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

Fatima Boutimah, Julia J.M. Eekels, Ying Poi Liu, Ben Berkhout *

Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, University of Amsterdam, The Netherlands

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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

* Corresponding author. Address: Laboratory of Experimental Virology, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam, K3-110, Meibergdreef 15, 1105 AZ, Amsterdam, The Netherlands. Tel.: +31 20 566 4822; fax: +31 20 691 6531.
E-mail address: b.berkhout@amc.uva.nl (B. Berkhout).

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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