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Anti-HIV-1 Activity of the Neurokinin-1 Receptor Antagonist Aprepitant and Synergistic Interactions with other Antiretrovirals

Mark M. MANAK¹, Dmitry A. MOSHKOFF¹, Lequan T. NGUYEN¹, John MESHKI², Pablo TEBAS³, Florin TULUC^{2,4}, and Steven D. DOUGLAS^{2,4}

¹SeraCare Life Sciences Inc., Gaithersburg, MD 20877

²Division of Allergy and Immunology, The Children's Hospital of Philadelphia Research Institute, Philadelphia, Pennsylvania 19104

³Division of Infectious Diseases, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

⁴Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

Abstract

Objectives—Neurokinin-1 receptor (NK1R) antagonists interfere with binding of neuropeptide substance P (SP) to NK1R and exhibit novel anti-HIV-1 activities. Since NK1R antagonists effectively penetrate the blood-brain barrier to reduce the inflammatory response within the brain, we wished to evaluate their potential as anti-HIV-1 candidates for targeting HIV-1 infections of the central nervous system.

Design—A series of small molecule agents were evaluated for anti-NK1R and anti-HIV-1 activity using peripheral blood mononuclear cells (PBMC). The most promising of these, aprepitant (Emend, Merck and Co. Inc.), was investigated for potential synergies with other antiretroviral drugs.

Methods—Anti-NK1R activity was tested by measuring intracellular calcium increase triggered by substance P. Anti-HIV-1 activity was evaluated by measuring p24 antigen in culture supernatants of PBMC following exposure to HIV. The concentration of drug which produced 50% reduction in intracellular calcium levels or viral production in 7-day PBMC cultures was

Author for correspondence and requests for reprints: Mark Manak, Ph.D. SeraCare Life Sciences 217 Perry Parkway Gaithersburg, MD 20877 Phone: 240.306.4109 Fax: 240.306.4209 mmanak@seracare.com.

Author Contributions: Mark M. MANAK, Principal Investigator headed up the study, designed experiments and lead author of the paper.

Dmitry A. MOSHKOFF, Assisted Dr. Manak in planning infectivity and synergy experiments.

Lequan T. NGUYEN, Executed all virus infectivity experiments, assisted in experimental design and particularly details of infection.

John MESHKI, Carried out the NK-1R activity studies.

Pablo TEBAS, Coordinated clinical studies of aprepitant and provided information on clinical utility of the drug. Provided guidance on in vitro studies.

Florin TULUC, Assisted Dr. Douglas in coordination of overall aprepitant program providing all drugs used in study and performing NK-1R activity studies.

Steven D. DOUGLAS Lead Investigator of aprepitant study for clinical evaluation and coordination of various aspects of investigation.

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determined. The combined effect of aprepitant with each of the major classes of anti-HIV-1 drugs was evaluated in synergy studies.

Results—Aprepitant had the highest anti-HIV-1 activity of the NK1R antagonists examined and was equally active against all major HIV-1 subtypes. Aprepitant acted synergistically with protease inhibitors (ritonavir and saquinavir), but not with nucleoside reverse transcriptase, non-nucleoside reverse transcriptase, or viral entry inhibitors.

Conclusion—The ability of aprepitant to penetrate the blood-brain barrier, its safety record as an FDA approved drug for reducing nausea and vomiting in chemotherapy, and synergistic activity with other anti-HIV-1 drugs make it a promising candidate for treatment of HIV infection.

Keywords

Human Immunodeficiency Virus Type 1 (HIV-1); Aprepitant (Emend, Merck and Co.); Antiretroviral drugs; Neurokinin-1 Receptor (NK1R) antagonists; Synergy

Introduction

The neuropeptide substance P (SP) modulates the interaction between the immune and nervous systems and is a major mediator of neurogenic inflammation [1,2]. The biologic responses to SP are mediated by neurokinin-1 receptor (NK1R), a G-protein coupled receptor. Drugs which interfere with binding of SP to NK1R reduce the inflammatory response [3]. SP enhances inflammatory cytokine (TNF- α , IL-1 and IL-6) production by immune cells such as macrophages through activation of NF- κ B [4]. Aprepitant (Emend, Merck & Co., Inc.), an NK1R antagonist, is approved by the FDA for the prevention of both acute and delayed chemotherapy-induced nausea and vomiting [5]. The SP-NK1R interaction is also important in viral infections, including HIV-1 infection of human immune cells. Infection of cells by HIV-1 requires the use of the chemokine receptors CCR5, CXCR4, or both (R5X4) [6]. The non-peptide SP antagonist CP-96,345 [7,8] and aprepitant [3,9,10], down regulate CCR5, the principal co-receptor for HIV-1 entry into macrophages, and inhibit HIV-1 infection of macrophages *in vitro* [8,9]. The addition of SP to macrophages *ex vivo* leads to enhanced HIV-1 expression in macrophages isolated from blood of healthy individuals [8,9,11–13] and activates HIV-1 replication in latently infected immune cells [14]. In nearly all individuals, the CCR5-tropic variants are responsible for transmission and establishment of infection, while the emergence of R5X4-tropic, and ultimately the shift to predominantly CXCR4-tropic variants, is frequently associated with disease progression and accelerated pathogenesis [6,15]. NK1R antagonists inhibit SP-induced T lymphocyte activation [16], and infection by HIV-1 [8].

In previous studies, aprepitant inhibited HIV-1 infection of monocyte-derived macrophages [8,9] and inhibited infection of AZT-resistant virus strains. Aprepitant treatment also led to decreased CCR5 expression on monocyte-derived macrophages [9]. In other studies, aprepitant acted synergistically with other antiviral agents, and significantly enhanced the anti-HIV-1 activity of the antiretrovirals zidovudine, efavirenz, indinavir and enfuvirtide in macrophages [10,11]. While either aprepitant or enfuvirtide treatment alone inhibited HIV-1 BaL infection of macrophages, the cells treated with both aprepitant and enfuvirtide had the lowest levels of HIV-1 reverse transcriptase (RT) among the cell cultures tested and at these concentrations exhibited no cytotoxic effects on macrophages. The results of these *in vitro* studies suggest that aprepitant can act synergistically with other antiviral drugs to give significantly greater inhibition of HIV-1 infection than either drug alone [9,10]. In this study, we tested additional SP antagonists for potential anti-HIV-1 activity *in vitro*, and examined the inhibition of infection by CCR5 vs. CXCR4 tropic HIV-1 isolates representing

a cross section of major world wide subtypes. Synergies with additional anti-HIV-1 drugs were further evaluated and strong synergies were found with protease inhibitors.

Materials and Methods

Infectivity studies

Inhibition of HIV-1 infectivity of PBMC *in vitro* was measured according to published methods [17]. Briefly, 96-well plates containing 200,000 PBMC per well and decreasing concentrations of drug were prepared. The PBMC used in these studies were obtained from uninfected healthy blood donors. Fresh blood samples were separated by Ficoll-Hypaque centrifugation, and PBMC were frozen in liquid nitrogen in media containing dimethyl sulfoxide (DMSO). Cells were thawed, washed and stimulated for 3 days with phytohemagglutinin (PHA). After a 1 hr pre-incubation in the presence or absence of varying concentration of drug, cells were infected with 200 TCID₅₀ of HIV-1 BaL (0.8 ng) or NL4-3 (3.5 ng) for a multiplicity of infection (moi) of 0.001. Infected cells were incubated for 7 days at 37°C in the same concentrations of anti-HIV-1 drug. Viral replication in PBMC from 3 different healthy donors was measured by quantifying p24 levels of culture supernatant following 7 days incubation at 37° C (Zeptometrix p24 ELISA). Serial dilutions of supernatant were tested in triplicate to establish a tissue culture infectious dose 50% (TCID₅₀). The inhibitory concentration of drug which resulted in 50% reduction of virus (IC₅₀) was calculated as the ratio of p24 production in test sample divided by the p24 production in the positive control. The cytotoxic concentration which resulted in 50% cell death in the absence of virus infection (CC₅₀) was determined from the OD readings of the MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) assay.

Synergy studies were performed in 96-well plates containing 2×10^5 PBMC per well and infected with BaL virus at moi = 0.01. Serial dilutions of aprepitant and HIV-1 antiviral drugs were added to the wells at 0, 0.1×, 0.5×, and 2× the TCID₅₀ for each drug. The plates were incubated for 5 days at 37° C and virus infection was determined by a tetrazolium assay in which 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) is metabolically reduced in viable cells to a water-soluble formazan product, phenazine methosulfate (PMS) [18].

Candidate antiretrovirals

A series of neurokinin-1 receptor antagonists, including aprepitant (Merck & Co, Inc., Whitehouse Station, NJ), L-733,060 (Sigma-Aldrich, St. Louis, MO), RP-67,580 (Tocris Bioscience, Ellisville, MO), L-759,274 (Merck & Co, Inc., Whitehouse Station, NJ), CP-96,345 (Pfizer, Inc, New York, NY), and CP-99,994 (Pfizer, Inc, New York, NY) were tested for potential *in vitro* antiviral activity against CCR5-tropic (HIV-1 BaL) or CXCR4-tropic (HIV-1 NL4-3) viral isolates.

The inhibitory activity of aprepitant was also tested against a panel of primary HIV-1 isolates (SeraCare Life Sciences, Inc.) grown in PBMC. These included a broad range of HIV-1 subtypes, representing both T cell tropic (CXCR4) and macrophage tropic (CCR5) primary isolates. Synergistic effects of combinations of aprepitant with representatives of each of the major classes of approved anti-HIV-1 drugs were evaluated for inhibition of HIV-1 BaL in PBMC.

Intracellular calcium measurements

U373MG astrocytoma cells were used to assess the potency of NK1R antagonists. Intracellular calcium recordings were performed as previously described [19]. Briefly,

U373MG cells were cultured in 96-well plates and incubated with Fura-2 AM (Molecular Probes, Eugene, OR) for 45 minutes at room temperature. Cells were washed with Hank's Balanced Salt Solution containing calcium chloride (1 mM) and intracellular calcium measurements were performed using Tsien's ratiometric method [16,20]. Peak intracellular calcium increases elicited by 100 nM SP were measured in the presence of different concentrations of antagonists (as indicated in each figure). The concentrations of antagonists were increased on a semi-logarithmic scale until the intracellular calcium response induced by 100 nM SP was completely blocked. Concentration-response curves were constructed and curves were fitted to data using the equation:

$$E = \min + \frac{\max - \min}{1 + \left(\frac{C}{IC_{50}}\right)^p},$$

where E is the measured effect (the increase in intracellular calcium concentration), max is the intracellular calcium increase measured in the absence of antagonist (the maximal response), min is baseline intracellular calcium level before addition of SP, C is the concentration of antagonist, and p is the slope of the concentration-response curve. Logistic curves were fitted to data and used to calculate the IC₅₀ values for each antagonist.

Results

Initial studies were performed in order to determine the effect of aprepitant on the infection of PBMC cultures with well characterized laboratory strains of HIV-1 with defined coreceptor tropism. For these studies, the viral isolate HIV-1 BaL was used as the reference CCR5-tropic virus, and the HIV-1 NL4-3 isolate as the CXCR4 tropic virus. The mean anti-HIV-1 activity (IC₅₀) of AZT on PBMCs obtained from three different healthy adult donors was 0.008 +/- 0.002 μM for HIV-1 BaL isolates and 0.005 +/- 0.004 μM for HIV-1 NL4-3 virus isolates. Similar studies with ddC showed IC₅₀ values of 0.011 +/- 0.004 μM for HIV-1 BaL, and 0.012 +/- 0.003 μM for HIV-1 NL4-3 virus. These results demonstrate that antiviral activities can be measured with reasonable precision, and that the nucleoside RT inhibitors AZT and ddC inhibit HIV-1 infection by both CCR5 and CXCR4 tropic viruses in the PBMC system. The assays have excellent reproducibility between runs and operators. We selected AZT as a control for subsequent studies because of its consistency of results, wide availability and extensive characterization in the literature [21,22].

The inhibitory effect of aprepitant on HIV-1 Infection was initially tested on human PBMC from 3 different donors' cells infected *ex vivo* with HIV-1 BaL and HIV-1 NL4-3 isolates. The average antiretroviral activity for aprepitant was IC₅₀ 5.4 μM against the BaL isolate, and 5.7 μM against the NL4-3 isolate. All 3 donors gave very similar results with both viruses (Fig. 1). The ave. concentration that resulted in 50% of virus inhibition (IC₅₀) was 6.4 μM for BaL and 8.0 μM for NL4-3. The cellular cytotoxicity in both cases was very low, with a CC₅₀ > 50 μM. Thus, aprepitant inhibits viral replication of both CCR5 and CXCR4 tropic viruses in PHA-stimulated PBMC, and has very limited cytotoxicity.

The effect of NK1R antagonists on SP-induced intracellular calcium increase in U373MG cells

In order to assess the potency of the NK1R antagonists selected for this study, we measured their ability to inhibit the intracellular calcium increase induced by SP in U373MG astrocytoma cells which endogenously express NK1R receptors [23]. Concentration-response curves (Fig. 2) were obtained by incubating different concentration of antagonist with U373 MG cells prior to addition of SP. Among the tested compounds, aprepitant,

L-733,060, CP-96,345, and CP-99,994 were most potent (Table 1), with IC₅₀ values below 100nM (0.1 μM), while RP-67,580 and L-759,274 were less potent with IC₅₀ values above 200 nM (0.2 μM).

Effect of NK1R antagonists on inhibition of HIV-1 infection in PBMC

A series of SP antagonists, including aprepitant, L733060, RP-67,580, CP-96345, and CP-99,994, were tested for potential *in vitro* antiviral activity against CCR5-tropic (BaL) or CXCR4-tropic (NL4-3) viral isolates. The ability of the NK1R antagonists to block HIV-1 BaL infection of PBMC was measured and the IC₅₀ concentrations determined as described above. The cytotoxicity was also measured using the MTS assay and expressed as CC₅₀. The results of these studies are shown in Table 1, which shows antiviral activity and toxicity of these drugs compared to that of AZT and ddC. The NK1R antagonists showed varying degrees of anti-HIV-1 activity, and did not have cytotoxic effect at concentrations as high as 25 μM. Aprepitant showed the highest antiretroviral activity (IC₅₀ 5.4 μM) of the NK1R antagonists tested, followed by L-733,060. The other NK1R antagonists had lower IC₅₀ values, or failed to show significant antiviral activity. The control drugs AZT and ddC performed as expected.

Efficacy against primary HIV-1 isolates in PBMC

Our results with NK1R antagonists confirm our previously published findings that aprepitant inhibits HIV-1 viral replication (8,9,10). In contrast to the results of previous studies in monocyte-derived macrophages, where aprepitant showed a strong inhibition of CCR5 virus infectivity, but lower inhibition by CXCR4 isolate [8], in PBMC, both the CCR5 (BaL) and CXCR4 (NL4-3) Isolates were inhibited to approximately the same extent. We further extended these studies to determine whether other HIV-1 isolates of varying tropism would be differentially inhibited by aprepitant. The inhibitory activity of aprepitant was tested against a panel of primary HIV-1 isolates cultivated in PBMC, which included representatives of a broad range of Subtypes, representing both T lymphotropic (CXCR4) and monocytoprotic (CCR5) primary isolates (Table 2).

The results of duplicate measurements are shown in Table 2 and indicate that aprepitant showed similar efficacy against each of the primary HIV-1 subtypes tested, including subtypes A, B, C, D, F, G, H, CRF01AE, CRF02AG, and Group O isolates when propagated in human PBMC. Furthermore, no significant differences were observed against T-lymphotropic (CXCR4) and M-tropic (CCR5) primary isolates. The IC₅₀ ranged between 3.86 and 19.86 μM in the various isolates, which is within a half log of the mean for all viruses and not significant. As shown in Table 2, the mean IC₅₀ values showed very similar results for virus categories with usage of CCR5, CXCR4 or R5/X4 co-receptors.

Synergy of aprepitant with antiretrovirals

An important consideration in studying any antiviral is the possible emergence of drug resistant strains and the requirement to switch therapies to alternate drugs. Current highly active antiretroviral therapies (HAARTs) often utilize combinations of drugs, and certain combinations are much more effective in reducing viral load than either drug alone. Combinations of drugs also greatly extend the time at which drug resistance is likely to emerge. Since the IC₅₀ dose of aprepitant is much higher than for many of the currently approved anti-HIV drugs, it was of particular interest to see whether aprepitant may act synergistically with other drugs, allowing better inhibition than by either drug alone.

Drug synergies studies were conducted in the PBMC system in assays employing aprepitant in combination with representatives of each of the major approved classes of HIV inhibitors. For these studies, we used the HIV-1 BaL strain propagated in PBMC at different

concentrations of aprepitant with currently approved antiretroviral drugs. The drugs evaluated in these studies represent a variety of different mechanisms of action, and are shown in Figure 3 along with their IC₅₀'s as determined in triplicate studies using our HIV-PBMC infection system.

Each drug was incubated alone or in combination with aprepitant at varying concentrations from 0 to $2.0 \times$ its previously determined IC₅₀. The results were scored as % inhibition of HIV-1 infectivity as shown in Fig. 3. Each chart in the figure shows the inhibition by the drug alone, or in combination with aprepitant. The first two panels show aprepitant alone for comparison. Synergistic effects were those in which the combination of drug showed a greater inhibition than simple additive effect. We observed significant synergistic effects for aprepitant with the protease inhibitors RTV and SQV, with a more than 2-fold greater inhibition when both drugs are used together at $0.5 \times$ and $2 \times$ TCID₅₀ than observed for either drug alone. A lesser degree of enhancement was also observed with nucleoside reverse transcriptase inhibitors AZT and 3TC. No significant synergies were observed with the NRTI, NNRTI and entry inhibitor drugs, and combinations of these drugs with aprepitant showed parallel levels of viral inhibition to that of the index drug alone. These *in vitro* PBMC results indicate that aprepitant is most effective in reducing HIV-1 infectivity if coupled with a protease inhibitor.

Discussion

These studies were designed to further characterize the *in vitro* anti-HIV-1 activity of aprepitant in a cultured PBMC system. Of several NK1R antagonists tested, all had low cytotoxicity (CC₅₀ > 25 μ M). Aprepitant exhibited the highest antiretroviral activity (IC₅₀ 5.4 μ M). Aprepitant had reproducible antiviral effect on a broad range of HIV-1 isolates in PBMC culture with notable efficacy (IC₅₀ 7.95 μ M $\pm$ 3.88) against primary infection by each major HIV-1 subtype (A, CRF02-AG, B, C, D, CRF02-AE, F, G, H and O), including CXCR4- and CCR5-tropic primary isolates propagated in PBMC, with low cytotoxicity > 50 μ M. Isolates which primarily utilize CXCR4 and CCR5 or both co-receptors were inhibited, with no apparent differences based on receptor usage. These results in PBMC are in contrast with those for monocyte-derived macrophages, where a strong preference on inhibition by CCR5 mediated infection was seen [10]. These studies suggest that inhibition of virus infectivity by aprepitant may function by more than one mechanism, depending on target cell. Strong synergistic effects were observed for aprepitant with the protease inhibitors RTV and SQV, but no significant synergies were observed with AZT, ddC, ddI, 3TC, NPV and T-20. This report extends our observations on the antiviral activities of the neurokinin-1 antagonist aprepitant. Previous studies demonstrated that aprepitant, as well as CP96,345, inhibit HIV-1 infection of monocyte-derived macrophages of HIV-1 R5-isolates (subtype A, F, and H), but showed a lower degree of inhibition for infection with the X4-tropic isolates (B and D subtype) [10]. Those studies showed that aprepitant treatment also inhibited HIV-1 infection of GAGO or JRFL HIV-1 strains in 7-day cultured microglia, while in the macrophage system, aprepitant enhanced the antiviral activity of T20, enfuvirtide, the HIV-fusion inhibitor. Thus although aprepitant may act by modulating CCR5 expression, the current studies show that additional antiviral effects not involving CCR5 may also operate in the PBMC system. We observed that the concentrations of NK1R antagonists required to induce inhibition of HIV-1 infection were higher than the corresponding concentrations required for inhibiting SP-induced intracellular calcium increase. It is known that the full length NK1R intracellular signaling mechanisms involve Gq activation and intracellular calcium increase [24]). This observation suggests that more complex mechanisms of virus inhibition than the simple blockade of the full length NK1R are involved. These mechanisms may be related to the modulation of expression of the HIV-1 co-receptors on the target cells [8] and they may require the engagement of the

truncated NK1R. This hypothesis is supported by the observation that the affinity of SP for the truncated receptor is about 10-fold lower than the affinity for the full length receptor [25]). Furthermore, interactions of the studied antagonists with molecular targets other than the NK1R cannot be excluded based on our observations, and they might contribute to the antiviral effect.

The present findings extend earlier observations and now demonstrate that aprepitant, L-733,060, RP-67,580, L-759,274, and CP-96,345 have low-potency antiviral effect against the HIV-1 BaL strain. Furthermore, we have shown that aprepitant is active against a broad range of HIV-1 isolates in peripheral blood mononuclear cell cultures and we have observed that there is synergy between aprepitant and the protease inhibitors in the PBMC virus culture system. The fact that this drug is already used clinically to control nausea in chemotherapy and has demonstrable anti-HIV-1 activity which is synergistic with other antiretrovirals, make it an excellent candidate as a potential anti-HIV-1 therapeutic agent for control of neurologic infections. The average peak levels of aprepitant (C max) after a single oral dose of 240 mg can reach values as high as 3.8 µg/ml (26), which is equivalent to 7 µM. This concentration is above the EC50 value determined in our study. Furthermore, it is reasonable to assume that higher doses of aprepitant will yield even higher plasma concentrations, which are expected to have measurable anti-HIV effect in vivo.

Furthermore, administration of aprepitant with a CYP3A4 inhibitor such as ritonavir, is expected to result in increased plasma levels of the drug due to inhibition of the induction phenomenon that has been reported with extended aprepitant administration (27,28). A phase 1B clinical trial evaluating the pharmacokinetic profile and safety of aprepitant in HIV-infected individuals is underway.

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Figure 1.

A. Viral replication in isolated PBMC was measured by assaying p24 in cell culture media collected from cells infected with either HIV-1 BaL or HIV-1 NL4-3, at varying concentrations of Aprepitant to determine the concentration at which virus replication is inhibited by 50% (IC₅₀). B. The 50% cytotoxic concentrations (CC₅₀) were determined in uninfected cells. All results represent the average of triplicate measurements.

Figure 2. Effect of NK1R Antagonists on SP-Induced Intracellular Calcium

Effect of NK1R antagonists on SP-induced intracellular calcium increase in U373MG astrocytoma cells. U373 MG cells were incubated in the presence of different concentrations of antagonist then substance P (100 nM) was added to medium while intracellular calcium concentrations were recorded as described under Materials and Methods. Curves were fitted to data by non-linear regression and they were used to calculate the EC_{50} values listed in Table 1.

Figure 3. Synergy Studies of Aprepitant with HAART Drugs

Synergy studies of aprepitant with known HAART drugs. Serial dilutions of aprepitant (0x, 0.1x, 0.5x and 2.0x IC_{50}) with the respective drugs were tested on PBMC infected with HIV-1 BaL virus. The 1 x IC_{50} values for each drug (in μM) are shown under each figure. Viral inhibition as measured by p24 after 5 days of infection is shown..

Table 1
NK1R Antagonist Potency Assessment

NK1R Antagonist Potency Assessment NK1R antagonist potency was determined in U373 MG cells. The drug concentration that causes 50% inhibition of intracellular calcium increase (IC_{50}) determined by non-linear regression for a series of NK1R agonists. Anti-HIV-1 activity of NK1R antagonists against BaL. The drug concentration that causes 50% inhibition of HIV-1 infection (IC_{50}) and 50% toxicity (CC_{50}) was determined for a series of NK1R antagonists. AZT and ddC served as controls.

Compound	Inhibition of $[Ca^{2+}]_i$ increase	Inhibition of HIV-1 replication	Cytotoxicity
	IC_{50} (μM)	IC_{50} (μM)	CC_{50} (μM)
Aprepitant	0.047	5.4	>50
L-733,060	0.04	5.6	>25
RP-67,580	0.29	11	>25
L-759,274	0.22	13.8	>25
CP-96,345	0.036	15.2	>25
CP-99,994	0.09	N/A	>25
AZT	N/A	0.011	>10
ddC	N/A	0.008	>10

N/A – no activity; ND – not determined.

Table 2

Anti-HIV-1 Activity of Aprepitant on PBMC Infection

Anti HIV-1 activity of aprepitant on PBMC infection by a series of HIV-1 subtypes including HIV-1 Circulating Recombinant Forms and HIV-1 Group O. Aprepitant is active against all isolates measured. No apparent differences between CCR5 and CxCR4 tropic isolates were found in these studies.

HIV-1 Subtype	Isolate ID	Country of Origin	NSI/SI (N/S)	Co-receptor	Aprepitant IC ₅₀ μ M	Mean IC ₅₀
A	UG275	Uganda	N	R5	5.65	7.46
A	I-2496	Ghana	N	R5	7.65	
CRF01_AE	ID17	Indonesia	N	R5	7.13	
CRF02_AG	DJ263	Djibouti	N	R5	11.7	
B	US2	USA	N	R5	3.86	
C	ZAM18	Zambia	N	R5	5.33	
F	BZ163	Brazil	N	R5	19.86	
G	199J	Zaire	NSI	R5	6.66	
H	BCF-KITA	Zaire	N	R5	5.97	
CRF01_AE	NP03	Thailand	S	X4	4.79	7.47
B	BZ167	Brazil	S	X4	6.37	
D	SE365	Senegal	S	X4	6.85	
D	UG270	Uganda	S	X4	9.82	
F	BCI-R07	Romania	S	X4/R5	9.53	8.84
O	BCF-06	Cameroon	S	X4/R5	8.15	