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Antiviral strategies combining antiretroviral drugs with RNAi-mediated attack on HIV-1 and cellular co-factors

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ABSTRACT

To improve the care of HIV-1/AIDS patients there is a critical need to develop tools capable of blocking viral evolution and circumventing therapy-associated problems. An emerging solution is gene therapy either as a stand-alone approach or as an adjuvant to pharmacological drug regimens. Combinatorial RNAi by multiplexing antiviral RNAi inhibitors through vector-mediated delivery has recently shown significant superiority over conventional mono-therapies. Viral as well as cellular co-factor targets have been identified, but they are generally attacked separately. Here, we hypothesized that a mixture of shRNAs directed against highly conserved viral RNA sequences and the mRNAs of cellular components that are involved in HIV replication could restrict mutational escape by enhanced synergistic inhibition. We screened for potent silencer cocktails blending inhibitors acting scattered along the viral replication cycle. The results show enhanced and extended suppression of viral replication for some combinations. To further explore the power of combinatorial approaches, we tested the influence of RNAi-mediated knockdown on the activity of conventional antiretroviral drugs (fusion, RT, integrase and protease inhibitors). We compared the fold-change in IC_{50} ($FCIC_{50}$) of these drugs in cell lines stably expressing anti-HIV and anti-host shRNAs and measured increased values that are up by several logs for some combinations. We show that high levels of additivity and synergy can be obtained by combining gene therapy with conventional drugs. These results support the idea to validate the therapeutic potential of this anti-HIV approach in appropriate *in vivo* models.

1. Introduction

Among the many steps of the human immunodeficiency virus type 1 (HIV-1) replication cycle that could theoretically be inhibited, five steps are targeted by the available 26 antiretroviral drugs (De Clercq, 2010): viral entry into the cell at the level of receptor binding and the subsequent membrane fusion process, reverse transcription, integration and proteolytic processing of the viral proteins. To treat HIV-1 infected patients, highly active antiretroviral therapy (HAART) regimens have been developed that usually consist of a triple combination of reverse transcriptase (RT), protease (PR), fusion/entry or integrase (IN) inhibitors. HAART achieved great clinical success, but it fails to provide a definite cure and viral clearance remains elusive (Bowman et al., 2009; Geeraert et al., 2008). The development of drugs that target different steps of the HIV-1 replication cycle remains important (Goldberg et al., 2012).

As a novel antiviral approach, the mechanism of RNA interference (RNAi) provides a promising genetic tool that enables the suppression of any viral or host cell function involved in the viral replication cycle (van Rij and Andino, 2006). RNAi can be induced by transfection of small interfering RNAs (siRNAs) or by short hairpin RNAs (shRNAs) that are intracellularly expressed from a gene cassette (Barichievy et al., 2009). Targeting of viral RNAs or the mRNAs encoding cellular co-factors imposes specific advantages and shortcomings. Host targeting may cause cytotoxicity, but one also cannot preclude adverse off-target effects of anti-HIV shRNAs. A major problem of virus targeting forms the selection of escape variants (Boden et al., 2003; Das et al., 2004; Westerhout et al., 2005). Promising anti-escape approaches include targeting of highly conserved and evolutionary restrained regions of the viral RNA genome (Nishitsuji et al., 2006; von Eije et al., 2008), the simultaneous use of multiple inhibitors in a combinatorial RNAi approach (Liu et al., 2008; ter Brake et al., 2006, 2008) or the use of RNAi reagents in combination with other RNA-based inhibitors (DiGiusto et al., 2010). Targeting of host factors may have a double advantage concerning viral escape. First, inhibition of an important

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co-factor will be effective against all viral variants in an infected individual and likely all HIV-1 strains and subtypes that circulate worldwide. Second, by targeting a cellular component that is critical for virus replication, theoretically the only viral escape route would be adaptation to an alternative cellular co-factor. Thus, it would seem important to target cellular factors or pathways that lack redundancy (Eekels and Berkhout, 2011).

RNAi does not allow an early attack on the RNA genome of the infecting virus particle (Westerhout et al., 2006), but such an early block is possible by RNAi suppression of cellular entry factors. The chemokine receptor 5 (CCR5) as HIV-1 receptor is a promising target because this protein is apparently not important for human physiology as demonstrated by individuals with a homozygous gene deletion that interrupts CCR5 protein expression. Furthermore, a proof of concept for this approach was obtained by bone marrow transplantation from such a CCR5-minus donor in the "Berlin" HIV-1 patient who subsequently did not need antiviral drugs to maintain an undetectable viral load (Hütter et al., 2009). This functional cure has spurred a search for other co-factors that are vital for HIV-1 replication, yet whose depletion does not have an impact on cell viability. This search included genome-wide RNAi screens (Brass et al., 2008; Zhou et al., 2008), but such transient assays with reporter genes in non-T cells are remote from the physiological setting. Some candidate host factors were subsequently tested for their antiviral activity in lymphocyte T cells (Eekels et al., 2011).

Co-factor silencing offers the ability to inhibit a broad range of additional viral replication steps. This could set the stage for a deeper understanding of viral dynamics. For instance, recent mathematical modelling predicted that HIV-1 decay dynamics depend on the stage of the viral replication cycle that is attacked, much more so than the actual drug efficacy (Sedaghat et al., 2008). A quantitative analysis also provided evidence for class-specific limitations of antiretroviral drug efficacy (Shen et al., 2008). The combinatorial antiviral approach is still considered a very prominent strategy for blocking the appearance of drug-resistant variants (Colman, 2009) and a recent study reinforced the importance of testing anti-HIV drug combinations in order to find synergistic drug pairs (Tan et al., 2012). Therefore extending our understanding of how the overall inhibitory efficacy depends on the different step/stage(s) targeted in the context of a multi-component antiviral strategy should be very useful.

We tested different combinations of three antiviral approaches that were previously tested individually: RNAi-mediated suppression of HIV-1 or cellular co-factors and conventional antiretroviral drugs. To date two relatively small studies have reported positive effects by combining transient RNAi knockdown of a viral component and small-molecule antiretroviral drugs, showing either a synergistic effect (Leonard et al., 2008) or an enhanced effect against drug-resistant HIV-1 strains (Huelsmann et al., 2006). We investigated here the additive efficacy of each shRNA type when combined with antiretroviral drugs belonging to specific drug classes. We intentionally chose shRNAs and antiretroviral drugs that act scattered along the HIV-1 replication cycle (Table 1).

2. Experimental/material and methods

2.1. shRNA constructs, antiretroviral drugs and cells

Anti-host shRNA constructs were described (Eekels et al., 2011). Anti-HIV shRNA constructs are based on lentiviral vectors (ter Brake and Berkhout, 2007; ter Brake et al., 2006). The shRNAs Gag-5, Pol-1, Pol-47, R/T-5 and Nef (renamed Gag5, Pol1, Pol47, RT5 and Nef respectively) are encoded in the JS1 vector, a third generation self-inactivating lentiviral vector with GFP reporter. The position of the target sequence on the HXB2 genome and the shRNA sequence is as follows: Pol1 (2328) ACAGGAGCAGAUGAUACAG; Pol47 (4963) GUGAAGGGGCAGUAGUAAU; RT5 (5970) AUGGCAGGAAGAAGCGGAG; Gag5 (1819) GAAGAAAUGAUGACAGCAU; Nef (9080) GTGCCTGGCTAGAAGCACA. These target sequences are highly conserved among HIV-1 isolates, with 100% identity in at least 75% of the 170 complete HIV-1 genomes, including all HIV-1 subtypes, present in the Los Alamos National Laboratory database (ter Brake et al., 2006). We obtained Raltegravir (RAL, MK-0518) from Bio-Connect Services, Lamivudine (3TC) from GlaxoWellcome, Indinavir (IDV) from Merck, and T1249 was synthesized (Eggink et al., 2009). T1249 was dissolved in double-distilled water, stored at −20 °C and diluted in Dulbecco's Phosphate-Buffered Saline (D-PBS) before use. Other drugs were dissolved in dimethylsulfoxide (DMSO) at 1 mM (RAL) or 10 mM (3TC, IDV) and stored at −80 °C. The drugs were diluted in D-PBS before use to reduce the DMSO concentration < 0.5% (vol/vol). The PM1 T cell line (Lusso et al., 1995) was grown in advanced RPMI 1640 medium with 1% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 5 mM L-glutamine.

2.2. Lentiviral vector production, CA-p24 ELISA and stable PM1 cell lines

The shRNA-expressing were produced as described (ter Brake et al., 2006) and virus production was monitored with a CA-p24 enzyme-linked immunosorbent assay (ELISA) (ter Brake et al., 2006). The transduction titer was measured via GFP expression. Transduction was performed at a multiplicity of infection (MOI) of 0.15 in a T25 flask seeded with 1×10^6 PM1 cells in a total volume of 5 ml to which the lentiviral vector was added for overnight incubation. Lentiviral vector transduction was performed as described for anti-host shRNAs (Eekels et al., 2011) and anti-HIV shRNAs (Liu et al., 2008). For generation of PM1 cell lines expressing both shRNA types (anti-host and anti-HIV), the anti-HIV shRNA expressing PM1 cells (sorted GFP-positive cells) were transduced with an anti-host shRNA lentiviral vector with subsequent puromycin selection.

2.3. Cell growth analysis and RT-qPCR

Growth of shRNA-expressing cells was monitored after a week of puromycin selection by daily cell counting for 5 days using FACS (Flowing software v2.2 http://www.flowingsoftware.com/). Measurements were performed as described (Eekels et al., 2011) and cell population doubling times were calculated based on the logarithmic growth phase using the Doubling Time Software v1.0.10 (http://www.doubling-time.com). For selected cell lines the knockdown efficiency of the targeted mRNA was measured by RT-qPCR and performed as previously described (Eekels et al., 2011).

2.4. HIV-1 infection of PM1-shRNA cells

The HIV-1 stock was produced by transfection of HEK293T cells with the molecular clone of the primary CXCR4-using HIV-1 LAI isolate (Peden et al., 1991). Cell-free viral stocks were passed through 0.45 μm pore-size filters. PM1-shRNA and control cells (500 μl cultures in 24-well plates, 1.5×10^5 cells/well) were infected with 500 μl of virus-containing medium. The viral input ranged from 0.015 to 0.15 ng of CA-p24 (intermediate and high viral dose respectively). Virus replication was monitored every 2 days by scoring syncytia formation and supernatant samples were taken for CA-p24 ELISA at the indicated times. For the 7 days experiment, cells were passaged on day 3. For the 25/16 days experiments, cells were passaged on day 3, 7, 10, 14, 17, 21, and 24. Relative CA-p24 values at peak of infection (day 6 or 7) were averaged from three

Table 1
HIV-1 replication steps targeted by the different type of inhibitors.

Step in the HIV-1 life cycle	Inhibitor type		
	ARV drug	Anti-HIV shRNA	Anti-host shRNA
1. Fusion	T1249		
2. Reverse transcription	3TC		
3. Nuclear import			IPO7
4. Integration	RAL		
5. Gene expression		Pol1, Pol47, RT5, Gag 5, Nef	
6. Virion assembly	IDV		HSPD1
7. Budding			ALIX
8. Maturation	IDV		
Unknown ?			ATG16

independent experiments after normalisation with respect to the control cell line (untransduced PM1 cells).

2.5. Antiviral assays for antiretroviral drugs on PM1-shRNA cells with dose–response curves

The 50% tissue culture infectious dose (TCID50) of the HIV-1 LAI isolate was measured in untransduced PM-1 T cells to standardize the infection across different drug treatments and cell types. Antiretroviral drug treatment was carried out in 96-well plates prefilled with 100 µl medium. We made 10-fold, 5-fold (RAL, 3TC, IDV) or 3-fold (T1249) serial dilutions in the first 11 columns. The last column received no drug as positive control. A mixture of 10,000 cells and 100 TCID50 of LAI virus in a total volume of 100 µl was distributed per well. For T1249, we first added 10 TCID50 LAI in 50 µl followed by a 30 min incubation at room temperature before addition of 50 µl of 10,000 pre-washed cells. The readout was performed at day 5 by CA-p24 ELISA. Dose–response curves of three independent experiments were generated using GraphPad Prism software v5.0. and by normalizing CA-p24 values without drug treatment to 0% (highest CA-p24 value) and the values with the highest drug concentration to 100% (maximal response). Data are presented as percentage of inhibitory activity. IC_{50} values are the best-fit values generated by fitting curves with a four parameters non-linear regression model and 95% confidence intervals (95% CI) are shown. IC_{90} were calculated with equation (1).

$$IC_{90} = \left(\frac{F}{100 - F}\right)^{\frac{1}{H}} \times IC_{50} \quad (1)$$

where F is the fraction of maximal response (for IC_{90}, $F = 90$) and H the Hill slope. Data represent the average values from at least three independent experiments. For every antiretroviral drug, fold-change in IC_{50} ($FCIC_{50}$) was calculated according to Eq. (2).

$$FCIC_{50} = \frac{[IC_{50}]_{transduced}}{[IC_{50}]_{untransduced}} \quad (2)$$

3. Results

3.1. Selection of host cell factors for gene knockdown

We first set out to optimize cellular co-factor silencing by means of shRNAs. In a recent study we performed a large-scale screen of 30 host factors that were silenced by shRNAs from the MISSION™ TRC-Hs 1.0 library (Eekels et al., 2011). For the present study, we chose four host factors for which strong HIV-1 inhibition was obtained with at least two shRNAs in the SupT1 T cell line: autophagy related 16-like 1 or ATG16L1 (hereafter ATG16) with a poorly defined function in HIV-1 replication (Brass et al., 2008), heat shock protein HSPD1 involved in virion assembly (Gurer et al., 2002), apoptosis-linked gene 2-interacting protein X (ALIX) that participates in virus budding (Strack et al., 2003) and importin 7 (IPO7) implicated in nuclear import of HIV-1 DNA genome (Ao et al., 2007) (Table 2). The previous screen was performed in the SupT1 T cell line that expresses the CXCR4 (X4) co-receptor. We now performed experiments with the PM1 T cell line that constitutively expresses both X4 and CCR5 (R5) co-receptors to facilitate the study of primary HIV-1 isolates that frequently use the R5 co-receptor.

3.2. Antiviral activity of anti-host shRNAs in stably transduced PM1 cells

We tested five different shRNAs per co-factor in order to maximize the chances for efficient co-factor knockdown in the PM1 cell line. Cells were stably transduced with shRNA-expressing lentiviral vectors from the TRC library at a relatively low MOI of 0.15 to obtain maximally a single integrated lentiviral vector per cell, to avoid shRNA overexpression and saturation of the RNAi machinery. The empty lentiviral vector (SHC1) was used as negative control. We thus generated 21 PM1 cultures upon puromycin selection. Several samples were excluded from further analyses because of strong shRNA-induced cytotoxicity: ATG16 shRNA 1 (or ATG16-1), HSPD1-5 and ALIX-3. The remaining cell samples were subsequently challenged with the HIV-1 LAI virus at intermediate and high viral dose (Fig. 1a, left and right panel, respectively).

Production of HIV-1 capsid protein (CA-p24) was measured in the culture supernatant over a period of 7 days and syncytia formation was monitored every other day. Relative p24 values at peak of infection are shown and were averaged from three independent experiments. Efficient HIV-1 replication was measured in the untransduced PM1 cells and the control SHC1 cells. At least one shRNA per co-factor exhibited some antiviral activity. ATG16-2 showed the highest inhibitory activity, even at high viral

Table 2
Cellular co-factors influencing HIV-1 replication.

Anti-host shRNA name	Cellular co-factor targeted	Function in HIV-1 replication	HIV-interacting protein	Reference
IPO7	Importin 7	Nuclear import of reverse transcription complex	Matrix/NC/Integrase/ Vpr	(Ao et al., 2007)
HSPD1	Heat shock 60 kDa Protein 1 (chaperonin)	Incorporated into virion through gag-interaction	Pr55/Integrase	(Gurer et al., 2002)
ALIX	Programmed cell death six interacting protein	Viral budding machinery	P6	(Strack et al., 2003)
ATG16	ATG16 autophagy 16-like (*S. cerevisiae*)	Autophagy factor	?[a]	(Brass et al., 2008)

[a] Function of ATG16 is not yet determined.

input. ATG-5 showed modest but reproducible antiviral activity combined with delayed syncytia formation. HSPD1-3 and -4 provide high anti-HIV activity at the intermediate viral dose, and the effect is fairly prolonged at high viral challenge for HSPD1-3. A moderate but still significant inhibition was scored for IPO7-1 and -3, which was sustained for IPO7-1 at higher viral challenge. For the ALIX target, only modest viral suppression was measured for two shRNAs (1 and 2), pronounced at intermediate viral dose and for ALIX-1 slightly sustained at higher viral dose. We selected seven anti-host shRNAs (ATG16-2 and -5, HSPD1-3 and -4, IPO7-1 and -3 and ALIX-1) for subsequent tests. It is noteworthy and reassuring that six out of these seven selected shRNAs were also scored as good antivirals in the previous screen in SupT1 cells (Eekels et al., 2011).

In order to monitor cytotoxicity of the selected shRNAs, the impact on cell proliferation was monitored by carefully counting the cell number during exponential growth using FACS analysis. The average doubling time measured in three independent experiments is presented in Fig. 1b. Most of the cell lines exhibited a doubling time of approximately 1.5 days that is similar to that of the control PM1 and SHC1 cells. We detected a slightly increased doubling time for HSPD1-4, IPO7-3 and ALIX-1. A doubling time of about 1.7 days was calculated for HSPD1-4 and IPO7-3. These shRNAs were removed from the study, except for ALIX-1 as the only effective ALIX inhibitor that enabled us to study the multiple-step targeting concept. We also measured the level of shRNA-induced mRNA knockdown by RT-qPCR (Fig. 1c). For each of the ATG16, HSPD1 and IPO7 co-factors, the most potent anti-HIV shRNA also triggered the most efficient mRNA knockdown. For ALIX-1 shRNA, we observed a very modest decrease of the target gene expression (3%), which may suggest that its inhibitory activity is due to a non-specific effect. For this reason, this shRNA was also removed from the study. Based on these combined results, we selected ATG16-2 and

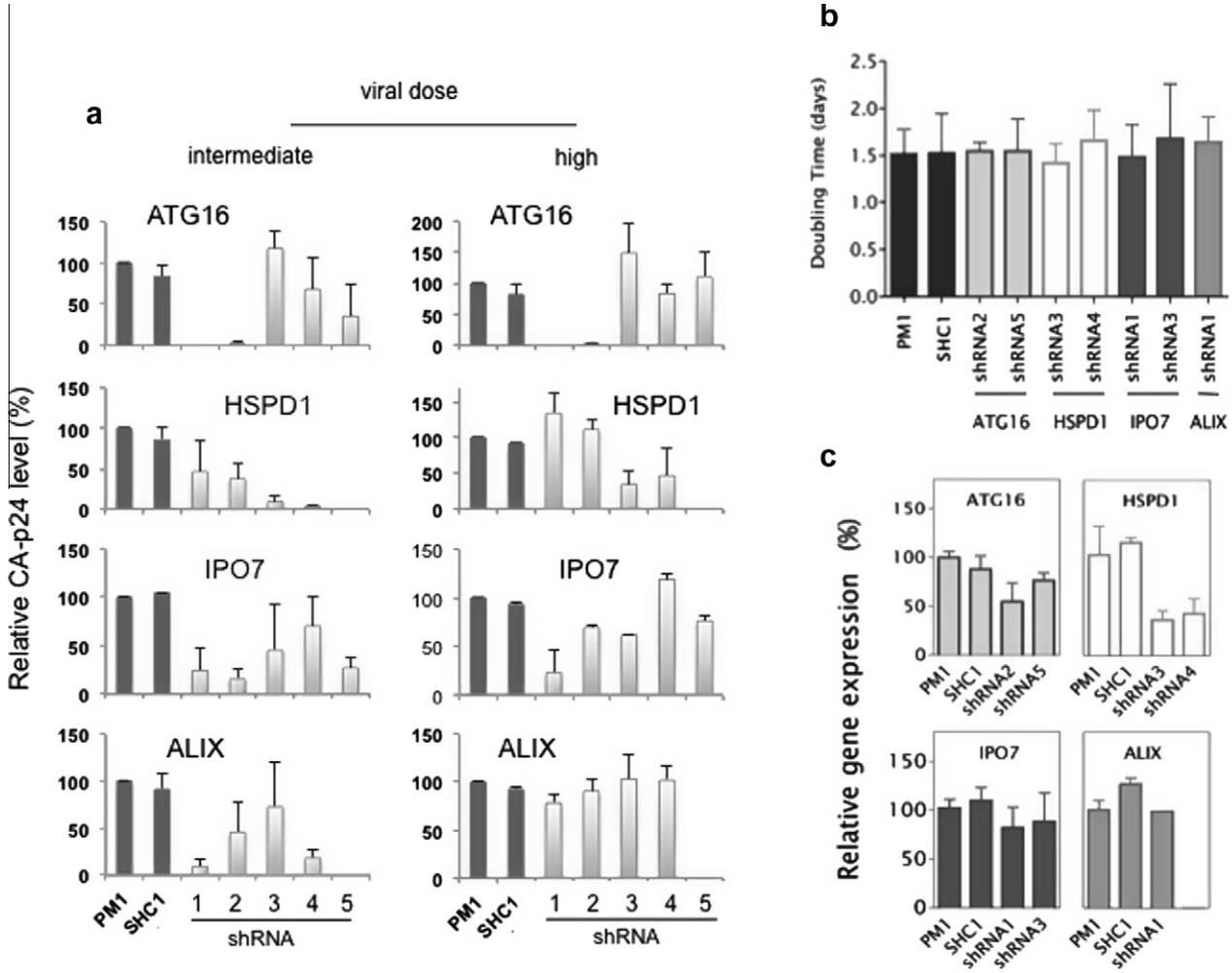

Fig. 1. Screening of antihost shRNAs. (a) Antiviral activity in PM1 cells stably expressing anti-host shRNAs. Twenty one shRNA-expressing cell lines were challenged with an intermediate (left panel) or high (right panel) viral dose of LAI virus and tested for their resistance to HIV-1 replication. From top to bottom, each panel corresponds to the knockdown of one host factor (ATG16, HSPD, IPO7 or ALIX) targeted by 4 to 5 different shRNAs (shRNA 1–5). The relative HIV-1 CA-p24 level compared to the control PM1 cells (untransduced) is shown at the peak of infection. Averages and standard deviation were calculated from three independent experiments. The controls, PM1 and SHC1, correspond to untransduced cells and cells transduced with the empty vector. (b) Scoring potential adverse effects of the shRNA-knockdown on cell growth. The cell lines that express the best HIV-1 inhibitors were tested for cytotoxicity. The cells were counted by FACS over a period of 5 days. The doubling time indicated in days is based on the logarithmic growth phase and was computed from three independent experiments for which the mean and the respective standard error are shown. The control cell lines PM1 and SHC1 are depicted in black. (c) Quantifying the mRNA knockdown triggered by the anti-host shRNAs. The mRNA expression level of each target in the shRNA-expressing cell line was measured by RT-qPCR. The control cell lines PM1 and SHC1 are depicted for each target. Results are depicted as percentage of relative gene expression and target mRNA was normalized based on the target and the beta-actin mRNA measured in the control SHC1 cell line. The mean and its standard error are from three independent experiments.

HSPD1-3 as good inhibitors and we added IPO7-1 as moderate antiviral.

3.3. Antiviral activity of anti-HIV shRNAs

We next validated several previously developed anti-HIV shRNAs upon stable transduction of the PM1 cell line. The Gag5, Pol1, Pol47 and RT5 shRNAs were designed to target highly conserved regions of the viral RNA genome and exhibited strong and durable antiviral activity in SupT1 cells (ter Brake et al., 2006, 2008). No adverse effects or reduced cell growth rates were observed in PM1 cultures for most shRNAs (results not shown), except for Gag5 that exhibited considerable cytotoxicity and that was consequently removed from the study. Cell toxicity was apparent for Gag5 by a dramatic delay of cell growth (e.g. yielding an intense pink color of the culture medium) and by visual inspection of the culture under the light microscope (few cells, cell death etc.). A parallel study in our laboratory confirmed the cytotoxicity of the Gag5 shRNA in a new competitive cell growth assay (Eekels et al., 2012a), which led to its removal from the translational track towards a clinical trial (Knoepfel et al., 2012). We instead included the combinatorial RNAi vector R3A that expresses the three non-toxic shRNAs Pol1, Pol47 and RT5. The cells were challenged with HIV-1 LAI and viral spread was monitored by measuring the CA-p24 levels over a 7-day period. All anti-HIV shRNAs demonstrated potent and durable inhibition at the intermediate virus dose (Fig. 2, left panel). The antiviral activity was maintained for RT5 and R3A when cells were infected with a high viral dose (right panel). These results confirm the antiviral potency of this anti-HIV shRNA set in PM1 cells, which enabled us to set up combinatorial antiviral strategies with anti-host shRNAs and antiretroviral drugs.

3.4. Combining anti-host with anti-HIV shRNAs

We generated PM1 cell lines that stably co-express different combinations of the previously identified anti-HIV and anti-host shRNAs. To do so, we transduced the anti-HIV cassette and sorted for GFP-positive cells, and then transduced the anti-host cassette followed by puromycin selection. In this way, eight cell lines were generated expressing a combination of an anti-host shRNA (ATG16-1 or HSPD1-3) and an anti-HIV shRNA (Pol1, Pol47, RT5 or Nef) and two control cell lines with the empty vector JS1 or both empty vectors (JS1 + SHC1). To replace Gag5, we included the potent antiviral shRNA Nef (Das et al., 2004).

Because we could partially overcome the suppressive effects of these antivirals in PM1 cells by the use of a higher input of challenge virus (see Figs. 1a and 2), this experimental system allowed us to measure additive inhibitory effects. We infected six independent cultures of each cell line with the HIV-1 LAI isolate. Infections were monitored by visual inspection for syncytia formation at day 10, which revealed no major differences between the replicates. Duplicate infections were analysed for CA-p24 production and one data set is presented in Fig. 3. In each panel, we compared the two single transduced cells (open symbols) with the shRNA combination (closed symbols). We used three control cells: untransduced PM1 cells, cells transduced with the empty JS1 vector and cells transduced with both empty vectors JS1 and SHC1. Potent HIV-1 inhibition was scored for all anti-HIV shRNAs and more moderate inhibition by all anti-host shRNAs, consistent with our previous observations. Most importantly, we observed additive inhibition by combination of the two strategies in seven out of eight cases: all combinations except HSPD1 with RT5. We observed an impact on virus-induced syncytia for all the shRNA combinations, ranging from a reduction (**) to the complete absence of syncytia (***). Potent virus inhibition correlated with the absence or the significant reduction of syncytia and cell death in the cultures. We obtained a durable and strong block of HIV-1 replication for up to 25 days in a few cultures (1 of 6 ATG16 + Pol47 cultures, 2 of 6 ATG16 + Nef cultures, results not shown). Noticeably, combinations that include the ATG16 inhibitor provided by far the strongest antiviral activity.

3.5. The impact of antiretroviral drugs in the presence of antiviral shRNA pressure

The anti-host or anti-HIV shRNAs might demonstrate additive or synergistic effects when combined with antiretroviral drugs belonging to a certain drug class that acts at a specific step of the HIV-1 replication cycle. To test this idea, we obtained dose–response curves for four different antiretroviral drugs: T1249 as fusion inhibitor, Lamivudine (3TC) as RT inhibitor, Raltegravir (RAL) as integrase inhibitor and Indinavir (IDV) as protease inhibitor (Table 1). We used eight PM1 cell lines expressing either an anti-host shRNA (ATG16-2, HSPD1-3 and IPO7-1) or an anti-HIV shRNA (Pol1, Pol47, RT5, Nef and R3A). Controls included untransduced cells (PM1) and cells transduced with JS1 and SHC1. We challenged the cultures with 100 TCID50 of the CXCR4-using LAI isolate and treated them with an antiretroviral drug. Viral spread was quantified by monitoring virus production level at 5 days post-infection. The inhibitory activity of the antiretroviral drugs was calculated by comparing virus production in the presence and absence of the specific antiretroviral drug. Upon this double shRNA-antiretroviral treatment different interactions were observed. Let us for example focus on the PM1-Pol1 cells treated with 3TC, RAL or IDV because

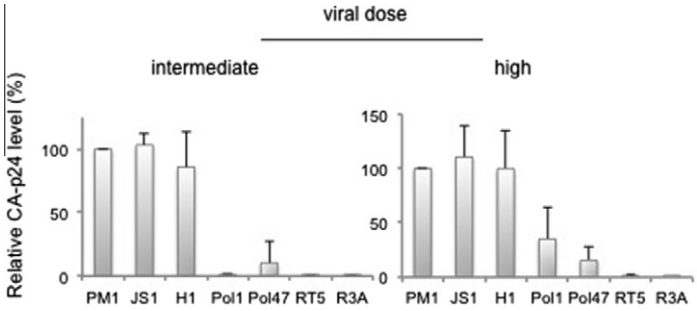

Fig. 2. Antiviral activity of stably expressed anti-HIV shRNAs. Cell lines expressing a single anti-HIV shRNA (Pol1, Pol47 or RT5) or a combination of the three shRNAs (R3A) were challenged with the HIV-1 LAI isolate and tested for their resistance to HIV-1 replication. The left and right panels represent respectively infections with intermediate and high HIV-1 dose. The relative HIV-1 CA-p24 level compared to that of the control PM1 cells was measured at the peak of infection. Values represent the averages and standard deviations from three independent experiments.

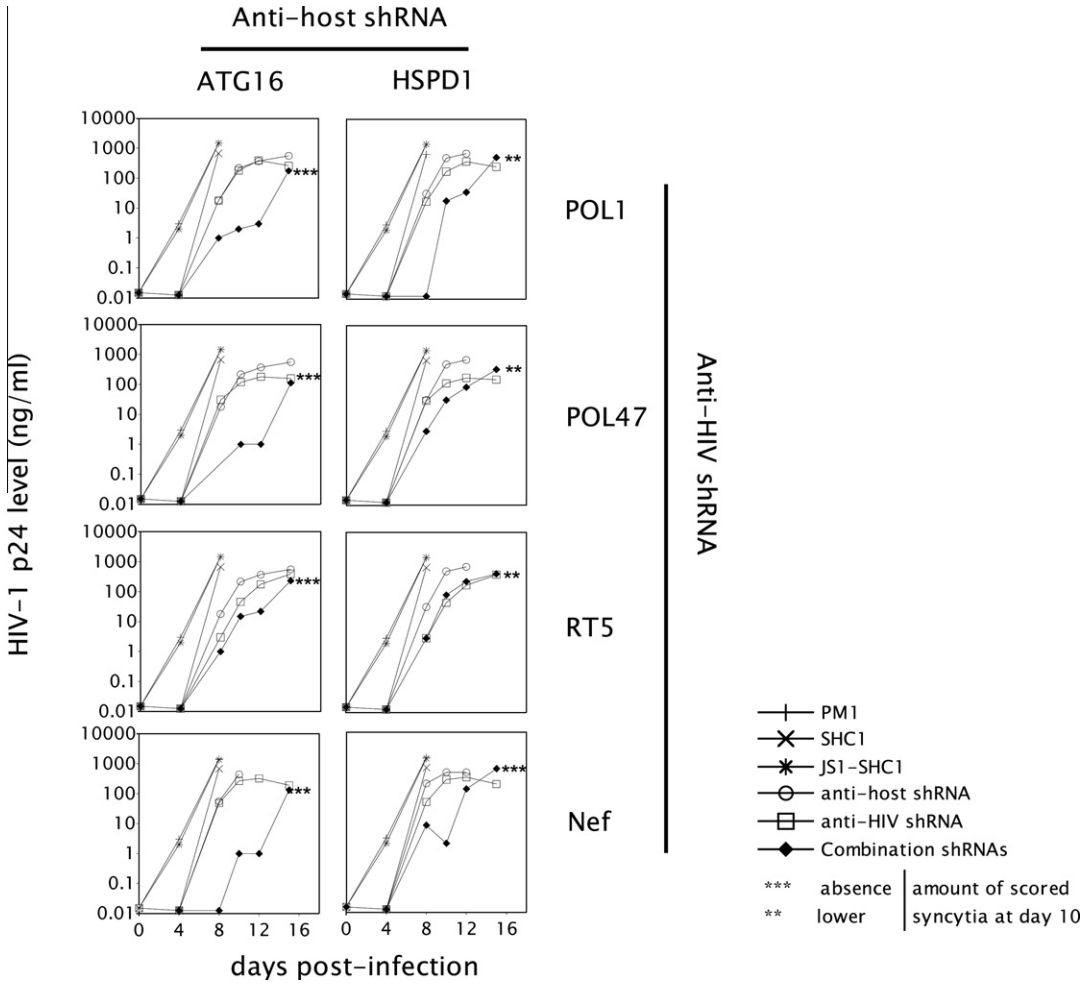

Fig. 3. Additive antiviral activity upon stable co-expression of anti-host and anti-HIV shRNAs. Cells co-expressing the best anti-host and anti-HIV shRNAs were challenged with LAI virus over a period of 15 days and tested for their resistance to HIV-1 replication. The left and right panels represent cell lines expressing the anti-host shRNA targeting ATG16 and HSPD1, respectively. The two control cell lines with the empty vector JS1 or both empty vectors (JS1 + SHC1) are also marked by cross and star symbols. The upper, upper-middle, lower-middle and lower panels correspond to cells co-expressing the anti-HIV shRNA Pol1, Pol47, RT5 or Nef, respectively. Virus replication was initiated with an intermediate viral input and the CA-p24 level was measured at several days post-infection. The absence (***) or the substantial reduction (**) of scored syncytia at day 10 is indicated on the graphs for the corresponding co-expressing cell line.

they exemplify three possible outcomes: no (left panel), moderate (right panel) or strong shift (middle panel) of the dose–response curve and the drug concentration that causes 50% inhibition (IC_{50}) of virus replication (Fig. 4). This example clearly illustrates the variation in inhibitory activity when shRNA Pol1 is combined with one of these antiretroviral drugs.

In total 32 drug/shRNA combinations were similarly evaluated and for all of them the measured IC_{50} and IC_{90} values are summarized in Table 3. To reveal additivity or synergistic effects of certain inhibitor combinations, we also plotted the calculated fold-change in IC_{50} (FCIC, Fig. 5). The different inhibitor combinations exhibited various responses with substantial differences of the IC_{50} and IC_{90} values compared to the control cell lines (Table 3).

About a third of the combinations (9) exerted a negative ($FCIC_{50} < 0$) or nearly neutral antiviral activity ($FCIC_{50}$ between 1 and 3). Two combinations scored a FCIC value < 0 that may suggest an antagonistic effect, two of these combinations involved 3TC or IPO7-1 shRNA, or both. Neutral combinations mainly involved 3TC (nearly all combinations) or T1249 (except in combination with ATG16-2) and the measured IC_{50} was nearly similar in all cases (see Table 3). Four combinations revealed a moderate additivity with a $FCIC_{50}$ between 4 and 7: RAL + ATG16-2, RAL + HSPD1-3, IDV + Pol1 and 3TC + Nef. Ten of the 32 combinations showed either a strong additivity with a $FCIC_{50}$ of at least one log ($10 < FCIC < 26$): anti-HIV shRNAs when combined with RAL (x4) and 3TC (x2). Very strong additivity with a $FCIC_{50}$ of two logs or higher was scored for combinations involving IDV (x4) and T1249 (x1). It is worth mentioning that no IC_{50} shift could be calculated in some cases (noted *n.d.* in Table 3), in particular when the antiviral activity of the shRNA alone was too strong (e.g. R3A). This problem could not be solved by increasing the viral input to 300 or 600 $TCID_{50}$. We were also unable to measure the IC_{50} shift when the additive effect of shRNA and antiretroviral drug was so strong that the shape of the dose–response curve was flattened or even had disappeared (IDV-Pol47, IDV-RT5 or IDV-Nef, T1249-ATG16-2 and all T1249-anti-HIV shRNA combinations). In both cases we considered the IC_{50} value to be too high to be measured in this experimental system. In order to display this category of combinations, we used a chart with non-linear *Y*-axis in Fig. 5. In some other cases, calculation of the 95% confidence interval (CI) for the IC_{50} value was ambiguous because of flattening of the dose–response curve (Table 3, e.g. ATG16-2-IDV and IPO7-T1249), corresponding to a strong alteration of the curve sigmoidicity or Hill slope.

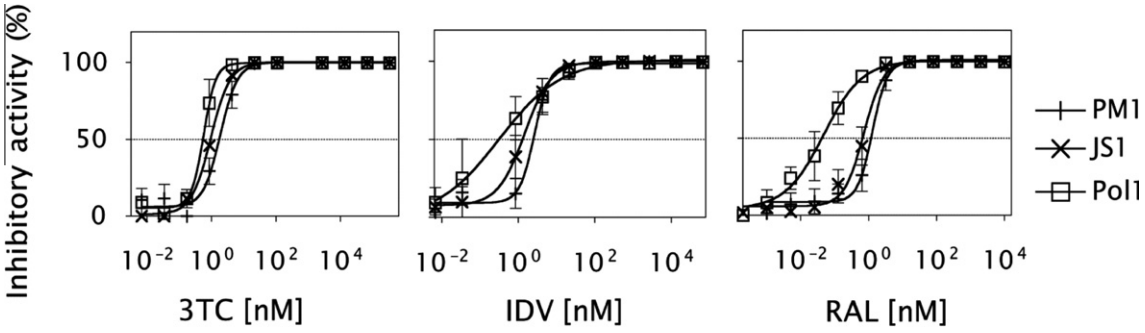

Fig. 4. Additive antiviral activity of shRNAs and antiretroviral drugs. The potential of having an additive effect of antiretroviral drugs was evaluated by determining the dose–response curve for drugs in cell lines expressing different shRNAs. As an example, the cell line expressing shRNA Pol1 is shown when challenged with 100 TCID50 of LAI virus in the presence of increasing concentration (nM) of RAL, 3TC and IDV. The graphs show the percentage of inhibitory activity of the antiretroviral drug. The horizontal bar emphasizes the shift observed in the IC_{50} between the control cell lines and the shRNA-expressing cell line.

Table 3
Antiviral activity (IC_{50} and IC_{90}) of T1249, 3TC, RAL and IDV in PM1 cells stably expressing anti-host or anti-HIV shRNAs.

		shRNA-expressing PM1 cells										
		Control PM1 cells			Anti-host shRNAs			Anti-HIV shRNAs				
Compound		Unt.[a]	SHC1[b]	JS1[b]	ATG16-2	HSPD1-3	IPO7-1	Pol1	Pol47	RT5	Nef	R3A
T1249	IC_{50} ng/ml	24	42.5	31.1	*n.d.*	14.2	78.5	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	95% CI[c]	7.8–74	12–153	24–41		10–19	*amb.*					
	IC_{90} ng/ml	0.01	0.02	0.02	*n.d.*	0.001	0.8	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
3TC	IC_{50} nm	1.9	4.1	0.9	1.5	0.7	2.9	0.6	1.1	0.2	0.4	*n.d.*
	95% CI	1.5–2.4	2.3–7.1	0.9–1.0	0.3–8.6	0.2–2.5	0.9–9.8	0.4–0.8	0.7–1.8	0.2–0.3	0.3–0.6	
	IC_{90} nm	27.4	28.5	4.1	62.3	13.7	55.5	1.6	2.6	1.1	1.4	*n.d.*
RAL	IC_{50} nm	1.3	0.5	0.8	0.2	0.3	0.5	0.05	0.07	0.1	0.08	*n.d.*
	95% CI	0.9–1.8	0.3–0.7	0.5–1.0	0.07–0.4	0.1–1.0	0.3–1.0	0.02–0.10	0.05–0.11	0.09–0.20	0.04–0.12	
	IC_{90} nm	3.9	1.8	3.5	3.1	3.2	2.5	0.9	0.4	0.6	0.4	*n.d.*
IDV	IC_{50} nm	2.0	2.1	1.5	0.003	0.9	1.6	0.3	*n.d.*	*n.d.*	*n.d.*	*n.d.*
	95% CI	1.3–3.2	1.3–3.5	1.0–2.2	*amb.*	0.4–1.8	0.8–3.3	0.1–5.1				
	IC_{90} nm	16.1	12.4	14.3	5.9	7.1	29.2	41.6	*n.d.*	*n.d.*	*n.d.*	*n.d.*

n.d. not determined because of strong inhibition.
amb. ambiguous curve fit: because of strong effect on the dose–response curve.
All compounds were evaluated at least in three independent experiments.
[a] Untransduced cells.
[b] SHC1 and JS1 correspond respectively to PM1 cells transduced with the empty control vectors SHC1 and JS1.
[c] The 95% confidence interval is shown underneath the IC_{50}.

4. Discussion

We show that new combinatorial approaches can provide enhanced suppression of HIV-1 replication. We combined three different classes of antivirals: anti-host shRNAs against cellular cofactors (HSPD1, IPO7 and ATG16), anti-HIV shRNAs against highly conserved regions of the HIV-1 RNA genome (Pol1, Pol47, RT5 and Nef) and clinically approved antiretroviral drugs (T1249, 3TC, RAL and IDV). Those inhibitors were carefully chosen to act at different steps of HIV-1 replication (Table 1) to increase the chance to detect additive or synergistic inhibition. From an initial set of 20 anti-host shRNAs we selected three potent shRNAs that exerted the strongest suppression of HIV replication (Fig. 1a), most effective knockdown of the targeted mRNA (Fig. 1c) and no serious effects on cell growth (Fig. 1b). Markedly, ATG16 and HSPD1 turned out to be good targets and ATG16 knockdown provided the strongest HIV-1 inhibition. Replicating virus emerged in 5 of 6 cultures, but we did not test whether this represents true viral escape or pseudo-escape caused by a sub-optimal viral blockade. The ATG16 protein is a component of the autophagy machinery that has a poorly-defined, but seemingly important role in HIV-1 replication (Eekels et al., 2012b; Espert et al., 2006, 2009; Kyei et al., 2009).

We screened for potent silencer cocktails with shRNAs against viral sequences and cellular components. Such a mixture could restrict mutational escape by enhanced synergistic inhibition. For the anti-HIV shRNAs, we made use of an in-house developed set of potent shRNAs that we first validated in the PM1 cell line (Fig. 2). A combination of ATG16 with an anti-HIV shRNA provided very strong HIV-1 inhibition up to 2 weeks post-infection, which was maintained at least 25 days in a few cultures (1 of 6 ATG16 + Pol47 cultures, 2 of 6 ATG16 + Nef cultures, results not shown). Combinations of HSPD1 and anti-HIV shRNAs yielded more modest additive activity in 3 of 4 cases (Pol1, Pol47 and Nef). The other combinations did not show any additive effects. These results indicate that the intrinsic antiviral activity of individual inhibitors is the most important property for selection in a therapeutic cocktail, rather than the actual replication step that is targeted. In general, we noticed that the level of virus inhibition with anti-host shRNAs is modest compared to anti-HIV shRNAs. Nevertheless, such moderately active shRNAs may still be useful in cocktails with two or three anti-HIV shRNAs, where it provides an additional anti-escape lock.

We also tested whether RNAi-mediated knockdown may influence the activity of antiretroviral drugs belonging to a specific class. We developed an experimental setting to monitor drug

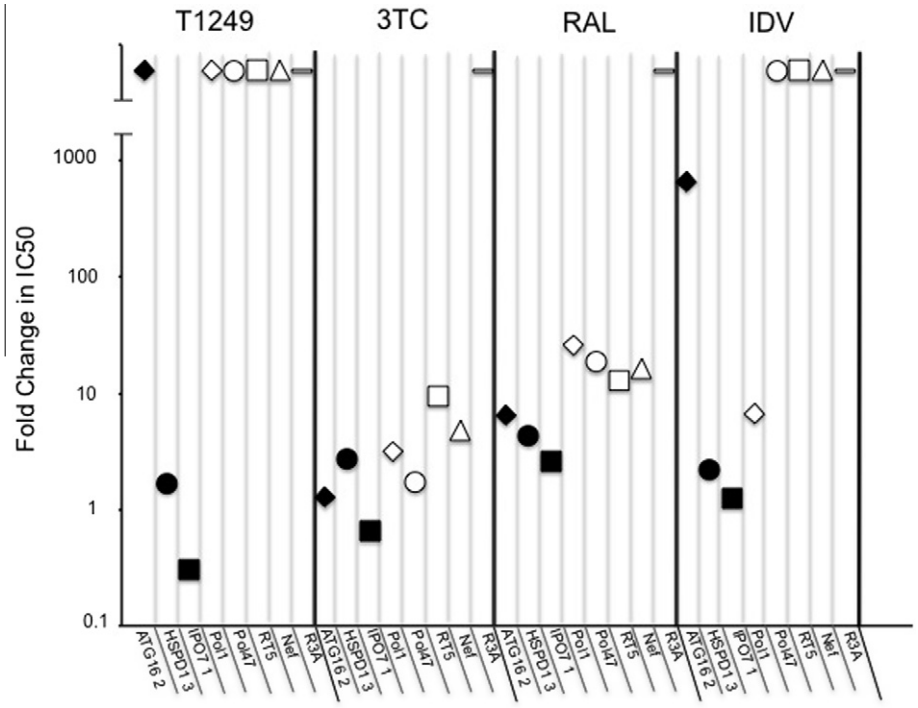

Fig. 5. The impact of antiretroviral drugs in the presence of antiviral shRNA pressure. IC_{50} was determined for each stable cell line expressing an anti-HIV or anti-host shRNA. Fold-change in IC_{50} ($FCIC_{50}$) was calculated as [IC_{50}] transduced cells/[IC_{50}] untransduced cells and is plotted along a logarithmic scale. Each vertical section corresponds to the tested drug, from left to right: T1249, fusion inhibitor; 3TC, lamivudine; RAL, raltegravir and IDV, indinavir. Within each drug section anti-host and anti-HIV shRNAs are indicated by a closed and an open symbol respectively, and with a distinctive shape: from left to right ATG16, HSPD1, IPO7 (anti-host shRNAs) and anti-HIV-Pol1, Pol47, RT5, Nef or R3A (anti-HIV shRNAs).

efficacy indicators (IC_{50} and IC_{90}) in PM1 cells that stably express the most potent anti-host or anti-HIV shRNAs and demonstrated that shRNA expression can dramatically affect drug efficacy (Fig. 5 and Table 3). In many cases, shRNA expression increased HIV-1 susceptibility to antiretroviral drugs, which translated into a lower IC_{50} (Table 3) and a greater fold-change in IC_{50} ($FCIC_{50}$) (Fig. 5). As a general trend, greater additivity was observed in combinations of drugs with anti-HIV shRNAs than anti-host shRNAs, regardless of the drug class. Among the anti-host shRNAs, ATG16 provided the strongest additive effect with T1249, RAL and IDV. Combination of HSPD1 and IDV, which both target virion assembly, provided very poor inhibition. Among the four anti-host shRNAs, ATG16 that plays a role in viral particle production in macrophages provided a remarkable strong additivity with IDV, which also affects virion assembly and maturation. This may cautiously suggest a potential benefit of co-targeting late steps of HIV-1 replication. For RAL, all combinations were effective regardless of the type of shRNA, but combination with Pol1 (RT target) and ATG16 provided the strongest inhibition. For some combinations, we scored a $FCIC_{50}$ increase ranging from several logs up to a complete disappearance of the dose–response curve (Fig. 5). The latter effect corresponds to an increase of the drug dynamic range to the infinite. We observed the strongest effects when shRNAs were combined with IDV. Interestingly, a recent study assigned a class-specific instantaneous inhibitory potential (IIP) to the most common antiretroviral drugs and that is an indicator of cooperativity (Shen et al., 2008). Protease inhibitors like IDV have been shown to have a very high IIP (between 2 and 10), which may relate to our findings.

To summarize, additive or synergistic anti-HIV effects were observed with combinations of shRNAs and small-molecule drugs. We showed that shRNA expression can strongly influence the activity of regular antiretroviral drugs. The multiplication of inhibitors targeting a single replication step yielded some prominent inhibitory effects. Leonard et al. reported that a combination of RNAi attack with antiretroviral drug did enhance the antiviral activity against WT and mutant virus (Leonard et al., 2008), in part by counteracting viral escape. We recently demonstrated that second-generation shRNAs can be combined with protease inhibitors to put pressure on virus evolution, resulting in a virus evolution block or the selection of less fit virus variants (Schopman et al., 2012). In an earlier study, the combination of two transfected siRNAs, targeting mRNAs encoding viral Gag or cellular CCR5, provided additive inhibition of infection of the HIV-1 BAL isolate in macrophages (Song et al., 2003). The current results confirm that a high degree of anti-HIV cooperativity between shRNAs and drugs can be achieved. As previously foreseen and discussed in the literature (Adamson and Freed, 2010; Rossi et al., 2007; Scherer et al., 2007), our study strongly supports the therapeutic interest of shRNA-drug combinatorial approaches.

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