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Synthesis, antiviral activity and resistance of a novel small molecule HIV-1 entry inhibitor

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ABSTRACT

One of the most critical requirements of the infection of the human immunodeficiency virus type 1 (HIV-1) is the interaction of its surface envelope glycoprotein gp120 with the cellular receptor CD4, which initiates virus entry to cells. Therefore, envelope glycoprotein gp120 has been validated as a potential target to develop HIV-1 entry inhibitors. Here we report the evaluation of a novel non-natural amino acid, termed 882376, reported earlier as a precursor of a CD4-mimetic miniprotein, as HIV-1 entry inhibitor. 882376 showed HIV-1 inhibitory activity against a large panel of primary isolates of different subtype. Moreover, genotyping of 882376 resistant HIV-1 virus revealed three amino acid substitutions in the gp120 including one in the CD4 binding site suggesting that this molecule may bind to gp120 and prevent its binding to CD4. Additional neutralization experiments indicate that 882376 is not active against mutant pseudoviruses carrying the amino acid substitutions S375H and S375Y located in the 'Phe43 cavity' which is the major site of CD4 binding, suggesting that this compound may interfere with the interaction between gp120 and CD4. The unnatural amino acid, 882376, is expected to serve as a lead for further optimization to more potent HIV-1 entry inhibitors.

1. Introduction

HIV-1 is the etiological agent of the Acquired Immunodeficiency Syndrome (AIDS). According to the World Health Organization (WHO) this disease ranked 6th among the 10 leading causes of death in the world in 2012 (http://www.who.int/mediacentre/factsheets/fs310/en/; accessed on August 26, 2015). Based on the 2014 Global Statistics from UNAIDS about 36.9 million people are living with HIV worldwide, about 2 million people were newly infected and 1.2 million people died from AIDS-related diseases world-wide (http://www.unaids.org/sites/default/files/media_asset/20150714_FS_MDG6_Report_en.pdf; accessed on 26th August, 2015). HIV-1 is a highly variable virus which mutates rapidly. The currently used treatment is termed the Highly Active Anti-Retroviral Therapy (HAART), which uses combination of drugs targeting different steps of the retrovirus life-cycle. There are five classes of drugs currently approved by the US FDA, which are usually used in combination, typically 2 nucleoside reverse transcriptase inhibitors (NRTIs) in combination with 1 non-nucleoside reverse transcriptase inhibitor (NNRTI), protease inhibitor (PI), integrase inhibitor (INI) or entry inhibitor.[1,2] To date only two entry inhibitors are available, Maraviroc which targets the co-receptor CCR5[3,4] and Enfuvirtide, a peptide drug which interacts with the N-terminal heptad repeat of gp41 of HIV to form an inactive hetero six-helix bundle preventing infection of host cells.[5,6] Despite the success achieved with the HAART the emergence of resistant viruses and the side effects of these therapies highlight the need of novel anti-retroviral agents to overcome these problems.

HIV-1 entry into the target cell is initiated by binding of the viral surface glycoprotein gp120 to the cellular receptor CD4, which induces the conformational change in gp120 that enable its binding to the cellular coreceptors CCR5 or CXCR4.[7,8] This binding in turn activates envelope glycoprotein gp41 which induces fusion of the viral and cellular membrane followed by the entry of the virus into the cells to initiate infection. One of the most important residues in the gp120/CD4 interaction is Phe43 of CD4, which deeply penetrates the cavity of gp120, referred to as the 'Phe43 cavity' and accounts for 23% of the total contact with gp120. The hydrophobic and highly conserved Phe43 cavity has been recognized as an attractive target for developing small mole-

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cule inhibitors[9–12] or CD4 mimetic 'miniproteins'[13,14] to fill the cavity and disrupt the gp120/CD4 interaction and prevent HIV-1 entry to cells. Recently, Morellato-Castillo et al. reported the structure-based design of CD4-mimetic miniproteins.[13] They modified the Phe43 of CD4 with 11 non-natural phenylalanine derivatives and one of the analogs M48U12 showed binding affinity of 8 pM with HIV-1 YU2 gp120 and showed low nM antiviral potency against several lab-adapted and primary HIV-1 isolates. The introduction of the unnatural phenylalanine group containing cyclohexylmethyl amine moiety rendered M48U12 a pico molar binder to gp120. Furthermore, M48U12 showed remarkable antiviral activity against HIV-1 laboratory strains, primary isolates and also transmitted/founder viruses. The X-ray structure of this peptidomimetics bound to gp120 revealed that the cyclohexylmethylamino phenyl moiety of the unnatural phenyl alanine residue occupies about 80% of the volume of the Phe43 cavity making it one of the best binders of gp120. Therefore, we are interested to find out the contribution of this unnatural amino acid in M48U12 on the antiviral activity and explore the possibility of using the unnatural amino acid and its analogs as lead for further optimization.

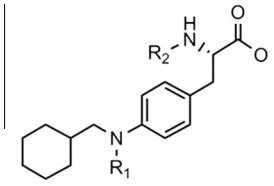
In this study we selected the unnatural amino acid, 882376, from the most potent inhibitor, M48U12, prepared its Boc and Fmoc substituted and other analogs and tested as HIV-1 entry inhibitors. To our surprise when we removed these two protecting groups we observed complete loss of antiviral activity. Since 882376 showed the best antiviral activity, we evaluated 882376 against a panel of primary HIV-1 Env-pseudotyped viruses from different subtypes. Moreover, we isolated 882376 HIV-1 resistant variants from HIV-1_{NL4-3} cloned virus obtained in the presence of escalating doses of 882376 to identify three amino acid substitutions in gp120 and two in the gp41 region.

2. Results

2.1. Chemistry

The synthesis of (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl) amino)-3-(4-((*tert*-butoxycarbonyl)(cyclohexylmethyl)amino)phenyl)propanoic acid (882376) was performed by following a reported method.[13] 882896 was prepared from 882376 by removing Fmoc by 20% piperidine in DMF (Fig. 1).

Analogs of (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-((cyclohexylmethyl)amino)phenyl)propanoic acid (883594, 883596, 883597, 883598, 883599 and 883600) and ((*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-((cyclohexylmethyl) amino)phenyl)propanoyl)-L-proline (902132, 902133, 902134, 902135, 902136 and 902137) were synthesized by using the synthesis outline depicted in Schemes 1 and 2, respectively. All synthesized compounds provided satisfactory analytical and spectroscopic data to confirm their structures. Since Boc group is located in the major binding site in the gp120 and is unstable in acidic and basic conditions we wanted to explore other groups to replace Boc and assess their structure activity relationship (SAR). We however, kept the Fmoc group in our set of compounds tested. Fmoc-containing unnatural amino acids have been reported to be good lead compounds against HIV-1 protease.[15]



882376: R_1= Boc; R_2= Fmoc
882896: R_1= H; R_2= H

Figure 1. The chemical structures of 882376 and 882896.

2.2. Evaluation of antiviral activity of a series of unnatural amino acids against HIV-1 pseudotyped with lab adapted HIV-1_{HXB2} ENV

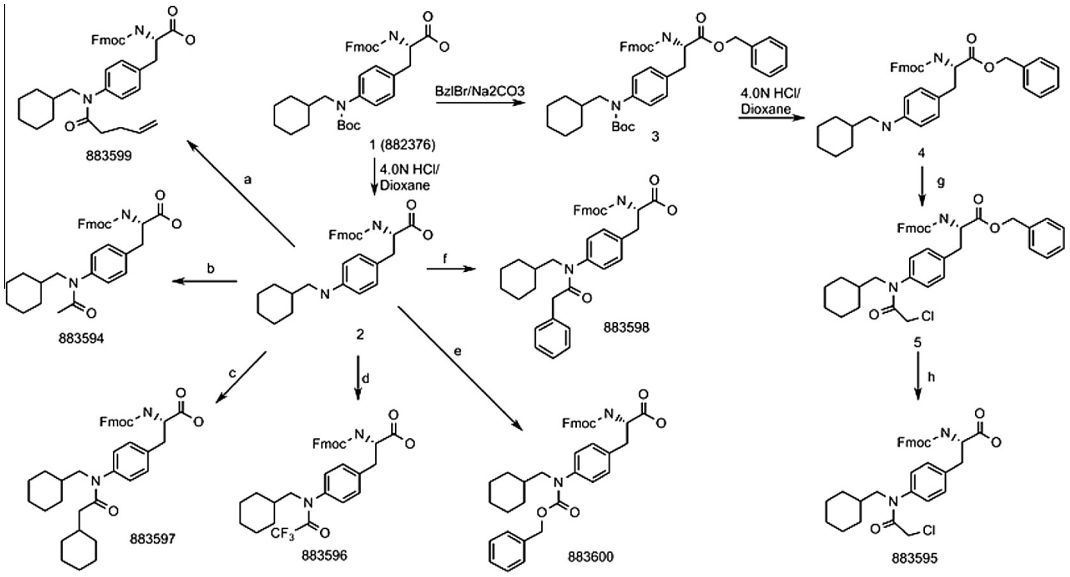
We first evaluated the HIV-1 inhibitory activity of a series of 15 unnatural amino acid analogs (Table 1) against HIV-1 pseudotyped with the HIV-1_{HXB-2} Env by using a single cycle infectivity assay. To this end, we infected TZM-bl cells with the above pseudovirus in the presence of increasing concentrations of the compounds. In parallel, we evaluated the cytotoxicity of the compounds. Our results indicated that 882376 displayed the best activity against HIV-1 with a calculated IC_{50} of 4.5 ± 0.4 μM and a CC_{50} > 50 μM (with 30% toxicity at this dose) while the respective unprotected form, 882896 (without BOC and Fmoc) showed no antiviral activity. Moreover, compounds 883597, 883598 and 883600 also displayed good anti-HIV-1 activity in the range 8.8–9.7 μM, however, they also showed higher toxicity than what we detected for 882376. When we replaced the Boc protecting group with an acetyl group (883594), the antiviral activity was also lost. Introduction of chloro acetyl group recovered some of the lost activity indicating that a bulkier hydrophobic group is favored in that position. We used chloro acetyl group just to understand the relative importance of bulkier groups despite the fact this moiety is known to be reactive group. Importance of bulky group is also evident in compounds 883597 and 883598 where a cyclohexylmethyl and benzyl groups, respectively, were used. Activity dropped substantially when a pent-4-enoyl group was used. This group has a more extended and flexible structure. The importance of bulky hydrophobic group was further evidenced in compound 883600 where a benzyl formate group was used. However, in Scheme 2 although different groups were used to generate SAR, the bulkier hydrophobic group did not make any difference in antiviral activity but notably the toxicity values were in general better.

2.3. 882376 prevents cell-to-cell fusion

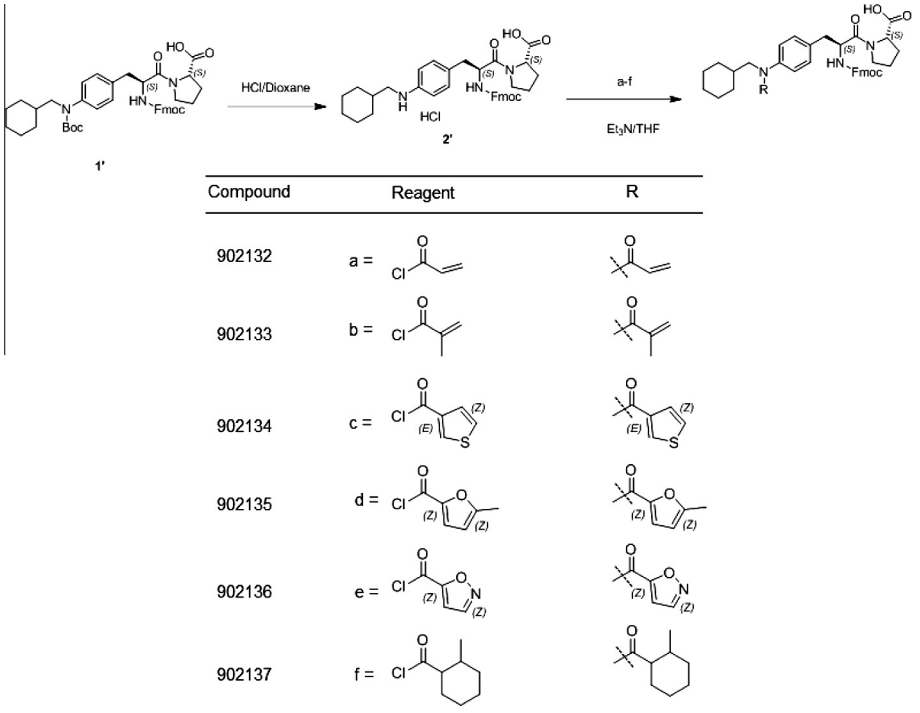
HIV-mediated cell-to-cell fusion is a significant mode of HIV-1 spreading from infected cells to uninfected cells. To investigate whether 882376 prevents HIV-1 spreading through cell-to-cell fusion we co-cultured MAGI-CCR5 cells with HL 2/3 cells in the presence of escalating concentrations of compounds for 24 h as previously reported.[16] We used BMS-378806 and NBD-556 which have been reported to inhibit cell–cell fusion[17,18] as controls. BMS-378806 and NBD-556 inhibited cell-to-cell fusion with an IC_{50} of 0.023 μM and 5.6 μM, respectively, while 882376 inhibited this process with an IC_{50} of 10 μM displaying an important HIV-1 entry inhibitor trait (Fig. 2).

2.4. 882376 does not enhance HIV-1 entry into CD4-negative cells

Previous studies have reported that the small molecule HIV-1 entry inhibitor NBD-556, which has been identified by our group about a decade ago,[18] can also act as CD4-agonist promoting CCR5 binding and enhancing HIV-1 entry into CD4-negative cells expressing CCR5.[10,19] To assess whether 882376 performs as a CD4-agonist or -antagonist, we infected CD4-negative Cf2Th-CCR5 cells with recombinant CD4-dependent HIV-1_{ADA} in the presence of escalating concentrations of compound. NBD-556 and BMS-378806 (a highly potent small-molecule HIV-1 entry inhibitor)[20] were used as control. As reported previously, NBD-



Scheme 1. Synthesis of compounds 883594–883600. Reagents and conditions: (a) pent-4-enoic anhydride/DIPEA in THF; (b) acetic anhydride/DIPEA in THF; (c) 2-cyclohexylacetic anhydride/DIPEA in THF; (d) trifluoroacetic anhydride/DIPEA in THF; (e) Cbz-Cl/DIPEA in THF; (f) 2-phenylacetyl chloride/DIPEA in THF; (g) Cl-CH_2CONa/IBCF/NMM in THF; (h) Pd/CH_2 in MeOH.



Scheme 2. Synthesis of compounds 902132–902137.

556 enhanced HIV-1 entry into CD4-negative cells while BMS-378806 as well as 882376 did not, suggesting that 882376 possesses CD4-antagonist properties (Fig. 3).

2.5. Evaluation of antiviral activity of 882376 against a panel of HIV-1 Env-pseudotyped reference viruses

The HIV-1 envelope glycoproteins are highly variable. Therefore, we decided to test the inhibitory activity of 882376 against a panel of HIV-1 Env-pseudotyped reference viruses of different subtypes in a single-cycle infection assay to assess its efficacy against a diverse set of HIV-1. We compared the antiviral activity of 882376 with the activity of the small molecule NBD-556 (most of this data was published recently[16]). Five representative dose-dependent inhibition plots of the neutralization assay of selected pseudoviruses [NIH # 11887 (clade A), # 11904 (clade A/D), # 11058 (clade B) #11909 (clade C) and # 11911 (clade D)] are shown in Figure 4. As previously reported[10,16] NBD-556 was active against all the viral subtypes tested showing poor activity against the viruses from subtype A and exhibiting an IC_{50} in the range of 1.9–27.8 μM (Table 2). 882376 was equally active against all the viruses tested displaying a better anti-HIV-1 activity profile than

Table 1
Antiviral activity (IC_{50}) and toxicity (CC_{50}) of unnatural amino acids in TZM-bl cells against HIV-1 pseudotyped with lab adapted HIV-1_{HXB2} ENV

Compound	IC_{50} (μM)	CC_{50} (μM)
882376	4.5 ± 0.4	~50
882896	>140	>140
883594	>50	>74
883595	14.3 ± 1.5	48 ± 2
883596	22 ± 1	>67
883597	8.8 ± 0.4	26 ± 3
883598	9.7 ± 0.4	36 ± 2
883599	23.9 ± 1.8	>69
883600	8.9 ± 0.6	35 ± 1
902132	12.3 ± 1.4	~50
902133	17.8 ± 1.7	~60
902134	16.8 ± 0.4	>42
902135	16.6 ± 1.8	>57
902136	15.9 ± 1.2	>58
902137	10.4 ± 0.7	24.5 ± 1.5

NBD-556. The IC_{50} of this compound was in the range of 1.4–12.5 μM with an overall mean of 5.17 ± 0.4 compared to the overall mean of NBD-556 of 7.28 ± 0.74 ($p < 0.05$) (Fig. 5). Moreover, 882376 as well as NBD-556 did not show activity against control virus A-MLV, suggesting that the inhibitory activity of this compound is also specific to HIV-1.

2.6. Selection of 882376 resistant viruses

To further confirm the mechanism of action of the 882376 we passaged the cloned HIV-1_{NL4-3} in Jurkat cells in the presence of escalating doses of the compound. We collected cellular and viral samples at 25 μM, 33 μM and 42 μM of 882376. The dose in culture could not be escalated beyond 42 μM due to toxicity. HIV-1_{NL4-3} virus obtained from 882376 cultures and virus obtained from untreated cultures were re-passaged in fresh Jurkat cells and tittered by infecting TZM-bl cells. The results suggested that the viruses released in the culture supernatant had infectivity rate very similar compared to the untreated control Wild Type (WT) virus (data not shown).

2.7. Identification and characterization of 882376-resistant mutants

To identify the amino acid mutations responsible for the HIV-1 resistance to 882376, we isolated genomic DNA from cellular samples. DNAs were sequenced and analyzed. We detected one amino acid substitution, K348E (frequency 1/7) in the sample obtained from the culture grown in the presence of 25 μM of 882376. By contrast, we identified 5 amino acids substitutions in the sample obtained from the culture grown in the presence of 33 μM of 882376 (Table 3): K348R located in the C3 that was different (K348E) in the sample from the 25 μM culture; S411G located in the V4; K432E located in the CD4 binding site; C604Y located in the immunodominant region of gp41 and C764R also located in the gp41 (Table 3). We detected the same amino acid substitutions in the sample obtained from the culture grown in the presence of to 42 μM of 882376 with a much higher frequency (7/11).

2.8. Impact of mutations on HIV-1 entry and resistance to 882376

As next step, we wanted to verify whether the above identified amino acid substitutions were responsible for the resistance of the HIV-1_{NL4-3} to 882376. We introduced single amino acid substitutions into the pHXB2-Env expression vector. We infected U87-CD4-CXCR4 cells with WT and mutant HIV-1_{HXB-2} luciferase-expressing pseudoviruses in the absence or in the presence of 882376. We used NBD-556 and BMS-378806 as controls. As reported in Table 4, we observed that the K348R substitution had virtually no effect on antiviral activity as indicated by the low IC_{50} detected that was similar to the IC_{50} obtained for the HIV-1_{HXB2} WT. The conservative substitution (K → R) supports this finding. Also, the substitution S411G only induced a 2-fold increase in 882376 IC_{50} and had no impact on NBD-556 and BMS-378806 IC_{50}s. By contrast, we observed that the amino acid substitution detected in the CD4 binding region K432E conferred resistance not only to 882376 which exhibited an $IC_{50} \geqslant 25$, about 6-fold the IC_{50} obtained for the HIV-1_{HXB2} WT but also to NBD-556 and BMS-378806. Moreover, one of the two substitutions detected in the gp41 region, C764R conferred about 3-fold increase of the IC_{50} of 882376 while the C604Y substitution detected in the highly conserved immunodominant region produced non-infectious virus when expressed as a single mutation. The substitution of Cysteine 604 with Tyrosine (C604Y) in the unprocessed gp160 disrupts the gp41 disulfide loop (C598/C604) which has been reported to be critical to the cellular protease furin recognition site of gp160[21] therefore, impedes the processing of gp160 resulting in non-infectious viral particles. When substitutions S411G and K432E were combined we noticed a slightly increase in viral infectivity suggest-

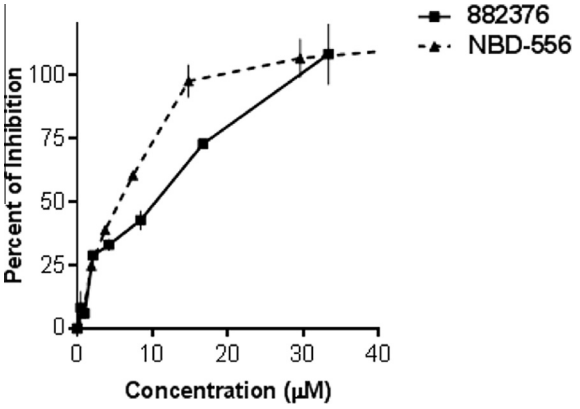


Figure 2. Cell-to-cell fusion inhibition assay. Inhibitory activity of 882376 and NBD-556 on HIV-1 mediated fusion of MAGI-CCR5 cells and HL2/3 cells. Experiments were performed in triplicate, and data is shown as mean ± standard deviation.

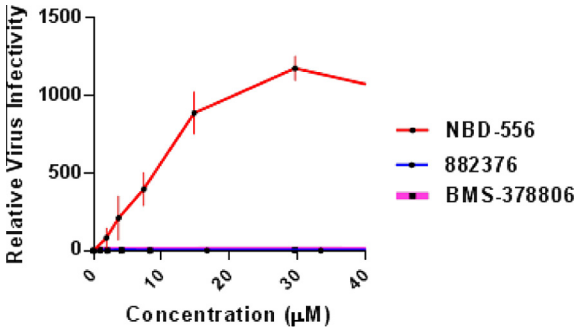


Figure 3. CD4-independent infectivity assay. CD4-negative Cf2Th-CCR5 cells were infected with CD4-dependent HIV-1_{ADA} in the presence of increasing concentrations of 882376, NBD-556 and BMS-378806. Relative virus infectivity specifies the amount of infection detected in the presence of the compounds relative to the infection detected in the absence of the compounds. Three independent experiments were performed in triplicate and the graph is representative of one experiment; the values represent the mean ± standard deviation.

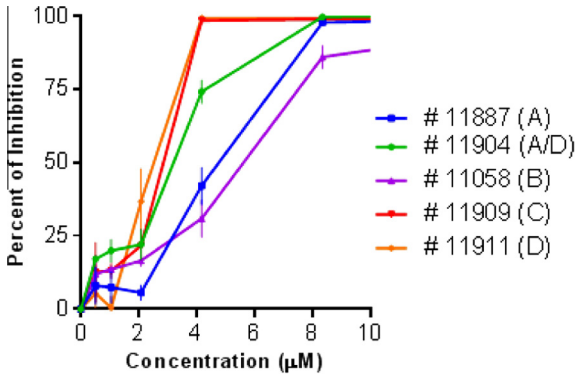


Figure 4. Representative dose-dependent inhibition curves of 882376 against ENV pseudotyped HIV-1. Experiments were performed in triplicate, and data is shown as mean ± standard deviation.

ing that these amino acid substitutions may be rescuing the virus (data not shown). To better understand the binding site of 882376 we also investigated its inhibitory activity against pseudoviruses carrying the S375H or S375Y mutation located in the Phe43 cavity. These mutations reduced the ability of 882376 to inhibit HIV-1 as evidenced by the 4-fold increase in IC_{50}. Furthermore, we tested 882376 with a set of mutated pseudoviruses based on BMS-378806 resistance study.[20] We selected the mutations that conferred the highest resistance of HIV-1_{LAI} to BMS-378806, the two single mutations M475I and M434I. As expected, our results indicated that these mutations induced resistance to 200 nM BMS-378806 and only the M475I induced resistance to NBD-556 but both mutants showed marginal resistance to 882376 (about 3-fold). Taken together these results suggest that 882376 may interfere with the gp120/CD4 interaction preventing HIV-1 infection.

2.9. Mutant viruses do not infect CD4-negative cells

To verify whether the mutated viruses acquired a CD4-independent phenotype that does not require interaction with CD4 for infection, we infected, in parallel, CD4-negative ACTOne-CXCR4 cells and CD4-positive U87-CD4-CXCR4 cells with the same amounts of mutant pseudovirus carrying a single amino acid substitution or with WT HIV-1_{HXB2} as a control. None of the mutant pseudoviruses acquired the ability to efficiently infect the CD4-negative ACTOne-CXCR4 cells (Fig. 6).

3. Discussion

Currently, after over 20 years of research, there is no effective HIV vaccine available. The HAART is the only available treatment which stabilizes the viremia but does not eradicate HIV and more importantly does not prevent the spread of HIV. Moreover, the emergence of recombinant and resistant viruses emphasizes the need of novel anti-retroviral agents. In this study, we investigated the effectiveness of 882376, a novel unnatural amino acid selected from the most potent inhibitor M48U12,[13] and its analogs as HIV-1 entry inhibitors that target and disrupt the gp120/CD4 binding. Single cycle neutralization experiments indicated that 882376 inhibits HIV-1 infection regardless of the co-receptor usage as demonstrated by its low micromolar antiviral activity against CXCR4-tropic (lab-adapted HIV-1_{HXB2} and HIV-1_{NL4-3}) and CCR5-tropic (primary isolates, Table 2) HIV-1 viruses. Additionally, 882376 showed broad spectrum inhibition against a diverse panel of HIV-1 Env-pseudotyped viruses exhibiting better activity against subtypes A, A/G and B viruses.

To further understand the possible implications of the amino acid mutations on the envelope glycoproteins by this unnatural amino acid we isolated and genotyped HIV-1 clones resistant to 882376. We identified three amino acid substitutions in gp120 and two in the gp41 region. The substitutions K432E located near the gp120/CD4 binding region conferred the highest resistance indicated by 6-fold increase in IC_{50} with respect to the WT (Table 4) suggesting that the gp120/CD4 binding region may be the target of

Table 2
Neutralization activity (IC_{50}) of 882376 against a panel of HIV-1 Env pseudoviruses

Subtype	NIH #	IC_{50} (μM) ± SD[a]		
		ENVs	NBD-556	882376
A	11887	Q259env.w6	9.5 ± 0.3	4.8 ± 0.5
	11888	QB726.70M.ENV.C4[c]	20.2 ± 6	5.5 ± 0.5
	11889	QB726.70M.ENV.B3	8.1 ± 1.5	12.5 ± 0.8
	11890	QF495.23M.ENV.A1[c]	13.6 ± 2.3	1.9 ± 0.2
	11891	QF495.23M.ENV.A3[c]	27.8 ± 9.3	2.7 ± 0.2
	11892	QF495.23M.ENV.B2[c]	20.4 ± 7	2.8 ± 0.3
	11899	1.QH359.21M.ENV.D1	3.6 ± 0.6	12 ± 1.3
		BG505-T332N[c]	11.5 ± 6	6.7 ± 2.3
		KNH1144[c]	20 ± 3.2	3.5 ± 0.4
A/D	11901	QA790.204I.ENV.A4[c]	12.6 ± 2.7	5.2 ± 0.9
	11903	QA790.204I.ENV.C8	9.7 ± 1.8	5.5 ± 0.5
	11904	QA790.204I.ENV.E2	15 ± 1.7	3.2 ± 0.06
A2/D	11905	QG393.60M.ENV.A1	6.9 ± 0.6	11.2 ± 2.5
	11906	QG393.60M.ENV.B7[c]	3.5 ± 0.8	10.3 ± 0.6
	11907	QG393.60M.ENV.B8	4.1 ± 1	5.8 ± 1.6
A/E	11603 (potential)	CRF01_AE clone 269[c]	10.7 ± 1.5	4.4 ± 0.5
		AA058	2.1 ± 0.3	3 ± 0.7
		CM244	4.2 ± 0.6	9.6 ± 0.4
A/G	11597	CRF02_AG clone 253	6 ± 1.7	3.9 ± 0.2
	11601	CRF02_AG clone 263	7.3 ± 1.7	6.1 ± 0.3
	11602	CRF02_AG clone 266[c]	4.6 ± 1.6	2.2 ± 0.3
	11605	CRF02_AG clone 278[c]	6.5 ± 1.1	3 ± 0.3
B		B41[c]	4.5 ± 2	9.4 ± 0.5
	11563	p1058_11.B11.1550[b,c]	1.9 ± 1	2.4 ± 0.1
	11578	pWEAUd15.410.5017[b]	4.3 ± 0.6	3 ± 0.3
	11018	QH0692, clone 42[c]	2 ± 0.6	1.4 ± 0.2
	11022	PVO, clone 4[c]	3.5 ± 0.7	3.2 ± 0.2
	11023	TRO, clone 11[c]	4.1 ± 0.6	3.2 ± 0.1
	11033	pWITO4160 clone 33[c]	4.8 ± 0.8	6.3 ± 0.3
	11035	pREJO4541 clone 67[c]	3.4 ± 0.2	2.8 ± 0.2
	11036	pRHPA4259 clone 7[c]	4.3 ± 0.5	4.1 ± 0.6
	11037	pTHRO4156 clone 18[c]	8.4 ± 2.4	4.2 ± 0.7
	11038	pCAAN5342 clone A2	4.1 ± 0.3	5.3 ± 0.5
	11058	SC422661.8	4.8 ± 0.7	5.7 ± 0.2
C	11306	Du156, clone 12[c]	5 ± 0.9	3.5 ± 0.3
	11307	Du172, clone 17[c]	4.8 ± 0.2	3.3 ± 0.2
	11308	Du422, clone 1[c]	6.1 ± 0.5	3.1 ± 0.1
	11309	ZM197M.PB7[c]	3.8 ± 0.3	4.8 ± 0.5
	11310	ZM214M.PL15[c]	4.1 ± 0.7	3.2 ± 0.2
	11311	ZM233M.PB6[c]	6.6 ± 0.8	6.9 ± 0.2
	11312	ZM249M.PL1[c]	6.1 ± 0.3	7.3 ± 0.2
	11313	ZM53M.PB12[c]	14.6 ± 1	3.2 ± 0.2
	11314	ZM109F.PB4[c]	6.2 ± 0.3	7.2 ± 0.5
	11315	ZM135M.PL10a[c]	2.2 ± 0.6	7.7 ± 1.1
	11316	CAP45.2.00.G3	6.2 ± 2.4	9.8 ± 1.5
	11317	CAP210.2.00.E8[c]	5.3 ± 0.6	3.6 ± 0.1
	11908	QB099.391M.ENV.B1[c]	5.7 ± 0.9	2.5 ± 0.2
	11909	QB099.391M.ENV.C8[c]	3.6 ± 0.9	2.7 ± 0.2
D	11911	QA013.70I.ENV.H1[c]	9.4 ± 2.6	2.4 ± 0.2
	11912	QA013.70I.ENV.M12[c]	5.4 ± 0.3	3.3 ± 0.1
	11916	QD435.100M.ENV.B5[c]	4.4 ± 1	4 ± 0.2
	11917	QD435.100M.ENV.A4	4.4 ± 0.7	8.4 ± 0.9
	11918	QD435.100M.ENV.E1	4 ± 0.4	10.2 ± 0.6
	Control	A-MLV	>50	>23

[a] The reported IC_{50} are means ± standard deviations ($n = 3$).
[b] R5X4-tropic viruses; all the rest were R5-tropic viruses.
[c] NBD-556 data previously published (Ref. 16).

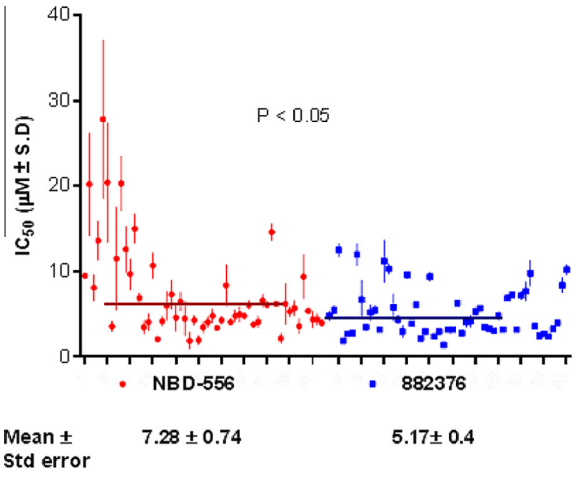


Figure 5. Comparison of IC_{50} obtained for NBD-556 and 882376 against a large panel of HIV-1 pseudoviruses from different clades. The significance is referred as the mean ± standard error.

Table 3
Amino acid substitutions detected by sequencing HIV-1_{NL4-3} resistant to different doses of 882376

Mutation	Frequency			Location
	25 μM	33 μM	42 μM	
K348R	(K/E) 1/7	1/6	7/11	C3
S411G	0/7	1/6	7/11	V4
K432E	0/7	1/6	7/11	CD4 binding site
C604Y	0/7	1/6	7/11	gp41 (immunodominant region)
C764R	0/7	1/6	7/11	gp41

The numbering was based on HIV-1_{HXB2} in the Los Alamos HIV database.

Table 4
Sensitivity of mutated-pseudoviruses to 882376

	882376 (μM)	NBD-556 (μM)	BMS-378806 (nM)
Toxicity CC_{50}	>50	>100	—
HXB2 WT IC_{50}	4 ± 0.9	7.4 ± 0.8	~1
882376 mutants			
K348R	5.6 ± 1	7.3 ± 0.2	~1
S411G	8.4 ± 1.6	7.4 ± 2.6	~1
K432E	⩾25	>30	~25
C764R	11.6 ± 0.2	7.8 ± 1.2	~1
S411G/K432E	12.2 ± 0.9	22.3 ± 1.2	~25
Phe43 cavity mutants			
S375H	>21	>20	>200
S375Y	>21	>30	>200
BMS-378806 mutants			
M475I	>15	>20	>200
M434I	15.6 ± 2.6	1.2 ± 0.8	~25

this compound. This finding was also supported by the poor antiviral activity of 882376 against two mutant pseudoviruses, M475I and M434I carrying amino acid substitutions located at or near the CD4 binding pocket, as shown by the 3-fold increase in IC_{50} with respect to the WT virus (Table 4). CD4 binds the HIV gp120 between the inner and outer domain creating a conserved cavity, known as the Phe43 cavity. Amino acid substitutions in this region especially the residue 375 (S/Y, S/H or S/W) may partially fill the cavity conferring resistance to neutralization by HIV-1 entry inhibitors that target the gp120/CD4 binding pocket. In fact, the substitution S375H was suggested to be responsible for the resistance of the CRF01_AE HIV-1 to a potent entry inhibitor, BMS-599793.[22]

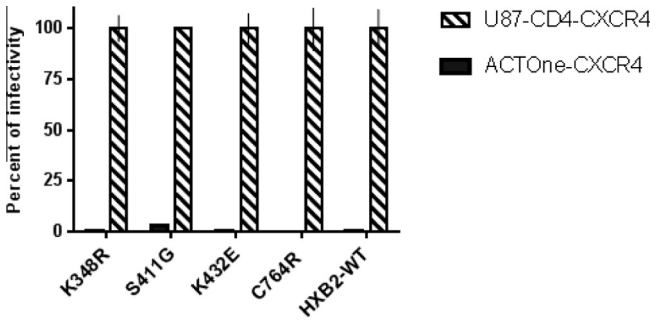


Figure 6. CD4-dependent (U87-CD4-CXCR4 cells) and CD4-independent (CD4-negative ACTOne-CXCR4 cells) infectivity assay of mutant and WT HIV-1_{HXB2} pseudoviruses. Infectivity of the pseudoviruses detected in the ACTOne-CXCR4 cells is reported as percentage of infectivity with respect to the infectivity detected in the U87-CD4-CXCR4 cells. Values represent the mean ± standard deviation of five independent experiments.

The S375Y substitution was shown to confer the highest resistance to entry inhibitors NBD-556, NBD-09027 and NBD-11008 as well as BMS-378806.[10] In this study we tested 882376 against two mutant pseudoviruses carrying single substitution S375H and S375Y. Also in these neutralization experiments we detected that the IC_{50} of 882376 against both mutants increased by about 4-fold with respect to the WT, again supporting the hypothesis that 882376 binds in the gp120/CD4 binding region. Another unique feature of this compound is that induces an amino acid substitution in a highly conserved region of the gp41.[21] This substitution C604Y by abolishing the disulfide loop of gp41 (C598/C604) inhibits the cellular protease furin to recognize its gp160 site and as consequence, induces the production of non-infectious viruses which carry an uncleaved gp160 incapable of binding the CD4 cellular receptor.

4. Conclusion

We can conclude that this unnatural amino acid 882376 showed a wide spectrum of inhibition against a diverse panel of HIV-1 viruses and although an X-ray structure of 882376 bound to gp120 core is not available our resistance studies and the experiments performed with the mutant viruses indicate that 882376 may target the CD4 binding site in gp120 and disrupts the gp120/CD4 interaction preventing HIV-1 infection. Therefore, 882376 has the potential as a lead compound for further optimization to more potent HIV-1 gp120 targeted entry inhibitors.

5. Experimental

5.1. Cells and plasmids

TZM-bl cells (a HeLa cell line that expresses CD4, CXCR4, and CCR5 and also luciferase under the control of the HIV-1 promoter),[23,24] HL 2/3 cells (from Drs. B.K. Felber and G.N. Pavlakis),[25] MAGI-CCR5 cells (from Dr. J. Overbaugh),[26] Jurkat cells[27] and U87-CD4-CXCR4 cells (from H. Deng and D. R. Littman)[28] were obtained through the NIH ARP. The CD4 negative Cf2Th-CCR5 cells were kindly provided by Dr. J.G. Sodroski.[29] The HEK 293T cells were purchased from ATCC. The ACTOne-CXCR4 cells, a modified HEK 293T cell line, expressing coreceptor CXCR4 without CD4 were purchased from Codex BioSolutions, Inc., Gaithersburg, MD.

The full-length, replication and infection-competent chimeric DNA pNL4-3 (from M. Martin),[30] the Env-deleted proviral backbone plasmids pNL4-3.Luc.R-.E-DNA (from N. Landau)[31,32] and the pSG3 DNA (from J.C. Kappes and X. Wu)[24,33] were obtained

through the NIH ARP. The Env-deleted proviral backbone plasmid pNL4-3KFS DNA was kindly provided by Dr. E. Freed from NIH/NCI.

The HIV-1 Env molecular clone expression vector pHXB2 (X4) DNA was obtained through the ARP from K. Page and D. Littman.[34] The Env expression vector pSVIIIenv-ADA DNA was kindly provided by Dr. J.G. Sodroski.[29] The HIV-1 Env molecular clones of gp160 genes for HIV-1 Env pseudovirus production were obtained as follows: the clones representing the standard panels A, A/D, A2/D and D and panel C (QB099.391M.ENV.C8, QB099.391M.ENV.B1) were obtained through the NIH ARP from J. Overbaugh.[35,36] The HIV-1 Env molecular clones of subtype A/G and the CRF01_AE clone 269 were obtained through the NIH ARP from D. Ellenberger, B. Li, M. Callahan, and S. Butera.[37] The AE clones AA058 and CM244 were kindly provided by Drs. R.J. McLinden and A.L. Chenine from US Military HIV Program, Henry M. Jackson Foundation (Silver Spring, MD). The HIV-1 Env molecular clones of standard reference subtype B SC422661.8, QH0692 clone 42, PVO clone 4 and TRO clone 11 were obtained through the NIH ARP from D. Montefiori, F. Gao, and M. Li. B.H. Hahn and J.F. Salazar-Gonzalez provided pWITO4160 clone 33, pREJO4541 clone 67 and pRHPA4259 clone 7. B.H. Hahn and D.L. Kothe provided pTHRO4156 clone 18 and pCAAN5342 clone A2.[24,33,38] The subtype B pWEAUd15.410.5017 and p1058_11.B11.1550 were obtained through the NIH ARP from Drs. B.H. Hahn, B.F. Keele and G.M. Shaw.[39] The subtype C HIV-1 reference panel of Env clones were also obtained through the NIH ARP from Drs. D. Montefiori, F. Gao, S.A. Karim and G. Ramjee (Du156 clone 12 and Du172 clone 17); from Drs. D. Montefiori, F. Gao, C. Williamson and S.A. Karim (Du422 clone 1), from Drs. B. H. Hahn, Y. Li and J.F. Salazar-Gonzalez (ZM197M.PB7, ZM233M.PB6, ZM249M.PL1 and ZM214M.PL15); from Drs. E. Hunter and C. Derdeyn (ZM53M.PB12, ZM135M.PL10a and ZM109F.PB4); from Drs. L. Morris, K. Mlisana and D. Montefiori, (CAP45.2.00.G3 and CAP210.2.00.E8).

The ENV pseudotyped genes of BG505.T332N, KNH1144 and B41 were kindly provided by Dr. J.P. Moore of the Weil Cornell Medical College, NY.

SV-A-MLV-env was obtained through the NIH ARP from Drs. N. Landau and D. Littman.

5.2. Synthesis

5.2.1. Synthesis of 882376 and 882896

The synthesis of **1** (882376) was performed by following a reported method.[13] 882896 was prepared from 882376 by removing protecting groups Boc and Fmoc by 4.0 N HCl in Dioxane and by 20% piperidine in DMF, respectively. The molecular mass of these molecules and all molecules reported subsequently were determined by low resolution mass spectrometry (LR-MS).

882376: ^{1}H NMR (400 MHz, CD_3Cl) δ: 7.766 (d, J = 6 Hz, 2H), 7.588–7.556 (m, 2H), 7.408–7.378 (m, 2H), 7.324–7.294 (m, 2H), 7.110 (s, 4H), 4.721–4.706 (m, 1H), 4.435–4.326 (m, 2H), 4.217–4.189 (m, 1H), 3.457 (d, J = 5.6 Hz, 2H), 3.153 (s, 2H), 1.663 (s, 4H), 1.447 (s, 9H), 1.255 (s, 4H), 1.131–1.117 (m, 3H). ESI-MS: 598.7 ($C_{36}H_{42}N_2O_6$, $[M+H]^+$).

882896: ^{1}H NMR (400 MHz, CD_3Cl) δ: 7.406 (s, 4H), 4.074–4.028 (m, 1H), 3.243–3.229 (m, 4H), 1.727–1.578 (m, 6H), 1.202–0.997 (m, 5H). ESI-MS: 276.4 ($C_{16}H_{24}N_2O_2$, $[M+H]^+$).

5.2.2. Synthesis of 2

A solution of **1** (1.5 g, 2.5 mmol) in 20 mL of 4.0 N HCl/Dioxane was stirred at room temperature for 2 h, the reaction mixture was monitored by TLC. The **1** was reacted completely then the reaction mixture was concentrated under vacuum to give **2**, which was used to next step without further workup.

5.2.3. General procedure for the synthesis of (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-((cyclohexylmethyl)amino)phenyl)propanoic acid derivative (883594, 883596, 883597, 883598, 883599, 883600)

To a solution of **2** (100 mg, 0.18 mmol) and DIPEA (65 µl, 0.37 mmol) in dry THF (4 mL) a solution of an acid anhydride or acid chloride (0.28 mmol) in dry methylene chloride (1 mL) was added dropwise with stirring at 0 °C in about 20 min. The reaction mixture was then allowed to warm up to room temperature and stirred for 2–3 h. It was then diluted with methylene chloride (50 mL) and washed with water (30 mL), saturated sodium bicarbonate (30 mL), brine (30 mL) and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo to give crude (*S*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-3-(4-((cyclohexylmethyl) amino)phenyl)propanoic acid derivative, which was purified by HPLC to obtain pure derivatives 883594 (33 mg, 96% purity), 883596 (54 mg, 98.8% purity), 883597 (73 mg, 99% purity), 883598 (54 mg, 99% purity), 883599 (25 mg, 98.9% purity), 883600 (53 mg, 97.9% purity).

883594: Yield: 33%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.896 (d, J = 7.2 Hz, 2H), 7.820 (d, J = 8.8 Hz, 1H), 7.656 (m, 2H), 7.675–7.629 (m, 2H), 7.357–7.316 (m, 4H), 7.138 (d, J = 8.0 Hz, 1H), 4.242–4.114 (m, 4H), 3.476–3.424 (m, 2H), 3.329–3.279 (m, 1H), 3.159–3.117 (m, 1H), 2.913–2.851 (m, 1H), 1.669–1.434 (m, 7H), 1.248 (m, 2H), 0.989 (s, 3H), 0.785–0.726 (m, 2H). ESI-MS: 540.7 ($C_{33}H_{36}N_2O_5$, $[M+H]^+$).

883596: Yield: 42.6%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.820 (d, J = 7.2 Hz, 2H), 7.752 (d, J = 8.8 Hz, 1H), 7.598–7.548 (m, 2H), 7.359–7.305 (m, 4H), 7.247–7.241 (m, 2H), 7.158 (d, J = 7.6 Hz, 1H), 4.180 (m, 1H), 4.098–4.045 (m, 3H), 3.469–3.417 (m, 1H), 3.107–3.063 (m, 1H), 2.860–2.798 (m, 1H), 1.480–1.331 (m, 5H), 1.238–1.166 (m, 2H), 0.932 (m, 3H), 0.781–0.706 (m, 2H). ESI-MS: 594.6, ($C_{33}H_{33}F_3N_2O_5$, $[M+H]^+$).

883597: Yield: 62.7%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.891 (d, J = 7.2 Hz, 2H), 7.842 (d, J = 8.8 Hz, 1H), 7.697 (d, J = 7.2 Hz, 1H), 7.627 (d, J = 7.2 Hz, 1H), 7.430–7.305 (m, 6H), 7.048 (d, J = 7.6 Hz, 1H), 4.272–4.232 (m, 1H), 4.110 (s, 3H), 3.417 (s, 1H), 3.325–3.274 (m, 1H), 3.173–3.131 (m, 1H), 2.900–2.837 (t, J = 12.6 Hz, 1H), 1.737–1.328 (m, 13H), 1.242 (s, 1H), 1.010 (m, 5H), 0.807–0.776 (m, 3H), 0.538–0.425 (m, 2H). ESI-MS: 622.8 ($C_{39}H_{46}N_2O_5$, $[M+H]^+$).

883598: Yield: 46.8%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.888 (d, J = 7.2 Hz, 2H), 7.832 (d, J = 8.8 Hz, 1H), 7.658–7.606 (m, 2H), 7.405–7.300 (m, 6H), 7.158–7.084 (m, 4H), 6.880 (d, J = 6.8 Hz, 2H), 4.287–4.229 (m, 1H), 4.114 (s, 3H), 3.498–3.446 (m, 2H), 3.358–3.146 (m, 4H), 2.920–0.859 (t, J = 12.2 Hz, 1H), 1.562–1.446 (m, 5H), 1.262–1.243 (m, 1H), 1.006–0.949 (m, 3H), 0.811–0.753 (m, 2H). ESI-MS: 616.8 ($C_{39}H_{40}N_2O_5$, $[M+H]^+$).

883599: Yield: 21.5%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.894 (d, J = 7.2 Hz, 2H), 7.825 (d, J = 8.8 Hz, 1H), 7.682–7.616 (m, 2H), 7.434–7.396 (m, 2H), 7.367–7.305 (m, 4H), 7.114 (d, J = 8.0 Hz, 1H), 5.567–5.500 (m, 1H), 4.790–4.727 (m, 2H), 4.278–4.220 (m, 1H), 4.151–4.105 (m, 3H), 3.330–3.278 (m, 1H), 3.166–3.124 (m, 1H), 2.908–2.846 (m, 1H), 2.098–1.855 (m, 4H), 1.550–1.432 (m, 5H), 1.243 (s, 1H), 0.999 (m, 3H), 0.842–0.708 (m, 2H). ESI-MS: 580.7. ESI-MS: 580.7 ($C_{36}H_{40}N_2O_5$, $[M+H]^+$).

883600: Yield: 42%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.892 (d, J = 7.2 Hz, 2H), 7.809 (d, J = 8.4 Hz, 1H), 7.673 (t, J = 6.8 Hz, 2H), 7.424–7.388 (m, 2H), 7.337–7.276 (m, 7H), 7.234–7.220 (m, 1H), 7.145–7.125 (d, J = 8 Hz, 2H), 5.073–5.000 (m, 2H), 4.210–4.136 (m, 4H), 3.383–3.330 (m, 2H), 3.123–3.080 (m, 1H), 2.891–2.829 (t, J = 12.4 Hz, 1H), 1.546–1.464 (m, 5H), 1.283–1.243 (m, 1H), 0.980–0.961 (m, 3H), 0.773–0.709 (m, 2H). ESI-MS: 632.4 ($C_{39}H_{40}N_2O_6$, $[M+H]^+$).

5.2.4. Synthesis of 3

To a solution of **1** (1 g, 1.67 mmol) and Na_2CO_3 (213 mg, 2.0 mmol) in DMF (10 mL), Benzyl bromide (240 μl, 2.0 mmol) was added dropwise with stirring at room temperature and stirred overnight at room temperature. Then the reaction mixture was diluted with DCM (100 mL) and washed with 5% H_2PO_3 (100 mL × 3), water (100 mL), brine (100 mL), dried over anhydride Na_2SO_4, the solvent was removed in vacuo to give 1.5 g of 3, which was used to the next step without further purification.

5.2.5. Synthesis of 4

A solution of **3** (1.5 g, 1.67 mmol) in 20 mL of 4.0 N HCl/Dioxane was stirred at room temperature for 2 h, the reaction mixture was monitored by TLC. After the reaction was complete, the reaction mixture was concentrated under vacuum to give **4**, which was used to the next step without further purification.

5.2.6. Synthesis of 5

To a solution $ClCH_2CO_2Na$ (932 mg, 8 mmol) in anhydrous THF (8 mL) isobutyl chloroformate (1.27 mL, 9.6 mmol) was added dropwise with stirring at 0 °C for 15 min, filtered and the filtrate was added to a solution of **4** (500 mg, 0.8 mmol) in anhydrous THF (4 mL), the reaction mixture was stirred at room temperature for 1 h, the solvent was removed in vacuo and the residue was dissolved in DCM (100 mL), washed with 5% H_3PO_4 (100 mL), brine (100 mL), dried over anhydride Na_2SO_4, the solvent was removed in vacuo to give 510 mg of **5**, which was used to next step without further purification.

5.2.7. Synthesis of 883595

A reaction mixture of **5** (510 mg, 0.77 mmol) and Pd/C (100 mg) in MeOH (10 mL) was stirred in an atmosphere of H_2 overnight, the reaction mixture was filtered to remove Pd/C, the solvent was removed in vacuo to give crude of 883595 (310 mg, HPLC purity: 86%), and which was purified by HPLC to give pure of 883595 (115 mg, HPLC purity is 99%).

883595: Yield: 37%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.895 (d, J = 7.2 Hz, 2H), 7.821 (d, J = 8.8 Hz, 1H), 7.679–7.625 (m, 2H), 7.434–7.366 (m, 4H), 7.343–7.301 (m, 2H), 7.222 (d, J = 8.0 Hz, 1H), 4.251–4.114 (m, 4H), 3.899–3.849 (m, 2H), 3.499–3.447 (m, 1H), 3.364–3.312 (m, 1H), 3.171–3.128 (m, 1H), 2.928–2.866 (m, 1H), 1.570–1.486 (m, 5H), 1.243 (s, 1H), 0.998 (m, 3H), 0.841–0.750 (m, 2H). ESI-MS: 575.1 ($C_{33}H_{35}ClN_2O_5$, $[M+H]^+$).

5.3. Synthesis of 1′

Fmoc-Pro-CTC resin (6.7 g, 2.0 mmol) was swelled in DMF (100 mL) for 2 h. The suspension was filtered. 20% piperidine in DMF (10 mL) was added into the resin. The suspension was kept at room temperature for 0.5 h while a stream of nitrogen was bubbled through it. Then the suspension was filtered. The resin was washed with DMF (6 × 100 mL). Fmoc-(*S*)-2-amino-3-(4-(*tert*-butoxycarbonyl(cyclohexylmethyl)amino)phenyl)propanoic acid (1.56 g, 2.6 mmol), HATU (0.936 g, 2.47 mmol), *N*-methylmorpholine (0.572 mL, 5.2 mmol) and DMF 20 mL were added into the resin. The suspension was kept at room temperature for 2 h while a stream of nitrogen was bubbled through it. When the ninhydrine test indicated the completion of the coupling, the peptidyl resin was washed with DMF (5 × 10 mL), MeOH (2 × 10 mL), DCM (2 × 10 mL) and MeOH (2 × 10 mL), then it was dried under vacuum overnight to give the peptidyl resin (7.5 g).

100 mL of AcOH/TFE/DCM (1:2:7) was added to the peptidyl resin (7.5 g) in a glass vessel. The mixture was stirred for 1 h. The suspension was filtered. The filtration was collected and the solvent was removed under vacuum to give **1′** as a white solid. 95% of purity by UV220 (1.3 g, yield: 94.9%).

5.3.1. Synthesis of 2′

To a solution of **1** (580 mg, 0.818 mmol) in HCl/dioxane (3.3 mol/L), the solution was stirred at room temperature for 2 h. The reaction completion was confirmed by TLC (DCM/MeOH/AcOH = 40:1:0.5). The solution was concentrated in vacuo to obtain **2′** (500 mg, yield = 100%).

5.3.2. Synthesis of 902132

(a) (18 mg, 0.252 mmol) was added in the mixture of a solution of **2** (75 mg, 0.126 mmol) and Et_3N (33 mg, 0.315 mmol) in dry THF (1.0 mL). The mixture was stirred at room temperature for 0.5 h. The completion of reaction was confirmed by TLC (DCM/MeOH/AcOH = 40:1:0.5). The solution was poured into water and extracted by ether. The organic phase was collected and washed by brine and dried over Na_2SO_4. Then it was concentrated and the compound was Prep-HPLC to get 902132 (32 mg) as a white solid.

Yield: 39%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.819 (d, J = 8 Hz, 3H), 7.643–7.570 (m, 2H), 7.356–7.322 (m, 3H), 7.264–7.227 (m, 2H), 6.966–6.882 (m, 2H), 5.993–5.951 (d, 1H), 5.816–5.749 (m, 1H), 5.295 (d, J = 10.4 Hz, 1H), 4.439–4.328 (m, 1H), 4.223–4.192 (m, 1H), 4.028 (s, 2H), 3.588–3.419 (m, 3H), 2.921–2.757 (m, 2H), 2.107–2.061 (m, 1H), 1.920–1.689 (m, 4H), 1.482–1.309 (m, 5H), 1.169 (s, 2H), 0.906–0.860 (m, 3H), 0.730–0.657 (m, 2H). ESI-MS: 649.8 ($C_{39}H_{43}N_3O_6$, $[M+H]^+$).

5.3.3. Synthesis of 902133

A solution of **2** (80 mg, 0.13 mmol) and Et_3N (33 mg, 0.315 mmol) in dry THF (1.0 mL), (b) (21 mg, 0.2 mmol) was added in the mixture. The mixture was stirred at room temperature for 0.5 h. The reaction completion was confirmed by TLC (DCM/MeOH/AcOH = 40:1:0.5). The solution was pour into water and extracted by ether. Collecting the organic phase was washed by brine and dried over Na_2SO_4. Then it was concentrated and the compound was Prep-HPLC to get 902133 (30 mg) as a white solid.

Yield: 35%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.899–7.851 (m, 2H), 7.729–7.655 (m, 2H), 7.440–7.404 (m, 2H), 7.346 (d, J = 7.2 Hz, 4H), 7.015 (d, J = 8 Hz, 2H), 4.832 (s, 1H), 4.705 (s, 1H), 4.449–4.414 (m, 1H), 4.292–4.269 (m, 1H), 4.125–4.080 (m, 3H), 3.384–3.333 (m, 2H), 2.955–2.786 (m, 2H), 2.160–2.132 (m, 1H), 2.016–1.870 (m, 4H), 1.588–1.463 (m, 5H), 1.245 (s, 4H), 0.977–0.718 (m, 6H). ESI-MS: 663.8 ($C_{40}H_{45}N_3O_6$, $[M+H]^+$).

5.3.4. Synthesis of 902134

(c) (40 mg, 0.25 mmol) was added in a solution of **2** (100 mg, 0.168 mmol) and Et_3N (43 mg, 0.42 mmol) in dry THF (1.5 mL). The mixture was stirred at room temperature for 0.5 h. The reaction completion was confirmed by TLC (DCM/MeOH/AcOH = 40:1:0.5). The solution was poured into water and extracted by ether. Collecting the organic phase was washed by brine and dried over Na_2SO_4. Then it was concentrated and the compound was Prep-HPLC to get 902134 (65 mg) as a white solid.

Yield: 55%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.897–7.831 (m, 3H), 7.714–7.656 (m, 2H), 7.439–7.402 (m, 2H), 7.343–7.304 (m, 3H), 7.230 (s, 1H), 7.121 (s, 1H), 6.999 (d, J = 4 Hz, 2H), 6.765 (d, J = 4.8 Hz, 1H), 4.429–4.418 (m, 1H), 4.295–4.264 (m, 1H), 4.117 (m, 3H), 3.666–3.598 (m, 2H), 3.506–3.457 (m, 2H), 2.955–2.766 (m, 2H), 2.181–1.841 (m, 4H), 1.585–1.464 (m, 5H), 1.245 (s, 1H), 1.036–0.772 (m, 5H). ESI-MS: 705.9 ($C_{41}H_{43}N_3O_6S$, $[M+H]^+$).

5.3.5. Synthesis of 902135

(d) (40 mg, 0.25 mmol) was added at −20 °C to a solution of **2** (100 mg, 0.168 mmol) and Et_3N (43 mg, 0.42 mmol) in dry THF (1.5 mL). The mixture was stirred at −20 °C-room temperature

for 0.5 h. The reaction completion was confirmed by TLC (DCM/MeOH/AcOH = 40:1:0.5). The solution was poured into water and extracted by ether. Collecting the organic phase was washed by brine and dried over Na_2SO_4. Then it was concentrated and the compound was Prep-HPLC to get 902135 (25.1 mg) as a white solid.

Yield: 21%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.890–7.843 (m, 3H), 7.692–7.656 (m, 2H), 7.415 (d, J = 8.0 Hz, 4H), 7.333–7.299 (m, 2H), 7.114–7.052 (m, 2H), 5.846 (d, J = 2.4 Hz, 1H), 5.411 (d, J = 2.8 Hz, 1H), 4.500–4.398 (m, 1H), 4.312–4.280 (m, 1H), 4.111–4.086 (m, 2H), 3.010–2.967 (m, 1H), 2.879–2.821 (m, 1H), 2.086 (s, 3H), 1.962–1.869 (m, 2H), 1.589–1.484 (m, 5H), 1.244 (s, 1H), 1.040–0.955 (m, 3H), 0.862–0.799 (m, 2H). ESI-MS: 703.8 ($C_{42}H_{45}N_3O_7$, $[M+H]^+$).

5.3.6. Synthesis of 902136

(e) (18 mg, 0.13 mmol) was added in a solution of **2′** (62 mg, 0.10 mmol) and Et_3N (25 mg, 0.25 mmol) in dry THF (1.0 mL). The mixture was stirred at room temperature for 0.5 h. The reaction completion was confirmed by TLC (DCM/MeOH/AcOH = 40:1:0.5). The solution was poured into water and extracted by ether. Collecting the organic phase was washed by brine and dried over Na_2SO_4. Then it was concentrated and the compound was Prep-HPLC to get 902136 (35 mg) as a white solid.

Yield = 51%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.352 (s, 1H), 7.897–7.852 (m, 3H), 7.711–7.652 (m, 2H), 7.437–7.305 (m, 6H), 7.108 (d, J = 8 Hz, 2H), 5.774 (s, 1H), 4.457–4.423 (m, 1H), 4.292–4.262 (m, 1H), 4.144–4.093 (m, 2H), 3.638–3.603 (m, 2H), 2.988–2.942 (m, 1H), 2.861–2.804 (m, 1H), 2.180–1.997 (m, 2H), 1.922–1.840 (m, 2H), 1.658–1.656 (m, 2H), 1.569–1.471 (m, 3H), 1.344 (m, 1H), 1.244 (m, 2H), 1.008–0.776 (m, 5H). ESI-MS: 690.8 ($C_{40}H_{42}N_4O_7$, $[M+H]^+$).

5.3.7. Synthesis of 902137

(f) (48 mg, 0.3 mmol) in dry THF (0.5 mL) was added dropwise in a solution of **2′** (150 mg, 0.25 mmol) and Et_3N (51 mg, 0.5 mmol) in dry THF (2.0 mL), at 0 °C. The mixture was stirred at 0 °C-room temperature for 0.5 h. The completion of reaction was confirmed by TLC (DCM/MeOH/AcOH = 40:1:0.5). The solution was poured into water and extracted by ether. The organic phase was collected and washed by brine and dried over Na_2SO_4. Then it was concentrated and the compound was prep-HPLC to get 902137 (56 mg) as a white solid.

Yield = 31%; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 7.893 (d, J = 7.2 Hz, 3H), 7.763–7.725 (m, 1H), 7.660 (d, J = 7.6 Hz, 1H), 7.416–7.322 (m, 6H), 7.017–6.975 (m, 1H), 4.482 (m, 1H), 4.397–4.387 (m, 1H), 4.304–4.014 (m, 2H), 3.508–3.163 (m, 3H), 2.992–2.819 (m, 2H), 2.338–2.143 (m, 2H), 2.017–0.830 (m, 3H), 1.555–1.447 (m, 8H), 1.346–1.244 (m, 5H), 1.015–1.005 (m, 6H), 0.862–0.679 (m, 6H). ESI-MS: 719.0 ($C_{44}H_{53}N_3O_6$, $[M+H]^+$).

5.4. Pseudovirus preparation

The pseudoviruses capable of single-cycle infection were prepared as previously described.[9] Briefly, HEK293T cells were transfected with a mixture of an Env-deleted proviral backbone plasmid pNL4-3.Luc.R.E DNA or pSG3 DNA and an Env expression vector by using FuGENE 6 (Promega). Amphotropic-MLV (A-MLV) pseudovirus was prepared by transfecting 293T cells with a mixture of the Env-deleted proviral backbone plasmid pNL4-3KFS DNA and A-MLV Env expression vector by using Lipofectamin 2000 reagent (Life Technologies). Pseudovirus titers to identify the 50% tissue culture infectious dose ($TCID_{50}$) by infecting the different cell types were determined as previously described.[9]

5.5. Evaluation of antiviral activity

5.5.1. Single-cycle infection assay in TZM-bl cells

The antiviral activity of the synthesized compounds against HIV-1 pseudotyped viruses expressing HXB-2 Env or a HIV-1 Env from the panel of standard reference was measured as previously described.[9] Briefly, TZM-bl cells were plated in a 96-well tissue culture plate at 10^5 cells/mL and incubated overnight. Fifty microliters of a test compound at graded concentrations was mixed with 50 μl of the HIV-1 pseudovirus. After incubation at 37 °C for 30 min, the mixture was added to the cells and incubated for 3 days. Cells were washed and lysed with 50 μl of the cell lysis reagent; 20 μl of lysate was transferred to a white 96-well plate and mixed with 100 μl of the luciferase assay reagent. We immediately measured the luciferase activity with a Tecan Infinite M1000 reader to calculate the percent inhibition by the compounds and IC_{50}s by using GraphPad Software, Inc, California.

5.5.2. Single-cycle infection assay in U87 cells

U87-CD4-CXCR4 cells were plated in 96-well plates at 10^4 cells/well and incubated overnight as previously described.[10] Briefly, pseudoviruses were pre-incubated with escalating doses of compounds for 30 min. The mixtures were then added to the respective cells and incubated for 3 days. Cells were washed and lysed with 40 μl of lysis reagent. Lysates were mixed with the luciferase assay reagent and the luciferase activity was measured as described above to calculate the IC_{50}.

5.5.3. Single-cycle infection assay in CD4-negative cells Cf2Th-CCR5

Cf2Th-CCR5 were seeded at 6×10^3 cells/well in a 96 well tissue culture plate and incubated at 37 °C overnight as previously described.[10,16] Briefly, 50 μl of a test compound at graded concentrations was mixed with an equal volume of the HIV-1_{ADA} recombinant virus. After incubation at 37 °C for 30 min, the mixtures were added to the cells and incubated for 48 h. Cells were washed and lysed with 40 μl of cell culture lysis reagent. Lysates were mixed with luciferase assay reagent and the luciferase activity was immediately measured as described above.

5.6. Cell-to-cell fusion

To evaluate the ability of 882376 to block cell-to-cell fusion mediated by HIV-1 we performed cell fusion assay as previously described.[16] MAGI-CCR5 cells (a HeLa cell clone expressing human CD4, both co-receptors CXCR4 and CCR5 and HIV-LTR-β-gal)[26,40] were used as target cells and HL 2/3 cells (a HeLa-derived cell line which express HIV-1_{HXB2} Env on the surface and Tat and other viral proteins in the cytoplasm and does not produce detectable amounts of mature virions)[25] were used as effector cells. Following fusion of the two cell types, Tat induces the expression of β-gal enzyme in the MAGI-CCR5 cells. Briefly, 1.5×10^4/well MAGI-CCR5 cells were incubated for 1 h with escalating concentrations of compounds then co-cultured with 7.5×10^3/well HL 2/3 cells for 24 h. β-gal expression was quantified with the Beta-Glo® Assay System (Promega) following the manufacturer's instructions. The percent inhibition and the IC_{50} values were calculated using the GraphPad Prism software.

5.7. Cytotoxicity assay

The cytotoxicity of test compounds in TZM-bl and U87-CD4-CXCR4 cells was measured by a colorimetric method using XTT (PolySciences, Inc., Warrington, PA) as previously described.[41] Briefly, 100 μl of the compound at graded concentrations was added to an equal volume of cells (10^5/mL) and incubated for

3 days. Four hours after the addition of XTT, the soluble intracellular formazan was quantitated colorimetrically at 450 nm. The percent cytotoxicity and the 50% cytotoxic concentration (CC_{50}) values were calculated.

5.8. Isolation and characterization of 882376 resistant viruses

To select HIV-1 resistant variants, Jurkat cells were infected with wild-type HIV-1_{NL4-3} in the presence of 25 and 33 μM of 882376. Viral replication was monitored by measuring p24 amounts in the culture medium by sandwich-ELISA. After 3 passages (10 days of culture) we detected small amounts of p24 in the cell culture medium of the cells treated with 25 μM of 882376 and after 6 passages (20 days of culture) p24 was detected in the culture medium of the cells treated with 33 μM of 882376. Thus, we escalated the concentration of the compound up to 42 μM. The cell cultures were continuously renewed by adding fresh cells during the procedure. We could not escalate the dose beyond 42 μM due to the toxicity of the compound. The cultures were expanded to collect cellular and viral samples at different doses. Viruses obtained from 882376 treated and untreated cultures were re-passaged in fresh Jurkat cells. Twenty hours post-infection the cells were washed with PBS and put back in culture. Six days later, the supernatants were filtered to remove cell debris and p24 was quantified by sandwich-ELISA. The viruses were normalized by p24 amounts and tittered by infecting TZM-bl cells to calculate $TCID_{50}$.

5.9. Genotyping of resistant and control viruses

To identify the amino acid substitutions in the gp160 responsible for conferring resistance to 882376, we extracted genomic DNA from the cells producing control WT virus and form the cells producing viruses resistant to 25 μM, 33 μM and 42 μM of 882376 by using a whole-blood DNA purification kit (Qiagen). The entire Env coding region was amplified by PCR using the Expand High-Fidelity DNA polymerase kit (Roche), cloned and sequenced. The viral DNA sequences were analyzed with the Geneious R8 software (Biomatters, New Zealand).

5.10. Drug sensitivity with ENV-mutated pseudovirus

Amino acid substitutions were introduced into the pHXB2-Env expression vector by site-directed mutagenesis (Stratagene) using mutagenic oligonucleotides and verified by sequencing the entire ENV gene of each construct. To measure the activity of the compounds against the pseudoviruses expressing different mutations, U87-CD4-CXCR4 cells were infected with the ENV-mutated pseudoviruses as described above.

5.11. Infectivity assay of CD4-negative cells

The infectivity assay of CD4-negative cells was performed as previously described.[10] Briefly, CD4-negative ACTOne-CXCR4 cells were plated at 10^4 cells/well in a 96-well tissue culture plate. The cells were infected with the wild-type (WT) HIV-1_{HXB2} and with HIV-1_{HXB2}-Env-mutant pseudoviruses expressing luciferase. In parallel, as a control, we infected U87-CD4-CXCR4 cells at 10^4 -cells/well with the same amounts of the respective pseudoviruses. After 2 days of incubation, the cells were washed and lysed. The luciferase activity was measured as described above. The infectivity of the pseudoviruses detected in the ACTOne-CXCR4 cells was reported as the percentage of infection relative to the infectivity detected in the U87-CD4-CXCR4 control cells.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.11.006. These data include MOL files and InChiKeys of the most important compounds described in this article.

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