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HIV-protease inhibitors block the replication of both vesicular stomatitis and influenza viruses at an early post-entry replication step

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ABSTRACT

The inhibitors of HIV-1 protease (PIs) have been designed to block the activity of the viral aspartyl-protease. However, it is now accepted that this family of inhibitors can also affect the activity of cell proteases. Since the replication of many virus species requires the activity of host cell proteases, investigating the effects of PIs on the life cycle of viruses other than HIV would be of interest. Here, the potent inhibition induced by saquinavir and nefdinavir on the replication of both vesicular stomatitis and influenza viruses is described. These are unrelated enveloped RNA viruses infecting target cells upon endocytosis and intracellular fusion. The PI-induced inhibition was apparently a consequence of a block at the level of the fusion between viral envelope and endosomal membranes. These findings would open the way towards the therapeutic use of PIs against enveloped RNA viruses other than HIV.

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Introduction

HIV protease inhibitors (PIs) are a family of small molecules specifically designed for blocking the activity of the HIV aspartyl-protease (for a review, see Flexner, 1998). The PI treatment leads to the block of HIV maturation with consequent release of non-infectious viral particles (Kaplan et al., 1993). PIs exert their inhibitory effect by disabling the enzyme before it can cleave the Gag–Pol polyprotein into its essential products. PIs, together with anti-HIV compounds targeting alternative steps of the virus life cycle, are part of the most advanced anti-HIV therapies allowing millions of infected people to co-exist with the virus experiencing a good quality of life. However, it is now clear that PIs can also directly or indirectly inhibit the activity of many cell proteases. In particular, PIs can inhibit the activity of both 20S (André et al., 1998) and 26S (Pajonk et al., 2002) proteasome subunits, as well as that of caspases (Badley, 2005), with alterations in the susceptibility to apoptosis stimuli. Both expression and release of matrix-metalloproteinases (MMPs) can be also targeted by PIs (Bourlier et al., 2005; De Barros et al., 2007) with consequences in the extra-cellular matrix modeling and, more in general, in the cell–cell communication. In addition, PIs have been found inhibiting cell signaling pathway involving both NF-κB (Dewan et al., 2009) and AKT (Kumar et al., 2009; Srirangam et al., 2006). From a clinical point of view, the PI treatment may induce beneficial effects other than those against HIV, as in the case of Kaposi sarcoma regression (Sgardari et al., 2002), but can also have detrimental consequences, as for the dyslipidemia occurring in PI-treated HIV patients (Calza et al., 2004).

To enter target cells, enveloped RNA viruses fuse their envelope either at the cell surface in a pH-independent way, or upon internalization in intracellular vesicles through a pH-dependent mechanism (Steven and Spear, 2006). In this latter case, low pH is required to induce the fusion between viral envelope and endosomal membranes, a phenomenon leading to the release of virion contents into cytoplasm. Although the viral fusion process is guided by the presence of viral envelope proteins, the contribution of cell proteins is critical. Using RNA interference as a tool for gene expression inhibition, it was demonstrated that several cell proteins are involved in the mechanism of entry of many viruses including HIV–1 (Brass et al., 2008; Nguyen et al., 2006), influenza (Hao et al., 2008), west Nile (Krishnan et al., 2008), Borna disease (Clemente et al., 2010), human hepatitis C (Ng et al., 2007), and vesicular stomatitis (VSV) (Pelkmans et al., 2005) viruses. Ideally, these cell products might represent potential antiviral therapeutic targets.

Here, the PI-induced inhibition of replication of both VSV and influenza virus is described for the first time. This was most likely a consequence of a PI-dependent block of the viral envelope fusion at the endosomes. Considering that PIs are well tolerated drugs in vivo, and that many relevant human pathogens belong to the family of RNA viruses infecting cells through an endocytic pathway, this finding would open the way towards a broader therapeutic use of PIs.

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Results

Ritonavir decreases the infectivity of VSV-G pseudotyped HIV-1

HIV virions emerging from cells treated with PIs remain immature viral particles as a consequence of the block of Gag polyprotein cleavage. Hence, PIs do not affect the amounts of HIV release from infected cells, but dramatically decrease their infectivity. Surprisingly enough, however, the HIV-1 release from cells infected with HIV-1 pseudotyped with the envelope protein from VSV (VSV-G) has been found inhibited by PIs in single cycle replication assays. In fact, when CEM<sub>GFP</sub> cells, i.e., a human T CD4<sup>+</sup> lymphoblastoid cell line expressing GFP under the control of HIV-1 LTRs (Gervaix et al., 1997), were infected with 100 ng HIV-1 CAp24 equivalent/10<sup>5</sup> cells of (VSV-G) Δenv HIV-1, decreased percentages of HIV-1 expressing cells were observed in ritonavir-treated cells as compared to control conditions (Fig. 1A). The outcome did not change by challenging the cells with doses up to 300 ng HIV-1 CAp24 equivalent/10<sup>5</sup> cells (not shown). Conversely, and as expected, the PI-treatment had no apparent effects on single cycle replication of non-pseudotyped HIV-1, as tested in PI-treated cultures of CEM<sub>GFP</sub> cells infected with wt HIV-1 (Fig. 1B). In these experiments, the presence of T-20 (i.e., a potent inhibitor of the HIV-1 Env-mediated fusion) (Kilby et al., 1998) added 16 h after challenge ensured that the wt HIV-1 replication was limited to a single cycle. As anticipated, ritonavir blocked the spread of wt HIV-1 in multiple-cycle replication assay (Fig. 1B).

To exclude that the observed effect was a consequence of the inhibition of some yet unidentified function of the HIV-1 protease at an early step of the virus life cycle, the assay was reproduced using a VSV-G pseudotyped, PI-resistant HIV-1 strain (here referred to as PM4). This viral mutant expresses a PI-resistant viral protease as a consequence of the four amino acid substitutions in the protease gene, i.e., A<sup>46</sup>I, I<sup>63</sup>F, V<sup>82</sup>T, and I<sup>84</sup>V (Condra et al., 1995). First, the real resistance to PIs of PM4 HIV-1 was checked by evaluating the percentages of CEM<sub>GFP</sub> infected cells 6 days after the infection in the presence or not of ritonavir (Fig. 1C). Then, CEM<sub>GFP</sub> cells treated with T-20 were infected with (VSV-G) PM4 HIV-1 in the presence or not of ritonavir. This experimental setting was representative of a single-cycle replication assay since T-20 disabled both HIV-1 Env-mediated viral entry and possible cell re-infections. Similarly to what observed with (VSV-G) Δenv HIV-1 strain, a significant inhibitory effect of ritonavir on the HIV-1 expression was detected in (VSV-G) PM4 challenged cells (Fig. 1D). This strongly suggests that the viral

![Fig. 1](#)

**Fig. 1.** Ritonavir inhibits the expression of (VSV-G) HIV-1. (A) PI-induced inhibition of the expression of (VSV-G) Δenv HIV-1. CEM<sub>GFP</sub> cells were pre-incubated with either 5 μM of ritonavir (RTV), the same volume of DMSO (DMSO), or left untreated, and then were infected with 100 ng HIV-1 CAp24 equivalent/10<sup>5</sup> cells of (VSV-G) Δenv HIV-1. The viral expression was evaluated in terms of GFP expression at the indicated days post infection. The results are given as mean values of percentages ±SD calculated from five independent experiments. Asterisks indicate p values < 0.05. (B) Ritonavir does not affect the HIV-1 single cycle replication, while blocking the viral spread within the infected cell culture. CEM<sub>GFP</sub> cells pre-incubated with 5 μM RTV, the same volume of DMSO, or left untreated, were infected with 500 ng HIV-1 CAp24 equivalent/10<sup>5</sup> cells of wt HIV-1. Infected cultures were washed 16 h later, and part of them were treated with 1 μg/ml of T-20. The viral expression was measured in terms of percentages of GFP<sup>+</sup> cells measured by FACS analysis 3 days post infection for T-20 treated cultures (left panel), and 6 days post infection for cells not treated with T-20 (right panel). The results are given as mean values of percentages ±SD calculated from three independent experiments. (C) PM4 HIV-1 strain resists the PI treatment. CEM<sub>GFP</sub> cells treated or not with 10 μM RTV were infected with 500 ng HIV-1 CAp24 equivalent/10<sup>5</sup> cells of PM4 HIV-1 and FACS analyzed 6 days later for the GFP expression. The results are given as mean values of percentages ±SD calculated from two independent experiments carried out with duplicated conditions. (D) PI-induced inhibition of the expression of (VSV-G) PM4 HIV-1. CEM<sub>GFP</sub> cells were pre-incubated with T-20 alone or in combination with either 5 μM RTV or the same volume of DMSO, and then infected with 100 ng HIV-1 CAp24 equivalent/10<sup>5</sup> cells of (VSV-G) PM4 HIV-1. The viral expression measured in terms of GFP expression was detected at the indicated days post-infection. The results are given as mean values of percentages ±SD calculated from three independent experiments. Asterisks indicate p values < 0.05.
Fig. 2. Effects of different PIs on VSV replication. (A,B) PI effects on single cycle replication of VSV. HeLa cells were treated overnight with the indicated concentrations of different PIs or equivalent volumes of DMSO, and then infected with 0.2 m.o.i. of VSV. After 1 h adsorption, cells were extensively washed and refed in the presence of PIs. Supernatants were harvested 8 h later, clarified, and the contents of infectious VSV were measured by the end-point dilution method. Ctrl: untreated infected cells. RTV: ritonavir; IND: indinavir; SAQ: saquinavir; NEL: nelfinavir; AMPR: amprenavir; ATAZ: atazanavir. The results are given as mean values of percentages ± SD calculated from six independent experiments. Single asterisks indicate \( p \) values \(< 0.05\). Double asterisks indicate \( p \) values \(< 0.01\). (C) PI-induced inhibition of VSV yield in multiple-cycle replication assay. HeLa cells were treated with PIs as indicated, and then challenged with 2-fold decreasing amounts of VSV starting to m.o.i. 2. The VSV-induced cytopathic effect was scored 24 h later. The results are given as fold-reduction of the end-point dilution as compared with untreated infected cells, and are given as mean values of percentages ± SD calculated from three independent experiments. Single asterisks indicate \( p \) values \(< 0.05\). Double asterisks indicate \( p \) values \(< 0.01\). (D) Dose–response effect of PIs on HeLa cell duplication. Cells were treated for 8 h with different concentrations of the indicated PIs, then labeled with CFSE, and afterwards incubated for additional 12 h. Finally, cells were harvested, and CFSE-related fluorescence evaluated by FACS analysis. In the shown experiment, the mean fluorescence intensity (MFI) of control cells at the end of labeling was 151. The results are given as means of MFI from duplicate conditions from a representative of two experiments. Asterisks denote over 20% of cell mortality. In the inset, both IC\(_{50}\) and CC\(_{50}\) for each PI are reported.
protease activity was not involved in the PI-induced inhibition of (VSV-G) HIV-1 expression.

Overall, these results can be a consequence of a still unrecognized inhibitory effect of PIs on some step of the viral entry driven by VSV-G.

PIs strongly inhibit VSV replication

Next, the possibility that the PI inhibitory effect was operative also against VSV was investigated. To this end, HeLa cells were pre-treated overnight with different concentrations of six PIs, i.e., ritonavir, indinavir, saquinavir, nelfinavir, amprenavir and atazanavir. Then, the cells were challenged with 0.2 m.o.i. of VSV. After 1 h of adsorption in a small volume, the viral inoculum was removed, the cells extensively washed, and incubated for additional 8 h in the presence of PIs. Afterwards, the supernatants were harvested and titrated for the amounts of infectious VSV. As depicted in Figs. 2A–B, a strong reduction of VSV replication (more than 2 logs) was observed when the cells were treated with 2.5 μM saquinavir and nelfinavir. The inhibitory effect appeared more potent (up to about 4 logs) by increasing the concentration of these PIs to 10 μM, and remained significant (i.e., more than 1 log) by increasing the m.o.i. up until 1 (not shown). No differences were observed when the PIs pre-treatment was carried out for 2, 4, 6, or 8 h (not shown). As calculated through a wide dose–response curve, the 50% inhibitory concentration (IC50) values of saquinavir and nelfinavir were 1.4 and 1.1 μM, respectively. On the other hand, significant inhibition of viral replication were detected in ritonavir and indinavir treated cells starting to the concentrations of 20–30 μM (Fig. 2B). The IC50 of ritonavir and saquinavir were 16 and 14 μM, respectively.

The idea that PIs negatively affect the VSV replication was further strengthened by performing a set of more stringent challenge experiments. In detail, HeLa cells were pre-treated with optimal concentrations of the most effective PIs, and then infected with 2-fold decreasing amounts of VSV starting to m.o.i. 2. The cytopathic effect was monitored 24 h post challenge. The outcome of this assay was scored in terms of the viral dilutions no more able to induce cytopathic effect. Notably, in these conditions an about 30-fold reduction of VSV infectivity was observed in cells treated with saquinavir or nelfinavir (Fig. 2C). Since the average replication time of VSV is 6–8 h (Whelan et al., 2004), in this experimental setting VSV was expected to complete at least three replication cycles in control cells.

Then, it was controlled whether the antiviral effect was a consequence of a general cytotoxicity of PIs. To this aim, cells were treated with different PI concentrations for 8 h, then labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and thereafter refed with complete medium in the presence of PIs. CFSE was expected to be equally distributed upon cell division, then resulting in a halving of the overall cell fluorescence after cell duplication. Cytotoxic effects were measured in terms of inhibition of the cell duplication as detectable by the impairment of the halving of the CFSE-associated cell fluorescence. Fig. 2D reports the mean fluorescence intensities (MFIs) measured in cell cultures treated with different concentrations of the PIs active against VSV replication. Block of cell duplication with cell mortality over 20% has been detected using 100 μM ritonavir, saquinavir, and nelfinavir, while indinavir showed a much lower cytotoxic effect. The 50% cytotoxic concentration (CC50) values of PIs (Fig. 2D, insert) were calculated as the doses inhibiting by 50% the fluorescence halving, expectedly a consequence of the block of the duplication in 50% of cells. The therapeutic indexes (i.e., CC50 divided by IC50) appeared higher than 22 for both saquinavir and nelfinavir. This is suggestive of specific antiviral effects for these PIs.

Taken together, these data reveal a previously unrecognized effect of PIs against VSV. In addition, these results support the idea that the PI-induced reduction of (VSV-G) HIV-1 expression is a consequence of the inhibition on some step of the viral entry involving the VSV-G envelope protein.

PIs inhibit the replication of H1N1 influenza A virus

To add relevance to our findings, the possibility that the inhibitory effect of PIs applied also to alternative virus species infecting by pH-dependent fusion was investigated. To this aim, the well characterized influenza virus/MDCK cell system was considered. MDCK cells were treated overnight with different PIs, and then challenged with m.o.i. 0.1 and 0.5 of the PR8 influenza virus. After challenge, the cells were extensively washed, and reseeded in medium without serum in the

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**Fig. 2 (continued)**

| PIs | IC50 μM | CC50 μM |
|-----|--------|--------|
| RTV | 16     | 41     |
| IND | 14     | 220    |
| SAQ | 1.4    | 51     |
| NEL | 1.1    | 25     |
presence of both PIs and tosylamido-2-phenyl ethyl chloromethyl ketone (TPCK)-treated trypsin. Twenty-four h later (i.e., the expected time for the completion of the replication cycle of influenza virus) (Sidorenko and Reichl, 2004; Smith and Ribeiro, 2010), the supernatants were harvested and titrated for the contents of infectious particles. A strong inhibition of the replication of influenza virus (up to 4 logs at the lower m.o.i.) has been detected with 10 μM saquinavir and 5 μM nelfinavir (Fig. 3A). Ritonavir inhibited the viral replication less efficiently (Fig. 3A), while indinavir, amprenavir and atazanavir appeared ineffective (not shown). In the interpretation of these data, it should be emphasized that the inhibitory effect of PIs cannot be a consequence of a PI-induced impairment of the activation of the hemagglutinin envelope protein (HA) of the influenza virus, which is a step required for productive infection (Steinhauer, 1999). In fact, influenza virus preparations used for challenges were exclusively recovered from the allantoic fluid of embryonated chicken eggs, where HA is cleaved by egg proteases. Consistently, TPCK-trypsin did not influence the infectivity of the virus preparations in single cycle replication assay (not shown).

The cell cytotoxicity of PIs active against influenza virus was then evaluated in MDCK cells similarly to what above described for HeLa cells (Fig. 3B). Block of cell duplication in the presence of cell mortality over 20% has been detected with 100 μM ritonavir and saquinavir, and with 50 μM nelfinavir. The therapeutic indexes appeared higher than 15 for both saquinavir and nelfinavir. The assay gave similar results when the cells were analyzed 24 h after CFSE labeling (not shown).

Taken together, these data suggest that PIs could target the replication of diverse enveloped viruses infecting by a pH-dependent endocytic pathway.

**PIs do not inhibit the replication of viruses infecting by a pH-independent mechanism**

Next, it was investigated whether the PIs most effective against VSV and influenza virus replication counteract also the replication of viruses infecting by a pH-independent mechanism. As a proof of principle, the PIs were assayed against the replication of Newcastle

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**Fig. 3.** Effects of PIs on influenza virus replication. (A) PIs negatively affect the influenza virus replication. MDCK cells were treated overnight with the indicated concentrations of PIs and then infected with the indicated m.o.i. of PR8 influenza virus. After 1 h adsorption, cells were extensively washed and refed in the presence of PIs and TPCK-trypsin. Supernatants were harvested 24 h post infection, clarified, and the contents of infectious particles were evaluated by the end-point dilution method. DMSO: cells treated with 1:250 diluted DMSO. Ctrl: untreated infected cells. The results are given as mean values of percentages ±SD calculated from four independent experiments. Single asterisks indicate p values <0.05. Double asterisks indicate p values <0.01. (B) Dose–response effect of PIs on MDCK cell duplication. The assay was performed as described for HeLa cells. In the shown experiment, the mean fluorescence intensity (MFI) of control cells at the end of CFSE labeling was 116. The results are given as means of MFI from duplicate conditions from a representative of two experiments. Asterisks indicate over 20% of cell mortality. In the inset, both IC50 and CC50 for each PI are reported.
Disease virus (NDV), i.e., a Paramyxovirus infecting upon fusion at the cell surface. MDCK cells were pre-treated with 2.5 to 10 μM saquinavir or nelfinavir, and then infected with 2-fold decreasing amounts of NDV starting to m.o.i. 1. The cytopathic effect was monitored 48 h post challenge. No significant reductions in the NDV infectivity have been noticed in PI-treated as compared to control cell cultures (Fig. 4). These results indicate that PIs do not affect the replication of viruses entering by a pH-independent way.

The inhibitory effect is operative when PIs are co-administered with the infecting virus

To identify the virus replication step targeted by PIs, the effects of PIs added before and/or after viral challenge were evaluated. The assays were carried out using both HeLa/VSV and MDCK/influenza virus systems. In a first set of experiments, the PI treatment was discontinued just before the virus challenge. Then, the cells were infected with VSV or influenza virus at m.o.i. of 0.2 and 0.5, respectively. After 1 h of adsorption, the cells were extensively washed and refed in the appropriate medium. In these conditions, no inhibition of the replication of both VSV and influenza virus was detectable (Fig. 5A).

Alternatively, PIs were added soon after the virus adsorption or at different times after the challenge within a timeframe of 8 h (for VSV infection) and 24 h (in the case of challenge with influenza virus), i.e. the respective replication times. The supernatants were harvested 8 h (for VSV) and 24 h (for influenza virus) post-infection, and titrated for the

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**Fig. 4.** PIs do not affect NDV replication. MDCK cells pre-treated with the indicated PIs were infected with 2-fold decreasing amounts of NDV starting to m.o.i. 1, and viral replication extents were evaluated in terms of cytopathic effect by the end-dilution method. The results are given as mean values + SD calculated from two independent experiments with triplicate conditions.

**Fig. 5.** PIs act on an early replication step of both VSV and influenza virus. (A) Effects of PI withdrawal after virus adsorption. HeLa and MDCK cells were treated or not with 5 μM saquinavir or nelfinavir, and challenged with 0.2 m.o.i. VSV or 0.5 m.o.i. influenza virus. After 1 h of adsorption, the viral inocula were removed, the cells were washed 5 times with 10× volumes of PBS, and infected cultures were refed with appropriate medium in the absence of the PI. Supernatants were harvested 8 h (for VSV infection) and 24 h (for influenza virus infection) later, and titrated for the respective virus contents by the end-point dilution method. The results are given as mean values of percentages + SD calculated from three independent experiments for both VSV (left panel) and influenza virus (right panel) challenges. (B) Effects on VSV and influenza virus replication of PIs added at different times after challenge. VSV (0.2 m.o.i.) and influenza virus (0.5 m.o.i.) were adsorbed on HeLa and MDCK cells, respectively. After 1 h at 37 °C, the viral inocula were removed, the cells were washed 5 times with 10× volumes of PBS, and then refed in appropriate medium. At this time, or at the indicated post-infection times, saquinavir or nelfinavir were added at the final concentration of 5 μM. After 8 h (for VSV infection) and 24 h (for influenza virus infection), supernatants were harvested, clarified, and then titrated for infectious virus contents. The results are given as percentages of inhibition as compared with PI-treated infected cell cultures where PIs were re-added after virus challenges. Mean values + SD from two independent experiments with triplicate conditions are reported.
Fig. 6. PIs affect neither attachment nor entry of viral particles. (A) Western blot analysis on 200 ng CAp24 equivalent of purified HIV-1 VLPs incorporating NefG3C-GFP and pseudotyped with either wt or fd VSV-G. Filters were incubated with the indicated Abs. The migrations of expected VLP products are indicated on the left side, whereas the molecular marker sizes are reported on the right. (B) Effect of PIs on the attachment on the cell membrane of HeLa cells of fluorescent (VSV-G) VLPs. Cells pre-treated with 10 μM saquinavir (PI), equal volume of DMSO, or left untreated (Ctrl) were challenged with NefG3C-GFP (VSV-G) VLPs at 4 °C, and, 1 h later, washed, fixed, and FACS analyzed. As control, cells were treated for 15 min with trypsin before FACS analysis. DMSO: cells pretreated with DMSO. SSC: side scatter. The results are representative of two independent experiments. (C) VLP endocytosis activity in PI-treated cells. Cells pre-treated with 10 μM saquinavir (PI), equal volumes of DMSO, or left untreated (Ctrl), were challenged with NefG3C-GFP (fd VSV-G) VLPs and incubated at 37 °C for 3 h. Thereafter, cells were treated with trypsin for 15 min at 37 °C, and then FACS analyzed. As control, the same procedure were applied on cells incubated at 4 °C. SSC: side scatter. The results are representative of four independent experiments. (D) Detection of the virion VSV-G envelope protein in HeLa cells challenged with VSV. Cells pre-treated with 10 μM saquinavir (PI), equal volume of DMSO, or left untreated (Ctrl), were challenged with VSV as above described. Thirty and 60 min after virus adsorption, the cells were washed and lysed. Cytoplasmic extracts were analyzed by western blot for the presence of both VSV-G and β-actin. The migration of relevant protein products is indicated on the left side, whereas the molecular marker sizes are reported on the right. The results are representative of two independent experiments.
amounts of infectious virus particles. It appeared that PIs inhibited the virus replication also when added early after challenge (Fig. 5B). The inhibitory effect dropped shortly in the case of VSV infection, and more gradually when cells were infected with influenza virus. Saquinavir and nelfinavir have shown similar inhibitory efficiencies (not shown).

These results indicate that the presence of PIs at early times after virus challenge is mandatory for the inhibitory effect, consistently with the idea that PIs target an early event of the replication cycle of both viruses.

**PIs affect neither binding nor endocytosis of viral particles**

The evidence that PIs inhibit viruses recognizing completely different strategies of nucleic acid replication, i.e., influenza virus,
VSV, and (VSV-G) HIV-1 in an HIV-1 protease independent manner, further supported the idea that PIs would influence some common early replication step, e.g., virus attachment, endocytosis, and/or endosomal fusion.

To dissect among these different possibilities, the intracellular fate of challenging virus was followed using GFP-labeled HIV-1-based VLPs (Muratori et al., 2006) pseudotyped with either wt or fusion-defective VSV-G (Fig. 6A). The fluorescence of these VLPs relies on the high incorporation levels of the product of fusion between GFP and a HIV-1 Nef mutant acquiring a palmitoylation site at its N-terminus as the consequence of the G to C substitution at the amino acid 3. First, the possible effects of PIs on the virus attachment on target cells were investigated. To this end, 10⁵ HeLa cells treated or not with PIs were challenged with 500 ng of (wt VSV-G) NefG3C-GFP VLPs for 1 h at 4 °C. Afterwards, the cells were extensively washed and FACS analyzed. To control that the cell-associated fluorescence was not a consequence of endocytosed VLPs, a part of the cell samples was treated with trypsin. It appeared that the treatment with PIs did not significantly affect the cell binding of fluorescent VLPs (Fig. 6B). This strongly suggests that PIs do not interfere with the binding of viral particles on the cell membrane.

Next, the influence of PIs on virus endocytosis was investigated. To this aim, GFP-fluorescent VLPs incorporating a VSVG mutant (here referred to as fd VSV-G) unable to support the pH-dependent fusion were used. The impaired fusion activity was a consequence of the A to K amino acid substitution at the position 133 (Fredericksen and Whitt, 1995). The use of this envelope protein mutant was associated with the trypsin treatment just before the FACS analysis to ensure that the cell-associated fluorescent signal exclusively referred to the intracellular accumulation of VLPs. HeLa cells treated or not with PIs were challenged with 500 ng HIV-1 Cap24 equivalents of (fd VSV-G) Nef⁶⁵C-GFP VLPs/10⁵ cells, and incubated for 3 h at 37 °C in the presence or not of PIs. Then, the cells were treated with trypsin, and the cell-associated GFP fluorescence was evaluated by FACS analysis. As control, VLP-challenged cells were incubated at 4 °C before the trypsin treatment. Again, no apparent differences in the cell-associated fluorescence were detected between control and PI-treated cells (Fig. 6C), suggesting that PIs have no influence on the efficiency of the endocytosis of viral particles.

This conclusion was enforced by the results obtained through an alternative experimental approach where control or PI-treated HeLa cells were infected with 0.2 pfu/cell of VSV. Thirty and 60 min later, the cells were extensively washed, treated with trypsin to leave out non-adsorbed viral particles, lysed, and analyzed by western blot for the presence of VSV-G. No significant reduction of the VSV-G specific signals was detectable at both time points in PI-treated cells as compared to control conditions (Fig. 6D). Considering that the neosynthesis of VSV-G starts at least 90 min post-infection (Rothman and Lodish, 1977), these results, consistently with the data obtained with fluorescent VLPs, suggest that PIs do not affect the endocytosis of viral particles.

The results from these experiments indicate that the inhibitory effect of PIs is not the consequence of impaired attachment and endocytosis of viral particles.

**Pls act at the level of intracellular viral fusion**

Next, the possibility that the inhibitory effect of PIs was a consequence of a defect in the endosome to cytoplasm virus delivery was investigated. To this end, the differences in the cell-associated fluorescence levels in cells internalizing wt or fd VSV-G pseudotyped Nef⁶⁵C-GFP VLPs were exploited. In fact, using the same experimental conditions described for the endocytosis assay, it was reproducibly observed that, early after VLP challenge, the mean fluorescence intensity (MFI) of cells challenged with (wt VSV-G) VLPs was near 2-fold higher than that of cells treated with (fd VSV-G) VLPs. More significantly, in the latter condition both percentages of fluorescent cells and MFI heavily dropped 16 h after challenge. On the contrary, both FACS parameters appeared only slightly reduced in cells challenged with (wt VSV-G) VLPs (Fig. 7A). These differences can be explained by the fact that, due to the inability of fd VSV-G to induce fusion and delivery of the VLP contents into cytoplasm, the Nef⁶⁵C-GFP molecules incorporated in (fd VSV-G) VLPs were addressed to rapid degradation into the endosomal/lysosomal compartment. Conversely, the efficient fusion between viral envelope and endosome membranes induced by wt VSV-G allowed the release of Nef⁶⁵C-GFP molecules into cytoplasm, where they are expected to be degraded with a kinetic much slower than that operating in endosomes/lysosomes.

Next, PI-treated or untreated HeLa cells were challenged with 500 ng HIV-1 Cap24 equivalent of either (wt VSV-G) or (fd VSV-G) Nef⁶⁵C-GFP VLPs/10⁵ cells, and 3 and 16 h later, the cells were treated with trypsin. FACS analysis of the cell-associated GFP fluorescence revealed that the PI treatment did not affect the fluorescence levels in cells challenged with (fd VSV-G) Nef⁶⁵C-GFP VLPs at both time points. On the contrary, after 3 h it was observed a relevant reduction in the MFI within PI-treated cells challenged with (wt VSV-G) Nef⁶⁵C-GFP VLPs as compared with untreated cells. More strikingly, a strong decrease of both MFI and percentage of fluorescent cells was detectable in PI-treated cells at 16 h...
post-challenge (Fig. 7B). As expected, among the different PI tested, saquinavir and nelﬁnavir produced the strongest inhibitory effect (data not shown).

Next, dose–response endocytosis assays using different concentrations of nelﬁnavir were carried out by analyzing the cell-associated fluorescence 16 h after the challenge. The results showed that, within the cells challenged with (wt VSV-G) Ne f G3C–GFP VLPs, the PI treatment reduced both MFI and percentages of ﬂuorescent cells in a dose-dependent manner. At the highest PI concentrations, both parameters reached levels similar to those detectable in cells challenged with (fd-VSV-G) Ne f G3C–GFP VLPs (Fig. 7C). Hence, it seemed that the PI treatment diverted the fate of endocytosed (wt VSV-G) towards that of (fd VSV-G) VLPs. This strongly suggests that PIs negatively affect the fusion of viral envelope with the endosomal membranes.

Together, these results support the idea that the inhibitory effect of PIs acts at the level of the delivery of the viral particles from endosomes to cytoplasm of infected cells.

PIs do not affect intracellular pH

A possible explanation for the apparent block of viral envelope fusion could be that PIs may increase the pH in endosomes in a way to inhibit the low pH-dependent conformational changes needed for both VSV-G and inﬂuenza HA to switch the fusion process. To test whether PIs affect intracellular pH, both HeLa and MDCK cells were labeled with LysoSensor Green DND-189. This reagent becomes ﬂuorescent only in acidic intracellular compartments, meanwhile exhibiting decreased ﬂuorescence intensity upon pH increase. Thus, it represents a useful reagent for detecting possible pH variations in endosomes. Cells were treated overnight with the PI doses most effective against VSV and/or inﬂuenza virus replication, then labeled with LysoSensor Green DND-189 for 30 min in the presence of PIs, and ﬁnally analyzed by FACS. As control, cells treated with bafﬂomycin A1, i.e., a powerful inhibitor of the vacuolar ATPase proton pump, were also tested. As depicted in Fig. 8, no signiﬁcant variations in the cell-associated ﬂuorescence have been detected in PI-treated cells as compared with control conditions, indicating that PIs do not affect the intracellular pH. These results strongly suggest that the PI-induced block of virus delivery in cytoplasm would not be a consequence of the increase of endosomal pH.

Discussion

Viruses hijack cell functions to replicate in host cells. Hence, the selective targeting of cell products supporting virus replication could represent a successful antiviral strategy. The results from the here described investigations indicate that drugs designed to counteract the activity of the HIV protease show a strong inhibitory effect against unrelated virus species likely by targeting yet unidentified protease

![Fig. 8](image-url) The PI treatment does not affect intracellular pH. Cells were treated overnight with the indicated concentrations of PIs or, as control, of bafﬂomycin A1, and then labeled with LysoSensor DND-189. After 30 min of incubation, cells were harvested, and the cell associated ﬂuorescence measured by FACS analysis. The results are reported as percent of the mean ﬂuorescence intensity (MFI) of control conditions, i.e., cell cultures with or without appropriate dilutions of DMSO, and were calculated as the mean values ±SD from two independent experiments with duplicate conditions.
activity[es] of target cells. The antiviral potency of the most effective PIs reached about 4 logs in the reduction of virus yields, with therapeutic indexes higher than 15. The PI-induced effect against VSV appeared comparable in magnitude to that observed in cell treated with type-1 interferons (Masters and Samuel, 1983). On the other hand, the potency of PIs against the influenza virus replication appeared comparable to that of most potent inhibitors. Among these, T-705 (Furuta et al., 2002) reduced the viral yield of the most susceptible influenza strain tested of at best 2.7 logs at the concentration of 5 μM upon MDCK cell challenge with 0.01 m.o.i. (Sleeman et al., 2010). DAS181, i.e. a sialidase fusion protein, blocked the influenza virus replication at sub-micromolar concentrations, however when MDCK cells were challenged with 0.001–0.005 m.o.i. (Triana-Baltzer et al., 2009). Conversely, micromolar concentrations of PIs induced more than 3 logs of reduction of the influenza virus yield from MDCK cells challenged with 0.5 m.o.i.

In the challenge experiments where PIs were added soon after the adsorption of VSV or influenza virus inocula, the antiviral effect appeared slightly reduced as compared with control conditions where PIs were maintained throughout. Considering also the results obtained in the endocytosis assays, it is conceivable that this subtle difference could be a consequence of the accession to cytoplasm of a small amount of viral particles during the adsorption time and before the PI treatment.

The possibility that PIs affect some virion structural component in a way to hinder the process of fusion in endosomes appeared unlikely. In fact, the treatment of 10-fold concentrated VSV or influenza virus preparations with up to 500 μM PIs for 30 min before challenge did not produce significant decrease of the viral yields (not shown). However, we cannot formally exclude that PIs inhibit some yet unidentified protease activity associated with VSV-G or influenza virions. In this regard, it has been proposed that PIs could negatively affect the chymotrypsin-like protease activity of the PA subunit of the RNA-directed RNA polymerase of influenza virus (Savarino, 2005). Furthermore, it was reported that nelfinavir reduces the replication of SARS coronavirus (Yamamoto et al., 2004) likely as a consequence of the accession to cytoplasm of a small amount of viral particles during the adsorption time and before the PI treatment.

The here reported findings would open the way towards preclinical assays designed to test the potency of PIs against in vivo infections sustained by Orthomyxov- and Rabies viruses. It will be also of interest extending the investigations on additional pathogenic enveloped viruses infecting by endocytosis.

Materials and methods

Virus preparations

VSV-G pseudotyped HIV-1 preparations were obtained from supernatants of 293T cells 48 h after co-transfection with a CMV immediate-early promoted VSV-G expressing vector and vectors expressing NL4-3 HIV-1, its Δenv derivative, or PM4 HIV-1 (molar ratio 1:5) performed by the Lipofectamine 2000-based method (Invitrogen). Supernatants wereclarified and concentrated by ultracentrifugation as described (Federico et al., 2001). Virus preparations were titrated by measuring HIV-1 Cαp24 contents by quantitative enzyme-linked immunosorbent assay (ELISA; Innogenetic). Both preparations and assays of VSV (Indiana strain) have been performed basically as described (Gresser et al., 1968). Briefly, high titer stocks of VSV have been prepared upon infection of HeLa cells with at low multiplicity of infection (m.o.i.), i.e. ~0.01 plaque forming unit (PFU)/cell. Supernatants were harvested 24 h later, clarified, stocked, and frozen. VSV titrations of high titer stocks have been carried out by plaque method. The A/Puerto Rico (PR)/8/34 H3N1 human influenza virus was grown in 11-day-old embryonated chicken eggs. The allantoic fluid was clarified by centrifugation at 5000 × g for 15 min at 4 °C. The virus was pelleted by centrifugation at 65,000×g for 1 h at 4 °C and resuspended in 1 ml of phosphate-buffered saline. Portions of the solution were stored as aliquots at −20 °C. Influenza preparations were titrated by standard plaque assay on MDCK cells. Virus titers for these stocks ranged from 1 to 8 × 107 PFU/ml. Preparations of the Hertz strain of NDV were recovered after 48 h of incubation in 9–11-day-old embryonated chicken eggs inoculated in the allantoic cavity. After harvesting, the allantoic fluid was clarified by centrifugation at 10,000 × g and titrated as infectious units/ml through the end-dilution method by assessing the cytopathic effect on MDCK cells 48 h post infection. The titer of the viral stock used was 2.1 × 108 infectious units/ml.

Cell cultures, infections, and titrations

CEM<sub>CFP</sub> cells were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat-inactivated fetal calf serum (FCS). HeLa, MDCK, 293T and 293/GPR inducible HIV-1 packaging cells (Sparacio et al., 2001) were grown in Dulbecco’s modified Eagle’s medium plus 10% FCS. Infections of CEM<sub>CFP</sub> cells with HIV-1 or pseudotyped derivatives were carried out by spinoculation at 400 × g for 30 min at room temperature (r.t.) using 200 ng and 50 ng Cαp24 equivalent of HIV-1 and (VSV-G) HIV-1/10<sup>5</sup> cells, respectively. Then, virus adsorption was prolonged for additional 2 h at 37 °C and, finally, cells were washed and refed with the complete medium. HeLa cells were infected with VSV by adsorbing the viral inoculum for 1 h at 37 °C in a small volume (e.g., 0.1 ml of serum free medium for 2 × 10<sup>5</sup> cells in 12 well plates). Thereafter, the cells were extensively washed, and refed with appropriate complete medium.
MDCK cells were used as targets of PR8 influenza virus. The challenges were carried out as for VSV infection, except that after virus adsorption, cells were refed with medium without serum in the presence of 1 μg/ml of TPCK-treated trypsin (Worthington Biochemical Corporation).

HeLa and MDCK cells served to titrate infectious VSV and influenza virus in supernatants harvested after challenge experiments. The titrations were carried out by the end-dilution method with triplicate conditions by challenging the cells with 3-fold scaled supernatant dilutions (in the presence of 1 μg/ml TPCK-trypsin for influenza virus titrations), and by evaluating the cytopathic effect after 36 h (for VSV) and 72 h (for influenza virus). Concentrated virus preparations previously titrated by the plaque assay were used as standards.

For NDV infections, MDCK cells were challenged with 2-fold decreasing viral dilutions starting to m.o.i. 1, and the cytopathic effect was assessed 48 h later.

T-20, ritonavir, indinavir, saquinavir, nelfinavir, amprenavir and atazanavir were obtained from the NIH AIDS Research and Reference Reagent Program.

**CFSE-based cell duplication assay**

Both control and PI-treated cells were labeled with 1 μM CFSE (Molecular Probes, Invitrogen) following the manufacturer's recommendations. A cell sample was immediately processed to determine the fluorescence levels at the zero time. Thereafter, the remaining cell cultures were refed with complete medium and, at the time of completion of one cell duplication (on the average, 12 h for both HeLa and MDCK cells at their logarithmic phase), were harvested, and the fluorescence measured by FACS analysis. Dead cells were identified upon labeling with 5 μg/ml of propidium iodide (Sigma-Aldrich).

**Preparation of fluorescent VLPs, challenge, and detection assays**

Fluorescent VLPs were obtained as previously described (Muratori et al., 2009). Briefly, 293/GPR HIV-1 packaging cells were co-transfected with vectors expressing the green-fluorescent protein (GFP) fused at its N-terminus with a $^{125}$I HIV-1 Nef mutant, together with a vector expressing wt or fusion-defective (fd) VSV-G (Fredericksen and Whitt, 1995). Supernatants were harvested 2 days later, concentrated by ultra-centrifugation on 20% sucrose cushion, and titrated for the HIV-1 Cap24 contents.

For HeLa cell challenge, 500 ng Cap24 equivalent of fluorescent (VSV-G) HIV-1 VLPs/10$^6$ cells were adsorbed for 1 h at 4 or 37 °C in a volume of 0.1 ml in 48 well plates. Thereafter, 0.1 ml of complete medium was added and, finally, the cells were extensively washed and analyzed by FACS. In the endocytosis assays, the FACS analysis was carried out after incubation with trypsin for 15 min at 37 °C.

**Western blot analysis**

Both cells and purified VLP preparations were lysed in PBS, 1% Triton X-100 in the presence of anti-proteolytic agents. For the preparation of cytoplast extracts, whole cell lysates were centrifuged at 6000 × g for 10 min at 4 °C, and the supernatants frozen at −80 °C. Aliquots of 200 ng HIV-1 Cap24 equivalent of VLPs and of 30 μg of total cell proteins were separated in 10% SDS-PAGE, and then transferred by electroblotting on nitrocellulose membranes (Sartorius AG) for 60 min at 100 V with a Bio-Rad transblot. Nitrocellulose membranes were blocked in 3% bovine serum albumin (BSA) fraction V (Sigma) in TTBS/EDTA (10 mM Tris–HCl, pH 7.4; 100 mM NaCl; 1 mM EDTA; 0.1% Tween-20) for 30 min at room temperature, then incubated for 1 h at r.t. with specific antibodies diluted in 1% BSA/TTBS-EDTA. The following Abs served for the revelation of both VLP and cell-associated products: ARP 444 sheep anti-Nef antisera from Mark Harris, University of Leeds, Leeds, UK; rabbit polyclonal anti-VSV-G Abs from Immunology Consultant Laboratories; monoclonal anti human β-actin from Amersham Pharmacia Biotech. Immune complexes were detected through horseradish peroxidase-conjugated goat anti-sheep, anti-rabbit (both from Calbiochem) and antimouse Abs (NEN), followed by enhanced chemiluminescence reaction (Euroclone).

**Detection of intracellular pH variations**

The Lysosensor probe DND-189 (Molecular Probes, Invitrogen) served to detect possible changes in the endosomal pH upon PI treatment. This is an acidotropic probe accumulating in acidic organelles which exhibits decreasing fluorescence upon pH increase. Cells pre-treated with PIs or, as control, with bafilomycin A1 (Sigma-Aldrich) were labeled with 1 μM of Lysosensor DND-189 in complete medium for 30 min in the presence of the drugs. Afterwards, cells were extensively washed, fixed, and fluorescence evaluated by FACS analysis.

**Statistical analysis**

When appropriate, data are presented as mean ± standard deviation values (SD). In some instances, statistical analysis was performed according to paired Student's t-Test, and confirmed using the non-parametric Wilcoxon rank sum test. P-values < 0.05 were considered significant.

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