Available online at www.sciencedirect.com

ScienceDirect

Virology 362 (2007) 38–49

VIROLOGY

www.elsevier.com/locate/yviro

HIV-1 integration is inhibited by stimulation of the VPAC2 neuroendocrine receptor

Payman Baradar Bokaei [a,d], Xue-Zhong Ma [b], Darinka Sakac [d], Donald R. Branch [a,b,c,d,*]

[a] *The Institute of Medical Science, University of Toronto, Toronto, Ontario, Canada M5G 2M1*
[b] *Departments of Medicine and Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada M5G 2M1*
[c] *The Division of Cell and Molecular Biology, Toronto General Research Institute, Toronto, Ontario, Canada M5G 2M1*
[d] *Research and Development, the Canadian Blood Services, Toronto, Ontario, Canada M5G 2M1*

Received 25 July 2006; returned to author for revision 11 October 2006; accepted 8 December 2006
Available online 25 January 2007

Abstract

Successful HIV-1 infection requires a number of specific stages leading to integration of the provirus. We previously suggested that members of the VPAC neuroendocrine receptor family may play a role in HIV-1 infection. We now show that stimulation of the VPAC2 receptor with specific agonists provides strong resistance to HIV-1 infection. Daily stimulation of VPAC2, but not VPAC1 or PAC1, resulted in up to 90% inhibition of X4 or R5 productive infections in either cell lines or PBMCs. VPAC2 agonist stimulation had no effect on cell surface co-receptors, the rate of apoptotic cells, or HIV-1 entry or reverse transcription of viral RNA. However, we provide evidence that VPAC2-specific agonists inhibit HIV-1 infection through an inhibitory effect on the ability of the HIV-1 cDNA to integrate into the host DNA. These data reveal that VPAC2 agonists are appropriate candidates for further study as possible treatments aimed at the amelioration of HIV/AIDS.

Keywords: VPAC2 receptor; HIV/AIDS; VPAC2 agonists; Helodermin; HIV integration

Introduction

It has long been appreciated that HIV-1 infection requires the expression of both CD4 and a chemokine co-receptor for infection of susceptible cells (Dalgleish et al., 1984; Alkhatib et al., 1996; Feng et al., 1996). More recent studies have shown that other receptors, such as DC-SIGN, may also play a role in HIV pathogenesis (Turville et al., 2003), while other investigators have begun to address in earnest the potential role of other cell surface molecules such as glycosphingolipids in HIV pathogenesis (Fantini et al., 2000; Puri et al., 2004; Lund et al., 2006). Furthermore, we have previously reported that a neuroendocrine receptor, VPAC1, may act to facilitate HIV-1 infection (Branch et al., 2002). Thus, the entire spectrum of host factors important for HIV infection remains incomplete.

Several receptors have been described (Ulrich et al., 1998) that interact with structurally-related regulatory neuroendocrine peptides including vasoactive intestinal peptide (VIP), secretin, and pituitary adenylate cyclase-activating polypeptide (PACAP) (Ulrich et al., 1998; Harmar et al., 1998). These neuroendocrine receptors belong to the class II (or group B) subfamily of 7-transmembrane G-protein-coupled receptors (Ulrich et al., 1998; Harmar et al., 1998). There are three members of this receptor family: VPAC1 (Sreedharan et al., 1993), VPAC2 (Svoboda et al., 1994) and PAC1 (Ulrich et al., 1998; Harmar et al., 1998). One or more of these receptors can be found in almost all human tissues including human immune cells and brain cells, both targeted by HIV-1.

Investigators have previously alluded to a possible role of neuroendocrine peptides in HIV infection (Pert et al., 1988). Early reports claimed that antibodies present in HIV infected individuals showed spectral and sequence homologies to VIP and peptides derived from HIV-1 gp120 that bound these antibodies (Veljkovic et al., 1992). Indeed, we have previously shown that using specific antibodies to VIP can recognize and

* Corresponding author. Canadian Blood Services/Toronto General Research Institute, 67 College Street, Toronto, Ontario, Canada M5G 2M1. Fax: +1 416 974 9757.
E-mail address: don.branch@utoronto.ca (D.R. Branch).

0042-6822/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2006.12.012

immunoprecipitate HIV-1 (Branch et al., 2002). It has also been shown, *in vitro*, that VIP and other VPAC1 and VPAC2 agonists, but not VPAC2-specific agonists, can directly activate HIV-1 transcription via the HIV-1 long terminal repeats (LTR) (Gilles et al., 1998) and we have recently shown that HIV-1 interacts with VPAC1 on the cell surface to transduce a facilitation signal for HIV-1 infection that can increase productive infection; we speculated that this may be through effects on integration/transcription (Branch et al., 2002).

Significant differences exist between VPAC1 and VPAC2, both in structure and function (Nicole et al., 1998; Xia et al., 1996; Tsutsumi et al., 2002; Bokaei et al., 2006). These two receptors, although having similar extracellular and transmembrane domain structure, have very different cytoplasmic tails with the potential for differential signaling. Indeed, there are reports that indicate opposing functions of these two receptors (Xia et al., 1996; Tsutsumi et al., 2002). Of significance is that agonist stimulation of VPAC receptors, including agonists that cross-react with VPAC1 and VPAC2, only activate LTR transcription through VPAC1 stimulation (Gilles et al., 1998). This possibility of differential signaling coupled with opposing functions involving VPAC1 and VPAC2 led us to hypothesize that, if VPAC1 stimulation results in facilitation of HIV-1 infection (Branch et al., 2002) and activation of LTR transcription (Gilles et al., 1998), then stimulation of VPAC2 may have an opposing functional outcome and result in inhibition of HIV-1 infection. Therefore, the aim of this current study is to test this hypothesis. Our results, presented herein, support this hypothesis as we show that by using specific agonists to stimulate VPAC2, we are able to significantly inhibit productive HIV-1 infection. Moreover, we show that this inhibitory effect is likely mediated by a profound negative effect on the ability of the viral cDNA to successfully integrate into the host genome.

Results

VPAC2 stimulation inhibits HIV-1 infection

To test the hypothesis of an inhibitory role of VPAC2 in HIV infection, we initially used helodermin, a 35 amino acid peptide originally isolated from the saliva of the Gila monster (*Helodermis suspectum*) that preferentially stimulates VPAC2 (Robberecht et al., 1996). We found that a single helodermin-pretreatment of Jurkat cells prior to HIV-1_{IIIB} infection resulted in a dose-dependent inhibition of productive infection (data not shown). Complete inhibition of HIV-1 infection was achieved, but this required relatively high concentrations of helodermin (10^{-4} M), the IC_{50} was 10^{-5} M. Furthermore, this inhibitory effect of helodermin was transient. Thus, when using a single, high-dose of helodermin, the inhibition of HIV-1 infection of Jurkat cells was greatest on day 2 (73%, $p=0.0001$) when compared to day 3 (42%, $p=0.04$) (Fig. 1A).

Hut78 cells expressing VPAC2 are resistant to HIV-1 infection

To ascertain whether the inhibitory activity of a single high-dose pre-treatment with helodermin was mediated by VPAC2,

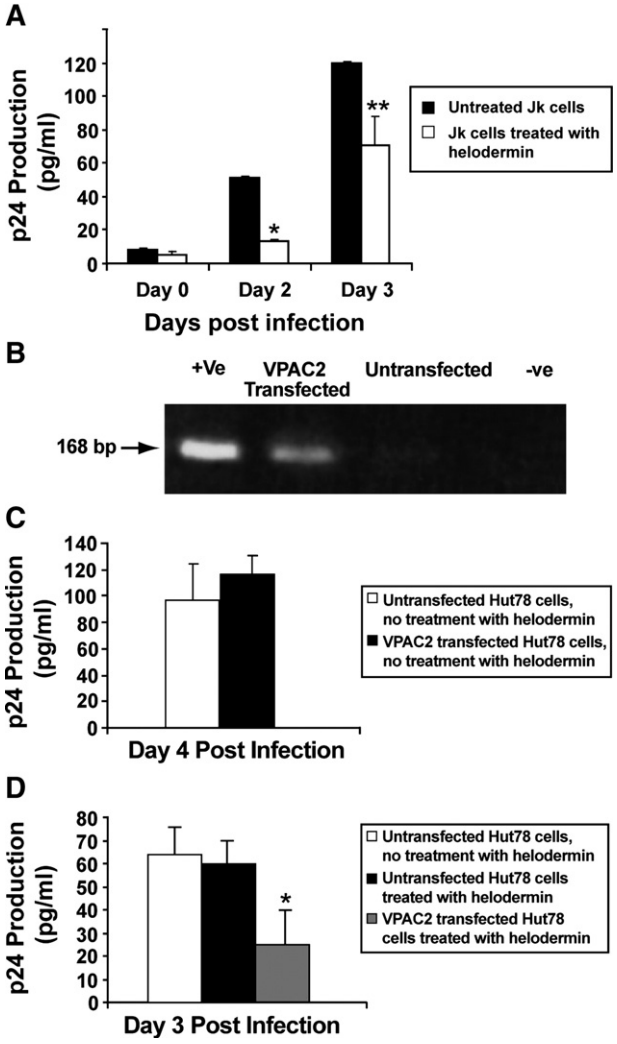

Fig. 1. Helodermin inhibits productive HIV-1 infection of cells expressing VPAC2. (A) Jurkat cells that express both VPAC1 and VPAC2 were treated with 5×10^{-5} M helodermin for 1 h prior to infection. Treated and untreated cells were washed and then infected with HIV-1_{IIIB} (moi, 0.1). Cells were washed and cultured for 4 days. The level of infection was measured by $p24^{gag}$ production at days 2 and 3, respectively. The results represent the mean pg/ml $p24^{gag}\pm$SEM from four separate cultures (*$p=0.0001$; **$p=0.04$). Similar results were obtained in six additional independent experiments. (B) Expression of VPAC2 mRNA in transfected and wild type Hut78 cells by RT-PCR. (C) Hut78 cells, untransfected or transfected with VPAC2 cDNA, were infected with HIV-1_{IIIB} and left untreated for 4 days. The level of productive infection was measured by $p24^{gag}$ production. (D) Hut78 cells, untransfected or transfected with VPAC2 cDNA, were untreated or treated with 5×10^{-5} M helodermin for 1 h prior to infection with HIV-1_{IIIB}, then washed and cultured for 3 days. The results represent the mean pg/ml $p24^{gag}\pm$SEM from four separate cultures (*$p=0.008$).

we used Hut78 cells transfected with VPAC2. Hut78 are infected with HIV-1 but do not express endogenous VPAC2 (Xia et al., 1996). Using a high expressing clone (Fig. 1B), we first confirmed that the level of infection for either transfected or untransfected Hut78 was equal when there was no helodermin (Fig. 1C). We next showed resistance to infection of Hut78 transfected to over-express VPAC2 when pre-treated with helodermin compared to helodermin-treated untransfected cells

($p=0.008$) (Fig. 1D). Thus, helodermin inhibition of HIV-1 infection appears to be mediated through the VPAC2 receptor.

Daily treatment with low-dose VPAC2 agonists sustains resistance to HIV-1 infection

To explore whether the transient nature of the inhibitory effect of VPAC2 agonist stimulation can be overcome, we used daily treatment with much lower concentrations of VPAC2 agonists. We now found that Jurkat cells treated daily with 10^{-9} M helodermin show ~90% inhibition of HIV-1 infection that is sustained over time post infection when compared to untreated cells ($p=0.002$)(Fig. 2A). To confirm that inhibition of HIV-1 infection is through VPAC2 receptor, additional high-affinity selective agonists to VPAC2 and other VPAC receptors were tested. We first tested R3P55 peptide and a control peptide called PAC10 that has no activity for VPAC receptors (Tsutsumi et al., 2002). Using daily treatment with 10^{-9} M of each peptide, there was no inhibition of HIV-1 infection using PAC10-treated Jurkat cells compared to untreated cells.

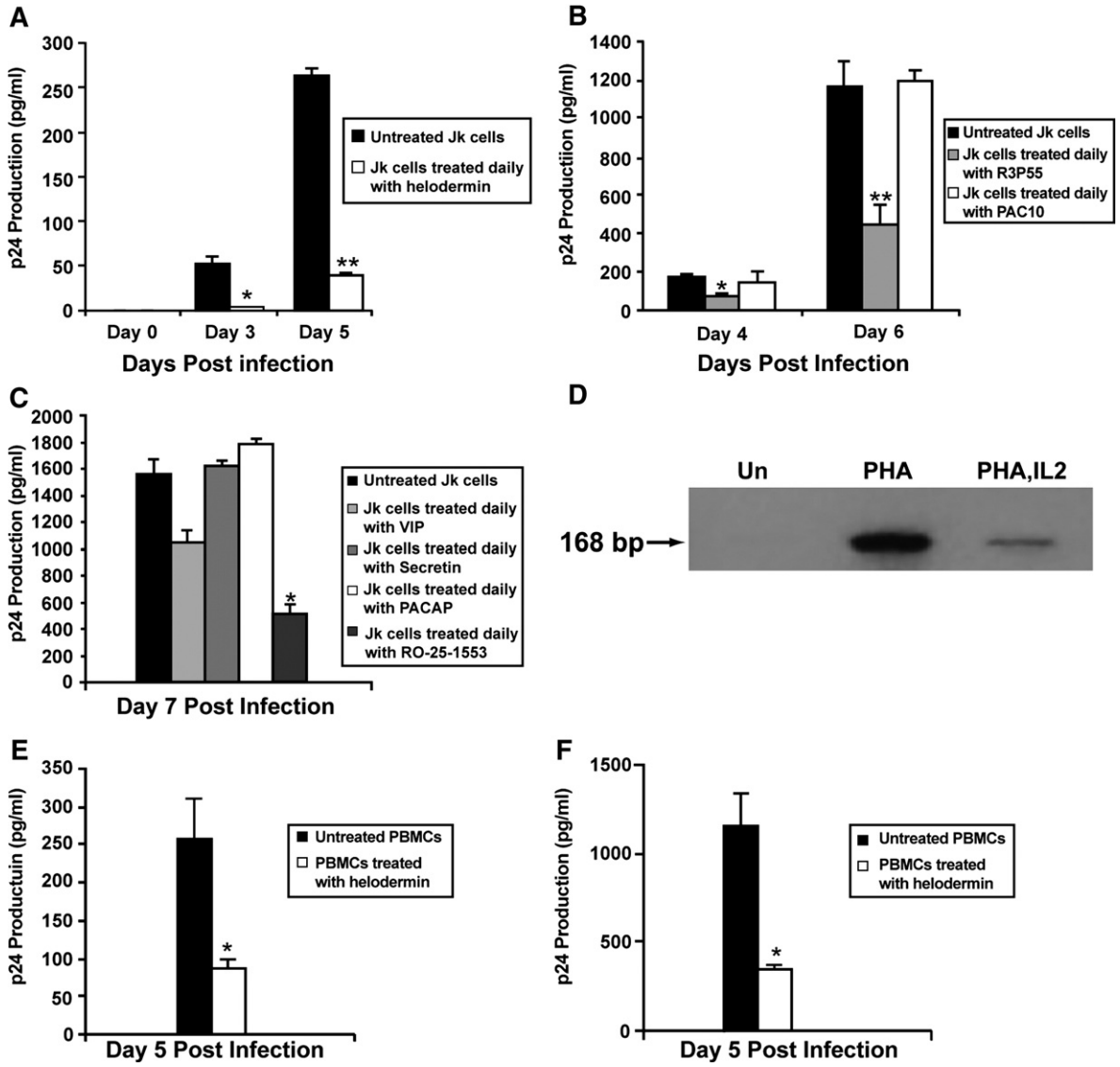

Fig. 2. Daily treatment of Jurkat or PBMCs with VPAC2-specific agonists results in inhibition of productive HIV-1 infection. Cells were pretreated for 1 h prior to infection with 5×10^{-5} M of each VPAC agonist and then washed. Cells were then infected with HIV-1_{IIIB} for 1 h, washed and cultured. Each day following infection, 10^{-9} M of each VPAC agonist was added to the culture. Samples were withdrawn at the indicated time points for measurement of productive HIV-1 infection by determination of $p24^{gag}$. (A) Effect of daily addition of helodermin. The results represent the mean pg/ml $p24^{gag}\pm$SEM from four separate cultures assayed on day 3 (*$p=0.03$) and day 5 (**$p=0.002$). (B) Effect of daily addition of R3P55 peptide (VPAC2 agonist) or PAC10 (control peptide). The results represent the mean pg/ml $p24^{gag}\pm$SEM from four separate cultures assayed on day 4 (*$p=0.06$) and day 6 (**$p=0.004$). (C) Effect of daily addition of VIP (VPAC1 and VPAC2 agonist), secretin (VPAC1 agonist), PACAP (PAC1 agonist) or R0 25-1553 (VPAC2 agonist). The level of infection was measured at day 7 of culture. The results represent the mean pg/ml $p24^{gag}\pm$SEM from four separate cultures (*$p=0.002$). (D) Expression of VPAC2 mRNA in resting and activated PBMCs by RT-PCR. (E) Effect of daily treatment with 10^{-9} M helodermin on infection with R5 (0.5 pg $p24^{gag}$) virus of PBMCs. Results represent the mean pg/ml $p24^{gag}\pm$SEM for four separate cultures assayed on day 5 (*$p=0.024$). (F) Effect of daily treatment of 10^{-9} M helodermin on infection with X4 (moi, 0.3) virus of PBMCs. Results represent the mean pg/ml $p24^{gag}\pm$SEM for four separate cultures assayed on day 5 (*$p=0.017$).

However, significant inhibition (75%; $p=0.004$) of HIV-1 infection with R3P55 was observed; this inhibitory effect was evident for at least 1 week post-infection (Fig. 2B). To further show specificity of the inhibitory activity, the effect of agonists specific for other members of the VPAC receptor family were tested. VIP, which can engage both VPAC1 and VPAC2 (Usdin et al., 1994), secretin which has a higher affinity for VPAC1 than VPAC2 (Gourlet et al., 1997a), $PACAP_{1-38}$ which has highest affinity for PAC1 (Ulrich et al., 1998; Arimura and Shioda, 1995) and R0 25-1553, highly specific for VPAC2 (Gourlet et al., 1997b) were tested. Daily treatment with 10^{-9} M of each of these peptides revealed significant inhibition of HIV-1 infection (75%; $p=0.002$) only with R0 25-1553. No inhibition was observed for cells that had been treated with PACAP or secretin (Fig. 2C). Although there was slight inhibition when VIP was used, this is likely because VIP interacts with both VPAC1 and VPAC2 with similar affinity (Ulrich et al., 1998; Svoboda et al., 1994; Gourlet et al., 1997a); thus, these results may indicate that the facilitating effect of VPAC1 (Branch et al., 2002) competes well with the inhibitory effect of VPAC2 or, relates to the relative receptor density on Jurkat cells. Taken together, these results indicate that the inhibitory effect on HIV-1 infection is specifically mediated through VPAC2. Furthermore, the inhibitory activity can be sustained by using daily treatments with low concentrations of VPAC2 agonists.

To study the effect of VPAC2 agonists on HIV-1 infection of primary cells, additional experiments were performed using human PBMCs treated with helodermin. It has been reported that resting $CD4^+$ cells express VPAC1 in high levels, while activated $CD4^+$ cells predominantly express VPAC2 receptors (Lara-Marquez et al., 2001); confirmed using RT-PCR (Fig. 2D). Thus, activated $CD4^+$ cells would be a good model for examining the effect of VPAC2 agonists on primary cell HIV-1 infection. Activated PBMCs were pretreated with 5×10^{-5} M helodermin, which was followed by daily treatments using 10^{-9} M helodermin. Fig. 2E shows the ability of helodermin to inhibit infection with R5 ($HIV\text{-}1_{Ba\text{-}L}$) ($p=0.024$). Inhibition of the infection is significant up to 5 days in culture. Fig. 2F shows VPAC2 agonists inhibit an X4 virus ($HIV\text{-}1_{IIIB}$) ($p=0.017$). These data indicate that the effect of VPAC2 stimulation is active for inhibition of normal peripheral blood HIV infection and is not strain specific.

VPAC2 agonists do not affect CD4 and co-receptor expression, cell growth or HIV-1 entry and reverse transcription

After pre-treatment of Jurkat cells with 5×10^{-5} M helodermin followed by daily treatment with 10^{-9} M for 2 days, FACS analysis to evaluate the level of cell surface expression of CD4 and CXCR4 showed no differences compared to untreated control cells (Fig. 3A). We also evaluated the number of viable and apoptotic cells and found no differences between untreated and helodermin-treated cells (Figs. 3B and C). In addition, we evaluated the level of receptor expression, apoptosis, and viable cells after 5 days. There was no difference between untreated and helodermin treated cells (data not shown).

To further investigate the mechanism by which VPAC2 agonists inhibit productive HIV-1 infection, we began an examination as to whether there is a block in one or more of the early stages of the HIV-1 life cycle. We first determined whether VPAC2 agonist stimulation results in inhibition of virus entry. We found that treatment of Jurkat with 5×10^{-5} M helodermin did not affect cellular entry when compared to untreated Jurkat cells (Fig. 4A). This was also true for Jurkat cells treated with the VPAC2-specific agonist R3P66 compared to Jurkat cells treated with the control peptide, PAC6 (data not shown). Furthermore, in order to investigate whether VPAC2 agonists have any effect on middle and late stages of reverse transcriptase (RT), we evaluated the level of gag and LTR/gag regions of HIV-1 cDNA in a time-dependent manner. The levels of HIV-1 cDNA were similar for untreated and VPAC2 agonist-treated cells (Fig. 4B). In addition, we used real-time PCR to detect the level of LTR/gag, the late stage of RT activity, for untreated and helodermin treated cells. Fig. 4C indicates that the level of HIV-1 cDNA was equal for untreated and VPAC2 agonist-treated cells; indicating that VPAC2 agonists have no effect on HIV-1 reverse transcriptase activity.

VPAC2 agonists inhibit reporter gene expression using pseudoenvelope-typed HIV-1

To continue our examination as to where in the HIV life cycle VPAC2 agonists exert their effect, we monitored the effects of helodermin on infection using the pseudoenvelope-typed viruses, VSV-G/NL4-3*luc* and JR-FL/NL4-3*GFP*. VSV-G/NL4-3*luc* virus does not require CD4 or co-receptors for infection and is replication deficient having a luciferase reporter gene inserted into the viral *nef* gene (Connor et al., 1995). JR-FL/NL4-3*GFP* carries a human R5 viral envelope and is also replication deficient having a green fluorescent protein (GFP) gene inserted into the viral nef gene. Thus, monitoring of luciferase production or GFP fluorescence will allow us to determine if the negative effect of VPAC2 agonists on productive HIV-1 infection occur post reverse transcription of the HIV-1 virus and at the transcriptional or integration stage. Using both pseudoenvelope-typed viruses will also allow an assessment of possible crosstalk required between viral envelope-specific cell surfaces receptor signaling and the VPAC2-stimulated signal for maximal inhibitory effect. We found that helodermin could inhibit luciferase production from Jurkat cells infected with the VSV-G/NL4-3*luc* virus (Fig. 5A). Similarly, using PBMCs, we found that helodermin treatment could inhibit the percent of cells expressing GFP following infection with JR-FL/NL4-3*GFP* (Fig. 5B). The inhibitory effect resulted in ~60% and ~90% inhibition of reporter gene detection, respectively. This inhibition occurs after one cycle of infection. Over time, this immediate effect may appear much stronger. Although it appears that when using the R5 pseudoenvelope-typed virus compared to the VSV-G pseudoenvelope-typed virus that there is increased inhibition, whether this is a result of crosstalk between signals sent through VPAC2 and the cell surface receptors recognized by the R5 envelope remains to be elucidated. Importantly, this

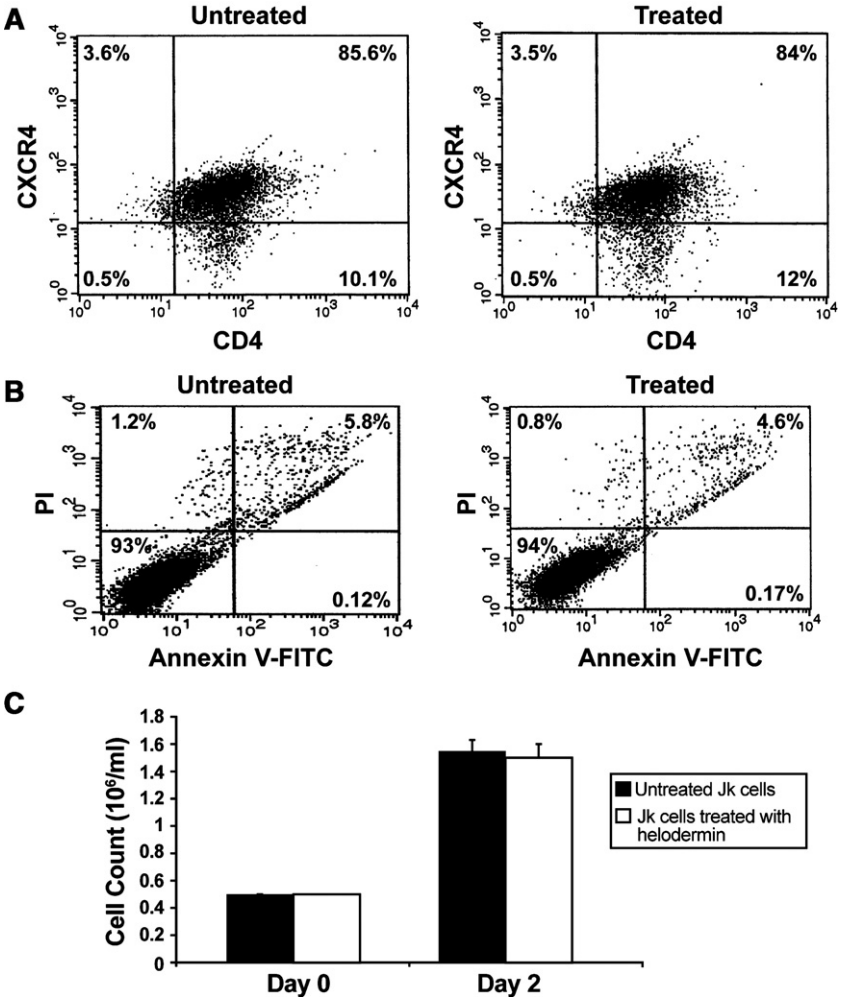

Fig. 3. Effect of daily administration of helodermin on CD4 and CXCR4 expression, apoptosis, and cell proliferation. Jurkat cells were pretreated with 5×10^{-5} M helodermin for 1 h, washed and then treated daily with 10^{-9} M helodermin for 48 h. (A) Two-color FACS analysis was used to compare CD4 and CXCR4 expression in helodermin-treated and untreated Jurkat cells. (B) FACS analysis for apoptosis, viable and dead cells was done with and without helodermin treatment after 48 h using propidium iodide (PI) and binding of Annexin V-FITC. (C) Effect of helodermin on cell growth was determined by counting cells that excluded Trypan blue using a hemocytometer. These results are representative of three independent experiments.

experiment provides evidence that the inhibition of productive HIV-1 infection by VPAC2 agonists likely occurs at either the integration or transcription stage of the HIV-1 life cycle or at a stage between the reverse transcription and integration stages.

VPAC2 agonists inhibit HIV-1 integration

To ascertain whether the inhibition of the production of luciferase and GFP was a result of VPAC2 effects on transcription/translation or on viral integration, we next examined the effect of VPAC2 agonist treatment on the integration of the HIV-1 cDNA into the host DNA. Jurkat cells were pretreated with 5×10^{-5} M helodermin for 1 h, washed and then infected with HIV-1_{IIIB}. Infected cells were then treated daily with 10^{-9} M helodermin (stimulates VPAC2) or secretin (stimulates VPAC1) in parallel with untreated Jurkat cells. Genomic DNA was extracted and subjected to nested *LTR-Alu* PCR. Final products were cut with Hinf I to give a specific predicted fragment size of 385 bp (Fig. 5C). The amplified products were run on a 1.5% agarose gel and subjected to Southern blotting with a radiolabelled HIV-1 specific probe. Fig. 5D reveals that cells that were treated with helodermin, but not secretin, had profoundly less, sometimes undetectable, levels of integrated HIV-1 cDNA.

Discussion

We present evidence showing that stimulation with specific agonists of the neuroendocrine receptor, VPAC2, results in a marked inhibition of productive HIV-1 infection. Moreover, our studies reveal that the effect of VPAC2-specific agonists on productive infection is to inhibit the cDNA integration of the HIV-1 provirus into the host genome. Thus, we show a means to inhibit HIV-1 integration that uses exogenous stimulation of a host cellular receptor by a potential pharmacological agent.

Our *in vitro* experiments have revealed that three well characterized stimulators of VPAC2, helodermin, RO 25-1553, and the R3P series of VPAC2 agonists, all inhibit productive

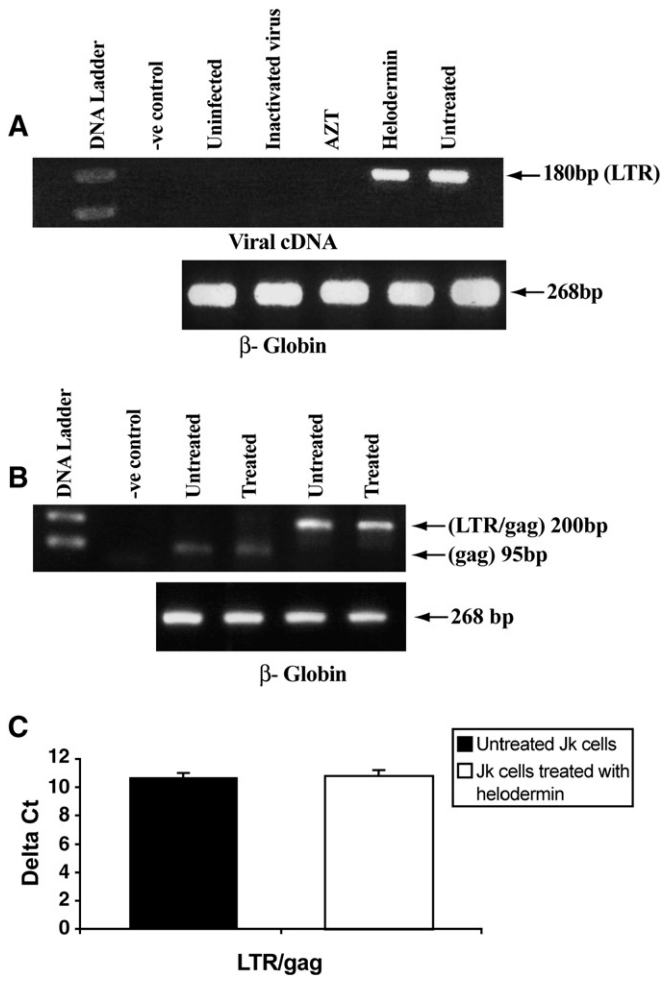

Fig. 4. VPAC2 agonists have no effect on HIV-1 entry or reverse transcriptase activity. For examination of the very early stage of viral entry (detection of LTR), Jurkat (Jk) cells were untreated or treated for 1 h with 5×10^{-5} M helodermin or 0.4 μM AZT and then infected for 1 h with HIV-1_{IIIB} (moi, 0.3) that had first been treated with DNAse I. Cells were then washed and trypsinized to remove virions attached to the cell surface, washed again and allowed to remain in culture for 30 min. Additional controls consisted of uninfected Jk cells and Jk cells infected with heat-inactivated, killed, HIV-1_{IIIB} virus. For the examination of later stages of viral reverse transcription (RT), untreated Jk or Jk cells treated for 1 h with 5×10^{-5} M helodermin were infected as above and cultured for 3 and 7 h for detection of gag and LTR/gag, respectively. Then, cell lysates were prepared and HIV-1 viral cDNA was detected by amplifying different regions of the HIV-1 genome in order to detect different stages of RT activity. Specific primers for LTR, gag, and LTR/gag regions of HIV-1 were used (see Materials and methods). (A) For detecting entry and the very early stage of RT activity, the HIV-1 LTR region was evaluated; Lane 1, DNA ladder; lane 2, −ve control (water); lane 3 (uninfected Jk); lane 4, Jk cells infected with heat-inactivated, killed, virus; lane 5, AZT-treated, infected Jk cells; lane 6, helodermin-treated infected Jk; lane 7, untreated infected Jk cells. The 180 bp fragment represents viral cDNA. As a control for RT-PCR, we used β-globin, represented by the 268 bp fragment (B) For middle and late stages of RT activity, gag and LTR/gag regions of viral cDNA for untreated and VPAC2 agonist-treated cells were evaluated. The 268 bp fragment represents the control comparison of the β-globin DNA level in analyzed samples. The results are representative of at least two independent experiments. (C) Real-time PCR was also performed for the LTR/gag region of HIV-1 cDNA. The same samples that had been used for PCR were used for this reaction. The threshold/cycle (Ct) values were determined for VPAC2 agonist-treated and untreated cells and normalized to Ct values for β-globin. The result is represented as the delta Ct, comparing the Ct of LTR/gag to the Ct for β-globin. Samples were run in triplicates.

HIV-1 infection of either X4 or R5 viruses. Initial studies found that a single bolus high-dose (5×10^{-5} M) of the VPAC2 agonist, helodermin, could inhibit HIV-1 X4 infection of a human T cell line. However, the inhibitory effect was transient, with productive infection being delayed in onset but eventually able to catch up to the untreated infection level. We surmised that the requirement for high concentration and the transient nature of the inhibition although, perhaps partly due to the agonist having a short half-life in culture, was likely due to desensitization of the VPAC2 receptor resulting in down-regulation of the receptor as has been reported for other agonist stimulators of 7-transmembrane G-protein-coupled receptors (Hirsh et al., 2005; Langlet et al., 2004) Thus, we reasoned that a physiological concentration of agonist using multiple treatments would be more effective at maintaining a sustained inhibitory activity over time. Indeed, using concentrations of VPAC2 agonists that were within the affinity constant range of VPAC2/ligand interaction (Gourlet et al., 1997a; Langlet et al., 2004), we found this approach very effective to sustain the inhibition of productive HIV-1 infection over time in culture. Although we examined the inhibitory activity of VPAC2 agonists using 10^{-9} M of agonist throughout the treatment regimen, we used 5×10^{-5} M agonist as the initial treatment followed by 10^{-9} M daily treatments because this higher initial treatment resulted in a more substantial sustained inhibition. The reason for this is unclear but may involve independent pathways that utilize high and low dose agonist as has been previously described (Sausville et al., 2003) or due to an initial requirement of a higher concentration to initiate the necessary signaling pathway that can then be sustained at lower concentrations. It is not unusual for clinical treatment regimens to begin treatments using a higher dose of therapy followed by lower maintenance doses (Vey et al., 2006; Sausville et al., 2003).

Using Jurkat cells that express both VPAC1 and VPAC2 (Branch et al., 2002), we were able to inhibit the infection through VPAC2-specific stimulation, even in the face of HIV-1 that we have previously shown can bind to VPAC1 and provide a facilitation signal to enhance infection (Branch et al., 2002). This result implies that the inhibitory signal sent through VPAC2 is dominant over the facilitation signal sent through VPAC1 when HIV-1 interacts with this receptor. Differences in inhibitory effect could also be explained by the density of the VPAC2 expression compared to that of VPAC1 on these cells. In primary cell infections, we use cells that have first been activated. This produces cells having maximum expression of VPAC2 and minimal expression of VPAC1 (Lara-Marquez et al., 2001; Bokaei et al., 2006) and overcomes any facilitation effects of the virus through VPAC1.

Our results clearly show that the mechanism of inhibition of productive HIV-1 infection is by a VPAC2-mediated blockade in the ability of the HIV-1 cDNA to successfully integrate into the host DNA. Furthermore, we show that this block occurs after full reverse transcription to viral cDNA. Future studies will examine VPAC2-mediated effects on the HIV-1 pre-integration complex and focus on the association of the p17 matrix and integrase proteins.

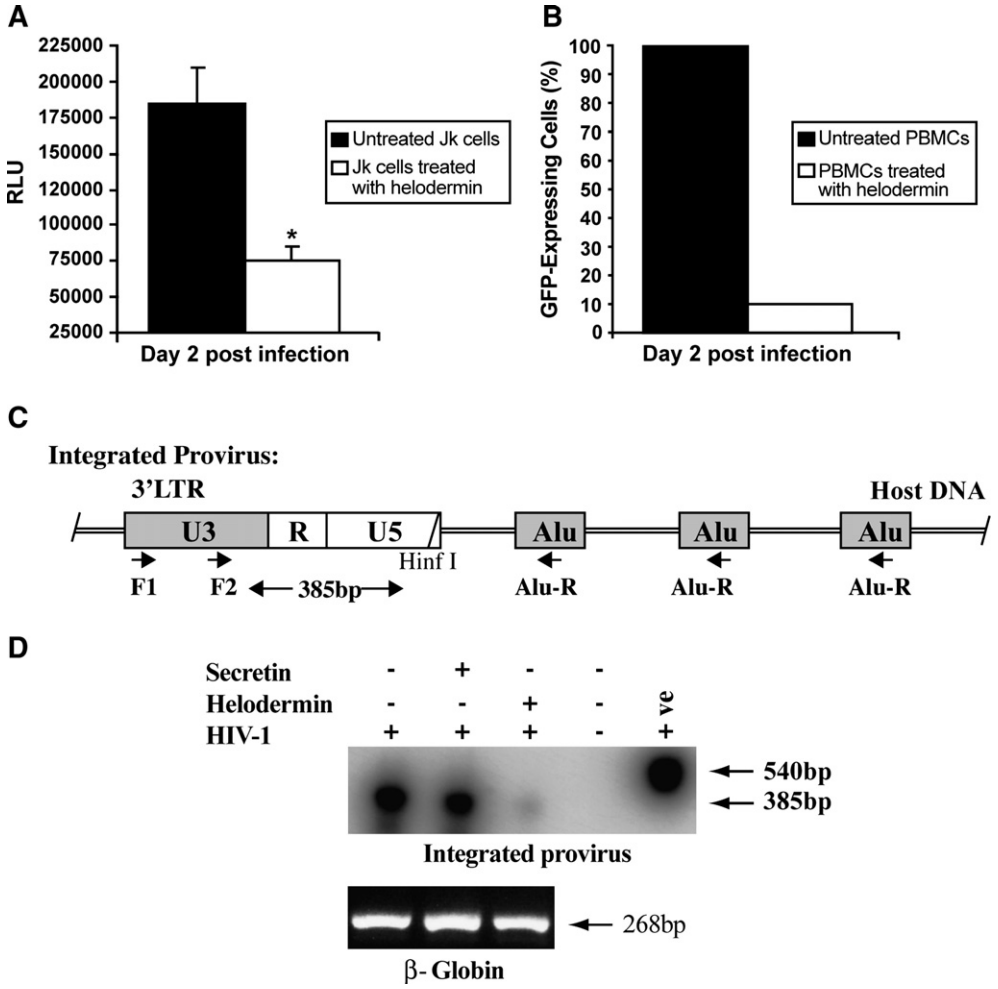

Fig. 5. VPAC2 agonists inhibit HIV-1 integration. (A) Jurkat cells were untreated or treated with 5×10^{-5} M helodermin for 1 h, washed, and then infected with 25 ng VSV-G/NL4-3*luc* pseudoenvelope-typed HIV-1 virus. After 24 h, the cells were treated again with 10^{-9} M helodermin for another 24 h. Then, cells were lysed and luciferase activity measured using a luminometer. Results represent the mean relative fluorescence units (RFU)±SD of triplicate cultures (*$p=0.017$). (B) Human primary PBMCs were activated with PHA (10 μg/ml) for 48 h. Activated cells were either untreated or treated with 5×10^{-5} M helodermin for 1 h, washed, and then infected with 400 ng JR-FL/NL4-3*GFP* pseudoenvelope-typed HIV-1 virus. After 24 h, the treated cells were again treated with helodermin (10^{-9} M) for another 24 h. Then, cells were tested for GFP reporter gene expression by FACS analysis, using untreated cells as representing 100% GFP-expressing cells to determine the percent of GFP-expressing cells in the helodermin-treated sample. To determine the effect of helodermin on integration of the viral cDNA, Jurkat cells were untreated or treated with 5×10^{-5} M helodermin or secretin for 1 h, washed, and then infected with HIV-1_{IIIB}. Cells were then treated with 10^{-9} M helodermin or secretin for 2 days and then processed for determination of integrated HIV-1 cDNA. (C) Schematic representation of the nested PCR strategy to determine the 385 bp fragment that represents integrated HIV-1 DNA. F1 and F2 are the first and second round PCR forward primers that recognize the 3′LTR region of HIV-1 while Alu-R represents the common reverse primer that is specific for human *Alu*-repeat sequences within the human genome. Hinf I indicates the approximate cut site to produce the 385 bp fragment to be confirmed by Southern blotting. (D) The amplified products were run on a 1.5% agarose gel and subjected to Southern blotting with a radiolabelled HIV-specific probe. A separate HIV-LTR fragment was subjected to separate PCR using primers corresponding to the HIV-1 LTR region to give a 540 bp fragment that was used as a Southern blot control. A 268 bp fragment represents the control comparison of β-globin DNA level in analysed samples. The result is representative of four independent experiments.

VPAC2 agonists have been developed for use in the treatment of diabetes (Tsutsumi et al., 2002; Yung et al., 2003) and perhaps will be shown useful in future therapies for cancer (Schulz et al., 2004). Helodermin, derived from the Gila monster lizard, has no human homologs (Pohl and Wank, 1998) and has never been proposed for use in humans; but, our results suggest that a possible use for this peptide could be in the treatment of HIV/AIDS. Another Gila monster-derived peptide, exendin-4, is currently receiving attention as a new therapy for diabetes (Dupre, 2005) and Alzheimer's disease (Perry and Greig, 2004). Thus, it is not inconceivable that helodermin or a related peptide may show efficacy in HIV therapy. Recently, a newly synthesized VPAC2 agonist has been described having even higher specificity and affinity for VPAC2 (Langer et al., 2004). Whether this newly reported peptide will show increased efficacy for prevention of productive HIV-1 infection remains to be determined.

It is interesting that we have now found VPAC2 stimulation to provide an inhibitory effect on HIV-1 infection. Previously, we found that stimulation of VPAC1 was able to facilitate HIV-1 infection and that the virus itself was able to interact with the VPAC1 receptor to initiate the facilitation signal (Branch et al., 2002). In that work, we provided evidence that the mechanism of facilitation was most likely through an increased ability for

the viral cDNA to integrate into the host genome. Now, we show that stimulation of VPAC2 inhibits HIV-1 infection by inhibiting viral cDNA integration. Thus, our findings represent another example of VPAC1 and VPAC2 having opposing functions.

In conclusion, we have further defined a potential role for VPAC neuroendocrine receptors in HIV infection. We have discovered that stimulation of the VPAC2 neuroendocrine receptor results in a strong negative effect on productive HIV-1 infection through its ability to greatly impede HIV-1 cDNA integration into the host DNA and that this effect is likely mediated through a specific signal being transduced after ligand/receptor interaction. Our discovery suggests that further studies may determine that VPAC2 agonists provide a novel and potentially potent therapy for HIV/AIDS that could be developed for future utility as a new drug-based treatment in the global fight against this terrible disease.

Materials and methods

Cells and growth assay

Sup-T1, Hut78, Jurkat E6.1 and 293T cells were purchased from American Type Culture Collection and maintained in complete medium (RPMI-1640/10% FBS; Sigma-Aldrich). Normal whole blood was obtained from volunteers after informed consent and peripheral blood mononuclear cells (PBMCs) were isolated using density gradients. Cell growth was determined by plating 10^7 cells into T25 flasks in complete medium and aliquots withdrawn over time and viable cells enumerated in the presence of Trypan blue dye using a hemocytometer.

VPAC1, VPAC2, and PAC1 receptor agonists

VIP, secretin, $PACAP_{1-38}$ and helodermin were purchased from American Peptide Company. RO 25–1553 was a gift from Dr. Patrick Robberecht (University of Brussels). R3P55, R3P66/BAY 55–9837, PAC10, and PAC6 peptides were provided by Dr. Clark Pan (Bayer Corporation). VIP can engage both VPAC1 and VPAC2 (Ulrich et al., 1998), secretin has a much greater affinity for VPAC1 than VPAC2 (Gourlet et al., 1997a), $PACAP_{1-38}$ has a high affinity for PAC1 (Arimura and Shioda, 1995), and helodermin (Robberecht et al., 1996), R0 25–1553 (Gourlet et al., 1997b), R3P66/BAY 55–9837 (Tsutsumi et al., 2002), and R3P55 (Yung et al., 2003) are specific for VPAC2.

Viruses and virus infection

HIV-1_{IIIB} (X4) and HIV-$1_{Ba\text{-}L}$ (R5) were from the NIH AIDS Research and Reference Reagent Program (Rockville) and used at a multiplicity of infection (moi) of 0.1 infectious virions (i.v.)/cell for X4 strain in cell lines and 0.3 moi for human PBMCs; for R5 viruses, 0.5 pg total $p24^{gag}/0.5\times10^6$ cells was used. T lymphocytes and monocytes were infected with X4 or R5 after activation of PBMCs as previously described (Lund et al., 2006). Initially, cell lines and activated primary cells were either untreated or pretreated once with 5×10^{-5} M agonist peptides for 1 h, washed, infected for 1 h with HIV-1_{IIIB} or HIV-$1_{Ba\text{-}L}$, washed again, and then cultured and monitored for viral infection. In later experiments, cells were pretreated and infected as previously stated but then, each day after infection; an aliquot of culture supernatant was removed for testing and replaced with an equal aliquot of medium containing peptides to give a final concentration of 10^{-9} M. HIV-1 infection was monitored by measurement of $p24^{gag}$ protein using ELISA (Beckman/Coulter) (Lund et al., 2006; Branch et al., 2002).

Cloning VPAC2 cDNA

VPAC2 mRNA was isolated from Sup-T1 cells by using QuikPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech Inc.) and cDNA made using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech Inc.). PCR was performed in a Perkin-Elmer Gene Amp PCR 9700 thermocycler using Olerup SSP PCR Master Mix with 1 unit of Taq polymerase (Genovison Inc). VPAC2 oligonucleotide primers were based on Genbank database; accession no. L36566: sense; 1F (5′-CACGCTGAGCTCGGGATGCGGA-3′) and antisense; 1R (5′-CTAGATGACCGAGGTCTCCGTTTG-3′). DNA fragments obtained by RT-PCR were directly cloned using the TA Cloning System (Invitrogen/Gibco Canada Inc). A full length VPAC2 sequence from TA clones was confirmed by the dideoxy method using reagents from Amersham Canada with the T7 and M13 primers.

Stable expression of VPAC2 in Hut78 cells

The cDNA encoding the full-length wild-type human VPAC2 was inserted into the mammalian expression vector pcDNA3.1 (Invitrogen). Transfection of Hut78 cells was carried out as previously described for VPAC1 (Branch et al., 2002). Stable clones expressing high levels of VPAC2 mRNA were selected using limiting dilution. VPAC2 protein expression was confirmed using immunoprecipitation with anti-Xpress tag of ^{35}S-biosynthetically labelled VPAC2 as previously described (Bokaei et al., 2006)(data not shown).

VPAC2 mRNA expression using RT-PCR

VPAC2 mRNA primers were: Forward primer, 5′-GTACTG-CATCATGGCCAACT-3′; Reverse primer, 5′-CTAAGTA-GAGCCTGGCCGC-3′. mRNA was isolated using Quick Prep Micro mRNA purification kit (Amersham Pharmacia Biotech Inc) from approximately 2×10^6 cells; 1 μg of mRNA was reverse transcribed to make cDNA using First Strand cDNA Synthesis kit (Amersham Pharmacia Biotech Inc). A plasmid containing a full-length VPAC2 insert was used as a positive control. PCR products were separated by electrophoresis through a 1.5% agarose gel and visualized by ethidium bromide.

Flow cytometry analysis

Two-color fluorescent antibody cell sorting (FACS) analysis of CD4 and CXCR4 expression was accomplished as described

previously for cell surface antigen detection (Hong et al., 2000). Briefly, 1×10^6 cells were incubated with 10 μl mouse anti-CXCR4 (12G5; NIH AIDS Research and Reference Reagent Program) followed by 2 μL fluoresceine isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Biosource, Camarillo, California, USA) and then with 10 μL phycoerythrin-labeled mouse anti-human CD4 (Serotec, Raleigh, North Carolina, USA). FACS was performed using two-color analysis on a flow-rate-calibrated (using BD CaliBRITE beads (Becton Dickinson)) FACSCalibur E4795 flow cytometer (Becton Dickinson, San Jose, CA, USA), and Cell Quest software for data analyses (Becton Dickinson).

Viral entry and reverse transcriptase kinetic activity

Detection of viral entry and intracellular HIV-1 cDNA following initiation of reverse transcriptase (RT) activation in infected cells were assessed by DNA-PCR using primers specific for the long terminal repeat (LTR), gag, and LTR/gag regions of HIV-1 as previously described (Taddeo et al., 1993; Baiocchi et al., 1997; Zack et al., 1990) with modification. HIV-1_{IIIB} was first treated for 30 min at 37 °C with DNAse I (Boehringer) to degrade any possible viral DNA contamination. In order to detect viral entry, initially, Jurkat cells were infected with the DNAse-treated HIV-1 for 1 h (moi, 0.3), washed, and the cells trypsinized to remove any virions attached to the cell surface. Additional controls for DNA contamination included cells treated with 3′-azido-3′-deoxythymidine (AZT)(Mercure et al., 1994) during the infection and Jurkat cells infected with heat-inactivated virus (65 °C for 3 h)(Taddeo et al., 1993). Subsequent studies of later stages of viral reverse transcription (gag and LTR/gag) (Baiocchi et al., 1997) were done using only virus that had been pretreated with DNAse I followed by trypsin treatment of the cells. These procedures for later stages of reverse transcriptase activity were evaluated after infection for 1 h, and then cells were washed and recultured in media for 3 and 7 h, respectively, in order to detect gag and LTR/gag regions. After washing, total cellular DNA was isolated by a quick lysis method using PUREGENE DNA purification kit (Gentra Systems) and different regions of HIV-1 cDNA were amplified by using PCR with specific primers. Briefly, DNA was denatured at 95 °C for 4 min, and amplified by 35 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and a final extension step of 7 min at 72 °C. The primers for amplification were: LTR: forward, 5′-GCTGGGGACTTTCCAGGGAG-3′ and reverse, 5′-AGGCAAGCTTTATTGAGGCTTAAGC-3′; gag: forward, 5′-GCGCGCACAGCAAGAGGCGA-3′ and reverse, 5′-GACGCTCTCGCACCCATCTC-3′; LTR/gag: forward: 5′-GGCTAACTAGGGAACCCACG-3′ and reverse: 5′-CCTGCGTCGAGAGAGCTCCTCTGG-3′. Amplified products were run on a 1.8% agarose gel and visualized by ethidium bromide. Cell lysates were also concomitantly amplified using primers specific for human β-globin as DNA control comparison. The primers for β-globin gene were: forward, 5′-CAACTTCATCCACGTTCACC-3′ and reverse, 5′-GAAGAGCCAAGGACAGGTAC-3′.

Quantitative HIV-1 DNA analyses

The level of HIV-1 cDNA LTR/gag was measured by quantitative real-time PCR as previously described (Victoria et al., 2003; Bouchonnet et al., 2005; Vitone et al., 2005) with modification. The same samples and specific primers were used for evaluation of LTR/gag as described above. In brief, reaction components were obtained from the LightCycler® FastStart DNA Master SYBR Green ^{PLUS} I Kit (Roche, Germany). Amplification, data acquisition and analysis were carried out by the LightCycler® instrument (Roche, Germany). The following reaction conditions were used: pre-incubation at 95 °C for 10 min, followed by 45 amplification cycles of denaturation at 95 °C for 10 s, annealing at 60 °C (LTR/gag) and 58 °C (β-globin) for 5 s, extension at 72 °C for 10 s, melting curve analysis 65 °C for 15 s and a continues acquisition mode of 95 °C with a temperature transition rate of 0.1 °C/s. The real-time PCR was performed in a final volume of 20 μl containing 1× Master SYBR green ^{PLUS} I buffer, 25 μM forward and reverse primers , and 2 μl of template DNA (100 ng/μl) plus no template as a control. The Light Cycler instrument, determines the threshold cycles (Ct), which is directly proportional to the $\log_{10}$ of the copy number of the input templates (Vitone et al., 2005; Kurmayer and Kutzenberger, 2003). An equal amount of DNA in untreated and VPAC2 agonist-treated samples was determined by spectrophotometry (Gibellini et al., 2004) and samples were run in triplicate. Results generated in Ct values were normalized to β-globin Ct levels and the deltaCt value determined.

Pseudoenvelope-typed HIV-1 viruses

To generate an amphotropic or R5 enveloped HIV-1-luciferase- or -GFP-containing recombinant virion, respectively, 293T cells were used and co-transfected with plasmids containing either an envelope gene or the HIV-1 genome lacking *env* but containing a luciferase or GFP gene as previously described (Connor et al., 1995; Branch et al., 2002; Serafini et al., 2004; Chang et al., 1999). In brief, 15 μg of plasmid HIV-1 NL4-3*luc* or HIV-1 NL4-3*GFP* (a kind gift from Dr. Veneet KewalRamani, New York Medical University, NY), lacking the *env* gene and in which the firefly luciferase (*luc*) or the green fluorescence protein (*GFP*) gene is inserted into the viral *nef* gene (Connor et al., 1995), and 10 μg of a plasmid containing the amphotropic envelope of the vesicular stomatitis virus (VSV-G *env*; a generous gift from Dr. Michel Tremblay, Quebec City, PQ) or a plasmid containing the human R5 envelope JR-FL (a gift from Vineet KewalRamani, New York Medical University, NY), were mixed with 875 μl of 0.25 M $CaPO_4$ and then added dropwise to 875 μl bubbling 2× BBS (0.28 M NaCl, 0.05 M BES, 1.5 mM Na_2HPO_4, pH 6.95). The mixture was then incubated at room temperature for 15 min and the solution was gently added to $2.5–3\times10^6$ 293T cells plated into 10 cm Petri dishes the day before transfection. Five hours post-transfection, cells were washed with PBS and incubated for 72 h in fresh DMEM medium with 10% FBS. The supernatant was

collected and centrifuged for 5 min at 300×*g* and then passed through a 0.45 μm filter and pelleted by ultracentrifugation over 20% sucrose for 1 h at 33,000×*g*. The virus-containing pellet was resuspended in TNE buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl), aliquoted and stored at −80 °C. Virion content was determined by $p24^{gag}$ ELISA. For infection using VSV-G/NL4-3*luc*, Jurkat cells were untreated or treated with 5×10^{-5} M helodermin for 1 h, washed, and then infected with the purified VSV-G/NL4-3*luc* pseudoenvelope-typed virus, 25 ng per sample, in RPMI medium (without phenol red) containing 10^{-9} M helodermin. After 24 h, the cells were again treated with 10^{-9} M helodermin and cultured for an additional 24 h. The cells were then lysed using the cell culture lysis reagent (CCLR, Promega Corporation, Madison, WI) as per the manufacturer's directions. 100 μl of luciferase assay substrate (Promega Corporation) was added to 20 μl of cell lysate and the luciferase activity immediately measured using a luminometer (Luminoskan Ascent, Thermo Electtron Corporation, Vantaa, Finland). For infection studies using JR-FL/NL4-3*GFP*, PBMCs were activated with PHA for 48 h and 10^6 untreated or cells treated with 5×10^{-5} M helodermin for 1 h were infected with 400 ng per sample of the pseudoenvelope-typed virus. After 48 h, the cells were washed with PBS (×4) and the level of GFP activity measured by FACS (Coulter-EPICS XL, Mississauga, Ontario Canada). The percentage of GFP-expressing cells measured in the infected but non-helodermin-treated cell population was considered 100%. The number of GFP-expressing cells in the helodermin-treated cell population was calculated as a percentage of the total number of GFP-expressing cells in the infected but untreated cell population.

HIV-1 cDNA integration

A modification of a previously reported (Daniel et al., 2003) nested LTR/Alu-PCR strategy was used to specifically detect integrated viral cDNA but not unintegrated forms. Briefly, 5×10^5 Jurkat cells per well in 12-well plates were left untreated or were pretreated for 1 h with 5×10^{-5} M of the agonist peptides. Cells were washed, infected with HIV-1_{IIIB} (moi, 0.1) for 1 h, washed again and then cultured. Cultures were treated daily with agonist peptides at a concentration of 10^{-9} M. At day 2 post-infection, cells were washed with PBS, lysed with 200 μl of lysis buffer containing 0.4 mg proteinase K and total genomic DNA collected in 50 μl of TE buffer (pH 8.3) with DNeasy Tissue Kit (Qiagen). The nested-PCR reactions contained 1× Olerup SSP PCR Master Mix with 1 unit of Taq polymerase (Genovison), 0.25 mM forward and reverse primers. 2 μl and 4 μl of DNA, in, respectively, 10 μl and 20 μl total volumes, was used for first- and second-round PCR, respectively, plus a no-template control. DNA concentration of samples was normalized by U.V absorbance and 400 ng DNA was used for the first round PCR. DNA was denatured at 94 °C for 4 min, then 35 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min; and a final extension step of 7 min at 72 °C. The sense and antisense primer pairs used were specific for the amplification of the HIV-1 LTR region and human *Alu* sequences, respectively. The primers used for the first round PCR were designed based on the published sequence in the Genebank database (accession no. NC001802) and were: Forward, LTRF1 (5′-GGGACTG-GAAGGGCTAATTC-3′); reverse, AluR (5′-TGCTGGGAT-TACAGGCGTGAG-3′). Products which were amplified in the first round of the PCR were subjected to a second round of amplification using: Forward, LTR F2 (5′- GAGAGCTG-CATCCGGAGTAC-3′) and reverse, AluR. LTR-Alu products generated from the second round of the nested-PCR were extracted with QIA quick PCR Purification Kit (Qiagen) and digested with the restriction endonuclease Hinf I (New England Biolabs) which recognizes a unique site in HIV-1 LTR region to yield a single internal integrated HIV DNA fragment from weak smear forms. 10 μl of the cut DNA with 2 μl loading buffer was run on a 1.5% agarose gel. A control for Southern blotting was 2 μl of an HIV-LTR cDNA which had been subjected to PCR using forward, LTR-F1, and reverse, LTR-R 5′- AGG-CAAGCTTTATTGAGGCTTAAGC-3′ corresponding to HIV-1 LTR region. After electrophoresis, the DNA was transferred to a nylon membrane (Hybond N+, Reche Diagnostic Co), fixed and cross-linked. The membrane was prehybridized with hybridization buffer at 42 °C for 1 h, followed by hybridizing for 24 h at 42 °C with a HIV-1 LTR-specific radioactive (^{32}P) probe which was generated by random priming labelling (Invitrogen/Gibco). The membrane was washed with hybridization washing buffer and then exposed to X-ray film (Hyperfilm MP). A single product of 385 bp indicated integrated viral cDNA while a control HIV cDNA product of 540 bp indicated that the radioactive probe and hybridization protocol is working. Although this approach relies on a specific distance between the LTR-Alu sequences, we have shown that this approach works in Jurkat cells and has a sensitivity of 1000 infected Jurkat cells. Cell lysates were also concomitantly amplified using primers specific for human β-globin as DNA control comparison.

Statistics

A paired Student's *t*-test was used for analysis of significance. A p value <0.05 was considered significant.

Acknowledgments

The authors acknowledge support for this work from the University of Toronto/Aventis-Pasteur Research Program (UOP-51600), the Ontario Ministry of Health AIDS Program Positive Action Fund (PA01127), the Canadian Institutes of Health Research University/Industry Matching Funds Program, and the "Skate the Dream" fund of the Toronto General and Western Hospital Foundation. We appreciate the provision of reagents used in this study that were provided by Drs. Patrick Robberecht, Clark Pan, Veneet KewalRamani and Michel Tremblay. We also thank Ramtin Rahbar, Ehtesham Baig, and Thomas Murooka for technical assistance. P.B.B. is a recipient of a Canadian Blood Services Graduate Fellowship Award.

References

Alkhatib, G., Combadiere, C., Broder, C.C., Feng, Y., Kennedy, P.E., Murphy, P.M., Berger, E.A., 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1 beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science 272 (5270), 1955–1958.

Arimura, A., Shioda, S., 1995. Pituitary adenylate cyclase activating polypeptide (PACAP) and its receptors: neuroendocrine and endocrine interaction. Front. Neuroendocrinol. 16 (1), 53–88.

Baiocchi, M., Olivetta, E., Chelucci, C., Santarcangelo, A.C., Bona, R., d'Aloja, P., Testa, U., Komatsu, N., Verani, P., Federico, M., 1997. Human immunodeficiency virus (HIV)-resistant CD4+ UT-7 megakaryocytic human cell line becomes highly HIV-1 and HIV-2 susceptible upon CXCR4 transfection: induction of cell differentiation by HIV-1 infection. Blood 89 (8), 2670–2678.

Bokaei, P.B., Ma, X.-Z., Byczynski, B., Keller, J., Sakac, D., Fahim, S., Branch, D.R., 2006. Identification and characterization of 5-transmembrane isoforms of human vasoactive intestinal peptide and pituitary adenylate cyclase activating polypeptide receptors. Genomics 88 (6), 791–800.

Bouchonnet, F., Dam, E., Mammano, F., de Soultrait, V., Hennere, G., Benech, H., Clavel, F., Hance, A.J., 2005. Quantification of the effects on viral DNA synthesis of reverse transcriptase mutations conffering human immunodeficiency virus type 1 resistance to nucleoside analogues. J. Virol. 79 (2), 812–822.

Branch, D.R., Valenta, L.J., Yousefi, S., Sakac, D., Singla, R., Bali, M., Sahai, B.M., Ma, X.Z., 2002. VPAC1 is a cellular neuroendocrine receptor expressed on T cells that actively facilitates productive HIV-1 infection. AIDS 16 (3), 309–319.

Chang, L.J., Urlacher, V., Iwakuma, T., Cui, Y., Zucali, J., 1999. Efficacy and safety analyses of a recombinant human immunodeficiency virus type 1 derived vector system. Gene Ther. 6 (5), 715–728.

Connor, R.I., Chen, B.K., Choe, S., Landau, N.R., 1995. Vpr is required for efficient replication of immunodeficiency virus type-1 in mononuclear phagocytes. Virology 206 (2), 935–944.

Dalgleish, A.G., Beverley, P.C., Clapham, P.R., Crawford, D.H., Greaves, M.F., Weiss, R.A., 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature 312 (5996), 763–767.

Daniel, R., Kao, G., Taganov, K., Greger, J.G., Favorova, O., Merkel, G., Yen, T.J., Katz, R.A., Skalka, A.M., 2003. Evidence that the retroviral DNA integration process triggers an ATR-dependent DNA damage response. Proc. Natl. Acad. Sci. U.S.A. 100 (8), 4778–4783.

Dupre, J., 2005. Glycaemic effects of incretins in Type I diabetes mellitus: a concise review with emphasis on studies in humans. Regul. Pept. 128 (2), 149–157.

Fantini, J., Hammache, D., Pieroni, G., Yahi, N., 2000. Role of glycosphingolipid microdomains in CD4-dependent HIV-1 fusion. Glycoconj. J. 17 (3–4), 199–204.

Feng, Y., Broder, C.C., Kennedy, P.E., Berger, E.A., 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane G protein-coupled receptor. Science 272 (5263), 872–877.

Gibellini, D., Vitone, F., Schiavone, P., Ponti, C., La Placa, M., Re, M.C., 2004. Quantitative detection of human immunodeficiency virus type 1 (HIV-1) proviral DNA in peripheral blood mononuclear cells by SYBR green real-time PCR technique. J. Clin. Virol. 29 (4), 282–289.

Gilles, A., Miquelis, A., Luis, J., Faure, E., 1998. Activation of transcription from the human immunodeficiency virus type 1 (HIV-1) long terminal repeat by the vasoactive intestinal peptide (VIP). Ital. J. Biochem. 47 (2), 101–110.

Gourlet, P., Vandermeers, A., Vertongen, P., Rathe, J., De Neef, P., Cnudde, J., Waelbroeck, M., Robberecht, P., 1997a. Development of high affinity selective VIP1 receptor agonists. Peptides 18 (10), 1539–1545.

Gourlet, P., Vertongen, P., Vandermeers, A., Vandermeers-Piret, M.C., Rathe, J., De Neef, P., Waelbroeck, M., Robberecht, P., 1997b. The long-acting vasoactive intestinal polypeptide agonist RO25-1553 is highly selective of the VIP2 receptor subclass. Peptides 18 (3), 403–408.

Harmar, A.J., Arimura, A., Gozes, I., Journot, L., Laburthe, M., Pisegna, J.R., Rawlings, S.R., Robberecht, P., Said, S.I., Sreedharan, S.P., Wank, S.A., Waschek, J., 1998. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. Pharmacol. Rev. 50 (2), 265–270.

Hirsh, L., Ben-Ami, I., Freimann, S., Dantes, A., Tajima, K., Kotsuji, F., Amsterdam, A., 2005. Desensitization to gonadotropic hormones: a model system for the regulation of a G-protein-coupled receptor with 7-transmembrane spanning regions. Biochem. Biophys. Res. Commun. 326 (1), 1–6.

Hong, C., Voralia, M., Bali, M., Sher, G.D., Branch, D.R., 2000. Irreversible loss of donor blood leucocyte activation may explain a paucity of transfusion-associated graft-versus-host disease from stored blood. Br. J. Haematol. 111 (1), 146–156.

Kurmayer, R., Kutzenberger, T., 2003. Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium Microcystis. Appl. Environ. Microbiol. 69 (11), 6723–6730.

Langer, I., Gregoire, F., Nachtergael, I., De Neef, P., Vertongen, P., Robberecht, P., 2004. Hexanoylation of a VPAC2 receptor- preferring ligand markedly increased its selectivity and potency. Peptides 25 (2), 275–278.

Langlet, C., Gaspard, N., Nachtergael, I., Robberecht, P., Langer, I., 2004. Comparative efficacy of VIP and analogs on activation and internalization of the recombinant VPAC2 receptor expressed in CHO cells. Peptides 25 (12), 2079–2086.

Lara-Marquez, M., O'Dorisio, M., O'Dorisio, T., Shah, M., Karacay, B., 2001. Selective gene expression and activation-dependent regulation of vasoactive intestinal peptide receptor type 1 and type 2 in human T cells. J. Immunol. 166 (4), 2522–2530.

Lund, N., Branch, D.R., Mylvaganam, M., Chark, D., Ma, X.Z., Sakac, D., Binnington, B., Fantini, J., Puri, A., Blumenthal, R., Lingwood, C.A., 2006. A novel soluble mimic of the glycolipid, globotriaosyl ceramide inhibits HIV infection. AIDS 20 (3), 333–343.

Mercure, L., Brenner, B.J., Phaneuf, D., Tsoukas, C., Wainberg, M.A., 1994. Effect of 3′-azido-3′-deoxythymidine and 2′,3′-didioxyinosine on establishment of human immunodeficiency virus type 1 in cultured CD8+ lymphocytes. Anthimicrob. Agents Chemother. 38 (5), 986–990.

Nicole, P., Du, K., Couvineau, A., Laburthe, M., 1998. Site-directed mutagenesis of human vasoactive intestinal peptide receptor subtypes VIP1 and VIP2: evidence for difference in the structure–function relationship. J. Pharmacol. Exp. Ther. 284 (2), 744–750.

Perry, T.A., Greig, N.H., 2004. A new Alzheimer's disease interventive strategy: GLP-1 Curr. Drug Targets 5 (6), 565–571.

Pert, C.B., Ruff, M.R., Hill, J.M., 1988. AIDS as a neuropeptide disorder: peptide T, VIP, and the HIV receptor. Psychopharmacol. Bull. 24 (3), 315–319.

Pohl, M., Wank, SA., 1998. Molecular cloning of the helodermin and exendin-4 cDNAs in the lizard. Relationship to vasoactive intestinal polypeptide/pituitary adenylate cyclase activating polypeptide and glucagon-like peptide 1 and evidence against the existence of mammalian homologues. J. Biol. Chem. 273 (16), 9778–9784.

Puri, A., Rawat, S.S., Lin, H.M., Finnegan, C.M., Mikovits, J., Ruscetti, F.W., Blumenthal, R., 2004. An inhibitor of glycosphingolipid metabolism blocks HIV-1 infection of primary T-cells. AIDS 18 (6), 849–858.

Robberecht, P., Gourlet, P., Vertongen, P., Svoboda, M., 1996. Characterization of the VIP receptor from SUP T1 lymphoblasts. Adv. Neurobionol. 6 (1), 49–57.

Sausville, E.A., Elsayed, Y., Monga, M., Kim, G., 2003. Signal transduction-directed cancer treatments. Ann. Rev. Pharmacol. Toxicol. 43, 199–231.

Schulz, S., Rocken, C., Mawrin, C., Weise, W., Hollt, V., Schulz, S., 2004. Immunocytochemical identification of VPAC1, VPAC2, and PAC1 receptors in normal and neoplastic human tissues with subtype-specific antibodies. Clin. Cancer Res. 10 (24), 8235–8242.

Serafini, M., Naldini, L., Introna, M., 2004. Molecular evidence of inefficient transduction of proliferating human B lymphocytes by VSV-pseudotyrped HIV-1-derived lentivectors. Virology 325 (2), 413–424.

Sreedharan, S.P., Patel, D.R., Huang, J.X., Goetzl, E.J., 1993. Cloning and functional expression of a human neuroendocrine vasoactive intestinal peptide receptor. Biochem. Biophys. Res. Commun. 193 (2), 546–553.

Svoboda, M., Tastenoy, M., Van Rampelbergh, J., Goossens, J.F., De Neef, P., Waelbroeck, M., Robberecht, P., 1994. Molecular cloning and functional characterization of a human VIP receptor from SUP-T1 lymphoblasts. Biochem. Biophys. Res. Commun. 205 (3), 1617–1624.

Taddeo, B., Federico, M., Titti, F., Rossi, G.B., Verani, P., 1993. Homologous superinfection of both producer and nonproducer HIV-infected cells is blocked at a late retrotranscription step. Virology 194 (2), 441–452.

Tsutsumi, M., Claus, T.H., Liang, Y., Li, Y., Yang, L., Zhu, J., Dela Cruz, F., Peng, X., Chen, H., Yung, S.L., Hamren, S., Livingston, J.N., Pan, C.Q., 2002. A potent and highly selective VPAC2 agonist enhances glucose-induced insulin release and glucose disposal: a potential therapy for type 2 diabetes. Diabetes 51 (5), 1453–1460.

Turville, S., Wilkinson, J., Cameron, P., Dable, J., Cunningham, A.L., 2003. The role of dendritic cell C-type lectin receptors in HIV pathogenesis. J. Leukoc. Biol. 74 (5), 710–718.

Ulrich, C.D., Holtmann, M., Miller, L.J., 1998. Secretin and vasoactive intestinal peptide receptors: members of a unique family of G protein-coupled receptors. Gastroenterology 114 (2), 382–397.

Usdin, T.B., Bonner, T.I., Mezey, E., 1994. Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. Endocrinology 135 (6), 2662–2680.

Veljkovic, V., Metlas, R., Raspopovic, J., Pongor, S., 1992. Spectral and sequence similarity between vasoactive intestinal peptide and the second conserved region of human immunodeficiency virus type 1 envelope glycoprotein (gp120): possible consequences on prevention and therapy of AIDS. Biochem. Biophys. Res. Commun. 189 (2), 705–710.

Vey, N., Bosly, A., Guerci, A., Feremans, W., Dombret, H., Dreyfus, F., Bowen, D., Burnett, A., Dennis, M., Ribrag, V., Casadevall, N., Legros, L., Fenaux, P., 2006. Arsenic trioxide in patients with myelodysplastic syndromes: a phase II multicenter study. J. Clin. Oncol. 24 (16), 2465–2471.

Victoria, J.G., Lee, D.J., McDougall, B.R., Robinson Jr., W.E., 2003. Replication kinetics for divergent type 1 human immunodeficiency viruses using quantitative SYBR green I real-time polymerase chain reaction. AIDS Res. Hum. Retroviruses 19 (10), 865–874.

Vitone, F., Gibellini, D., Schiavone, P., Ponti, C., La Placa, M., Re, M.C., 2005. Quantitative DNA proviral detection in HIV-1 patients treated with antiretroviral therapy. J. Clin. Virol. 33 (3), 194–200.

Xia, M., Gaufo, G.O., Wang, Q., Sreedharan, S.P., Goetzl, E.J., 1996. Transduction of specific inhibition of HuT 78 human T cell chemotaxis by type I vasoactive intestinal peptide receptors. J. Immunol. 157 (3), 1132–1138.

Yung, S.L., Dela Cruz, F., Hamren, S., Zhu, J., Tsutsumi, M., Bloom, J.W., Caudle, M., Roczniak, S., Todd, T., Lemoine, L., MacDougall, M., Shanafelt, A.B., Pan, C.Q., 2003. Generation of highly selective VPAC2 receptor agonists by high throughput mutagenesis of vasoactive intestinal peptide and pituitary adenylate cyclase- activating peptide. J. Biol. Chem. 278 (12), 10273–10281.

Zack, J.A., Arrigo, S.J., Weitsman, S.R., Go, A.S., Haislip, A., Chen, I.S., 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 61 (2), 213–222.