluation of the antiviral activity of reference compounds in the replicating virus/PBMC assay

The dose–response curves for all compounds were generated using a predetermined virus concentration of 10^{-4} MOI. Each combination of replication-competent virus (RV) and compound was tested in triplicate in two independent experiments. EC50 values are depicted in set-up 3 of Fig. 3(a–e).

Overall, infection of PHA/IL-2 stimulated BC1 with replicating viruses resulted in higher EC50 values as compared to the PV assays. For T-20, the EC50 values between the different viruses were within one log difference. In contrast, up to 2 log differ-

ences in antiviral activity were found for TAK779 with EC50 values varying between 54 nM (SF162), 396 nM (VI829-1), and 4671 nM (VI525-1) indicating a subtype or isolate related effect. AMD3100 had a very similar antiviral activity against CA10-3 (EC50 of 1000 nM) and MN (EC50 of 522 nM). Remarkably, the antiviral activity of BMS806 was very low. This compound was unable to inhibit the replication of the two X4 viruses (MN and CA10-3) even at the highest concentration (10,000 nM) used. BMS806 showed a mean EC50 value of 3735 nM against VI525-1 and 3587 nM against VI829-1. For SF162 the experiment with BMS806 was not done because PBMCs from donor BC1 were exhausted. Using cells from another donor an EC50 of 363 nM was found (data not shown).

The EC50s of AZT and Efavirenz were independent of the virus subtype and AZT was about 10 times less potent than Efavirenz, with a mean EC50 of 406 nM (range 89–932 nM) versus 14 nM (range 4–31 nM), respectively. Furthermore, as compared to the PV/PBMC assay the EC50 values obtained with the replication-competent virus were one log higher for AZT (mean EC50 of 406 nM versus 12 nM) but only slightly higher for Efavirenz (mean EC50 of 14 nM versus 5 nM).

3.7. Inter-assay comparison of EC50 values

As compared to the replication-competent viral-PBMC assay, both pseudovirus assays, PV-Ghost and PV-BC1, were more sensitive to all of the antiretrovirals tested ($P = 0.013$ and $P = 0.015$, respectively). To analyze the correlation between the EC50 results in the various assays, linear regression analysis on the $\log_{10}$ transformed data was performed using JMP Version 5.0 software. A significant linear relationship was found between both PV assays ($P < 0.0001$, $R^2 = 0.75$). The intercept of the linear regression was not significantly different from zero and its slope was not significantly different from unity ($P > 0.05$). For all practical purposes one can state that PV/Ghost was not significantly different from PV/BC1. Furthermore, the results from the RV/BC1 assay can be significantly predicted from the PV/Ghost assay ($P < 0.0001$, $R^2 = 0.61$) as well as from the PV/BC1 assay, although to a lesser degree ($P < 0.0001$, $R^2 = 0.55$) (Fig. 4(a–c)).

3.8. Analysis of new BMS806 related compounds interacting with the CD4 binding site

Since strong, subtype related differences were seen with the BMS806 compound, we analyzed five new BMS806 related products for their potential to block PV infection. In addition to the five previously used PVs an extra panel of four R5-using PV constructs belonging to subtype A (VI2809 and VI191), B (VI886-1), and CRF01_AE (VI1888) were tested using Ghost cells as targets. Efavirenz was included as a control in all experiments. Two of the compounds (BA1 and BA2) showed similar potency as BMS806 while the 3 other compounds (BA3–5) were less potent. However, none of the tested compounds could inhibit infection with VI1888 (R5, CRF01_AE), even at the highest concentration (10,000 nM) tested (Table 1). The differences in potency cannot only be attributed to mutations in the BMS806 binding pocket itself (Madani et al., 2004). Sequences outside the binding pocket are probably also involved. However, to further unravel this phenomenon, modelling, which is beyond the scope of this manuscript, is needed. Nevertheless it is clear that a pseudoviral-based assay can provide useful information about the potency of a compound.

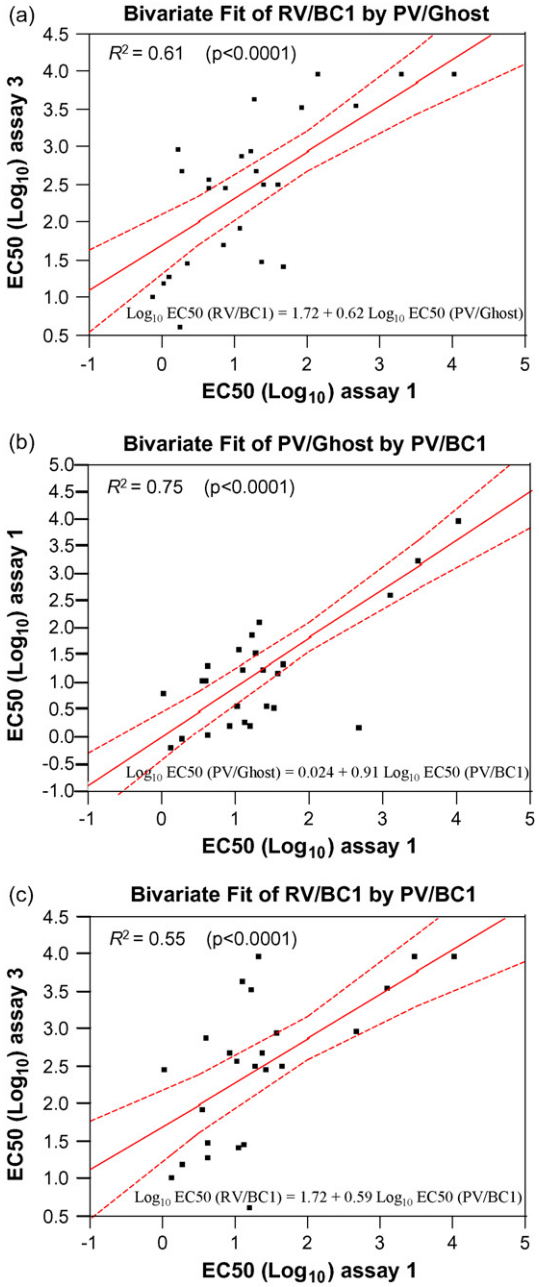

Fig. 4. Bivariate fit analysis of the $\log_{10}$ transformed EC50 values between all assays. For comparison the EC50 ($\log_{10}$) values from one assay are plotted against the EC50 ($\log_{10}$) values from another assay. Linear regression analysis was carried out using JMP Version 5.0 software (SAS Institute Inc., Cary NC, 2002). For each comparison the equation and the *R*-squared value is given.

4. Discussion

In order to evaluate the antiviral activity of new anti-HIV drugs including entry and fusion inhibitors, a robust and high

Table 1
Antiviral activities (EC50) of BMS806 related compounds

Compound	VI 2809 A; CCR5	VI 525-1 A; CCR5	VI 191 A; CCR5	VI 886-1 B; CCR5	SF162 B; CCR5	VI 829-1 C; CCR5	MN B; X4	VI 1888 CRF01; R5	CA10-3 CRF01; X4
BA 1	96	413	883	0.6	4.6	84	612	>10,000	1,634
BA 2	332	325	144	1	7.9	39	885	>10,000	6,697
BA 3	>10,000	>10,000	>10,000	106	625	>10,000	>10,000	>10,000	>10,000
BA 4	>10,000	7,338	>10,000	44	96	>10,000	>10,000	>10,000	>10,000
BA 5	>10,000	>10,000	>10,000	8764	>10,000	>10,000	>10,000	>10,000	>10,000
BMS 806	1,779	458	804	2	4	80	137	>10,000	1,979
Efavirenz	4	2	9	2	2	1	1	4	1

Pseudovirus of seven CCR5 and two CXCR4 using constructs, generated as described in Section 2, and using Ghost cells were used to generate dose–response curves for BMS806 and 5 BMS806 analogues (BA1–5). Efavirenz was used as a control. Experiments were done in triplicate. In between brackets: subtypes and co-receptor usage of the pseudoviruses.

throughput standardized assay able to measure relatively small differences in antiviral activity is needed. From a technical point of view an HIV-1 Env pseudovirus (PV) based assay fulfils these criteria. Moreover, Env-PV vectors can be made relatively rapidly starting from both plasma as well as primary cells, allowing researchers to make a battery of vectors covering all important subtypes and CRFs. Though assays based on replication-competent viruses in combination with peripheral blood mononuclear target cells are more biologically relevant, they are less flexible and more complicated to interpret. The data described in this paper provide a side-by-side comparison of the antiviral activity, measured as 50% effective concentrations (EC50), of six reference compounds using two pseudoviral based assays and an assay based on replication-competent virus.

To exclude differences related to quasi-species present in a primary virus isolate, a panel of 2 reference strains (MN and SF162) and 3 biological cloned viruses was used. Furthermore, cryopreserved peripheral blood mononuclear cells (PBMCs) from one buffy coat donor were used in most experiments.

Based on the results it is concluded that a clear correlation exists between the EC50 values of both PV assays as well as with the replication-competent virus assay. As a consequence, PV assays in combination with a continuous cell line like Ghost cells can help researchers studying subtle differences while optimizing compounds which are still in an early phase of development.

An interesting observation was that only two (SF162 and MN) out of the 5 PV used did not have to be concentrated to infect PBMCs. This might be due to the accessibility of CD4i epitopes, and by extension to the co-receptor binding site for both viruses. Earlier reports (Hsu et al., 2003; Xiang et al., 2002) indicated that T-cell line-adapted virus strains have an open structure, making gp120 more accessible. Another explanation might be that, differences in expression levels of CD4 and both chemokine receptors (CCR5 and CXCR4) on target cells have an impact on the infection rate and might explain the differences in RLUs seen between Ghost cells and PBMCs, respectively. These differences in expression levels can have an impact on the antiviral activity of compounds interacting with the receptors. This could be similar to a study reported by Choudhry et al. (2006), where it was shown that the CCR5 expression level correlated with the activity of neutralizing antibodies. Therefore, for compounds selected for further development, a biologically more relevant assay using replication-competent virus in combination with PBMCs should be performed to obtain a more complete picture of the antiviral activity of a compound.

From our initial analysis it was apparent that the potency of the BMS806 compound was subtype dependent, which is in agreement with previously published results (Lin et al., 2003). Using an extended panel of PVs, the antiviral activity of BMS806 and 5 related (BA1-5) compounds was examined in the Ghost assay (Table 1). Two of these compounds showed a potency similar to BMS806 but none, including BMS806, were able to inhibit the pseudoviral infection of VI1888 (CRF01_AE, R5) even at the highest concentration (10 μM) tested. Based on known mutations in the CD4 binding pocket interacting with BMS806 it was not possible to clarify the lack of potency against

both CRF01_AE (CA10-3 and VI1888) as well as against one (VI2809) of the three subtype A pseudoviruses.

It remains extremely important to develop new HIV therapeutics and microbicides. Existing and new compounds can be evaluated on PV derived from either plasma RNA or cellular proviral DNA from both acute (seroconvertors) and chronic infections. Moreover, pseudoviral constructs derived from other biological fluids (e.g. breast milk, semen) can provide very useful information regarding the potency of antiretroviral compounds on viruses involved in transmission.

Currently there are quite a number of pseudoviral constructs available through both the NIH AIDS Research & Reference Reagent Program and the National Institute for Biological Standards and Control which can help investigators in their endeavours to make potent entry inhibitors against the whole spectrum of subtypes and CRFs.

In summary, Env pseudotyped viruses are a useful tool to study interactions between envelopes and compounds interacting with the entry process.

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