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Molecular strategies to design an escape-proof antiviral therapy

Ben Berkhout *, Rogier W. Sanders

Laboratory of Experimental Virology, Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, The Netherlands

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ABSTRACT

Two antiviral approaches against the human immunodeficiency virus type 1 (HIV-1) were presented at the Antivirals Congress in Amsterdam. The common theme among these two separate therapeutic research lines is the wish to develop a durable therapy that prevents viral escape. We will present a brief overview of these two research lines and focus on our efforts to design an escape-proof anti-HIV therapy. The first topic concerns the class of HIV-1 fusion inhibitors, including the prototype T20 peptide and the improved versions T1249 and T2635, which were all developed by Trimeris–Roche. The selection of T20-resistant HIV-1 strains is a fairly easy evolutionary process that requires a single amino acid substitution in the peptide binding site of the viral envelope glycoprotein (Env) target. The selection of T1249-resistant HIV-1 strains was shown to require a more dramatic amino acid substitution in the viral Env protein, in particular the introduction of charged amino acid residues that cause resistance by charge-repulsion of the antiviral peptide. The third generation peptide T2635 remains active against all these HIV-1 escape variants because the charged residues within this peptide are "masked" by an introduced intra-helical salt bridge. This charge masking concept could facilitate the future design of escape-proof antiviral peptides. The second topic concerns the mechanism of RNA interference (RNAi) that we are currently employing to develop an antiviral gene therapy. One can make human T cells resistant to HIV-1 infection by a stable RNAi-inducing gene transfer, but the virus escapes under therapeutic pressure of a single inhibitor. Several options for a combinatorial RNAi attack to prevent viral escape will be discussed. The simultaneous use of multiple RNAi inhibitors turns out to be the most effective and durable strategy.

Contents

1. HIV-1 and AIDS

Over 33 million persons are currently infected with HIV-1. This virus causes a chronic infection that ultimately leads to AIDS and death. Much progress has been made in the past 25 years to develop an effective antiviral therapy. Disease progression can be halted effectively with antiviral drugs, and in particular a combinatorial approach can avoid the evolution of drug-resistant HIV-1 variants. Problems associated with such drug regimens include serious toxicity during long-term follow-up. In the absence of any breakthrough at the anti-HIV vaccine front, one should continue to think

* Corresponding author.
E-mail address: b.berkhout@amc.uva.nl (B. Berkhout).

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about the design of more durable therapeutic measures. We will discuss the concept of novel entry blockers of the fusion inhibitor peptide class that are specifically designed to prevent viral escape. In addition, we will discuss attempts to develop an RNAi-based gene therapy against HIV-1, which should durably protect cells of the immune system that are susceptible to virus infection.

2. HIV-1 entry into the cell

The original antiviral drugs target the HIV-1 reverse transcriptase and protease enzymes, but more recently drugs have been developed that target the process of virus entry into the cell. Maraviroc is an entry inhibitor that acts as CCR5 receptor antagonist. Another subclass of entry inhibitors consists of the fusion inhibitors that prevent fusion of the viral and cellular membranes, a process that is required for the intracellular release of the viral RNA genome and the enzymes needed for genome replication. We and others studied whether HIV-1 can become resistant to these fusion inhibitors, and the causal molecular mechanisms provided useful insight for the design of improved drugs with anti-escape properties.

A more detailed introduction on the entry mechanism is important to understand the mechanism of action of fusion inhibitors. The viral protein that is targeted is the envelope glycoprotein (Env) trimer on the outside of the virion particle that consists of three globular surface gp120 subunits and three trans-membrane gp41 subunits. Upon binding of Env to the CD4 receptor on the host cell, the conformation of Env changes such that it can bind to a second receptor, either CCR5 or CXCR4. This interaction triggers additional conformational changes in both the gp120 and gp41 subunits that activate the fusion machinery of gp41. A trimeric core is formed by the first helical repeat domains (HR1) of three gp41 subunits and the hydrophobic N-terminal fusion peptide of gp41 inserts itself into the membrane of the target cell. Next, the second helical repeat domains (HR2) fold onto the grooves of the HR1 core to form a very stable six-helix bundle structure, and the energy released in this process triggers the fusion of the juxtaposed viral and cellular membranes.

3. Three generations of HIV-1 fusion inhibitors

Peptides based on the HR2 domain of viral fusion proteins are effective inhibitors of virus entry (Bosch et al., 2004; Pyrc et al., 2006; Liu et al., 2004; Porotto et al., 2007; Wang et al., 2003; Zhu et al., 2005). Most HIV-1 fusion inhibitors are peptides that mimic the HR2 domain of the viral gp41 protein and that act by competitively binding to the HR1 core, thereby preventing the binding of HR2 and the formation of the six-helix bundle and fusion of the viral and cellular membranes (Fig. 1). The first generation of this class of peptide inhibitors is called T20 or enfuvirtide, which has been approved for clinical use (Kilby et al., 1998). In succession to T20 the second generation peptide fusion inhibitor T1249 was developed that contains an optimized amino acid sequence with increased antiviral potency (Lalezari et al., 2005). The third generation inhibitor T2635 contains several adjustments like introduced salt bridges that stabilize the α-helical structure of the peptide. These modifications led to a greatly improved half-life in serum and also boosted the antiviral activity that depends on the α-helical conformation of the peptide (Dwyer et al., 2007). These first, second and third generation peptides (T20, T1249 and T2635, respectively) were developed by Trimeris–Roche.

4. How HIV-1 escapes from fusion inhibitors

Resistance to T20 usually maps to the 36–45 region of the peptide binding site in the HR1 domain of the viral gp41 Env protein, with position 38 being the hotspot for resistance. This insight was obtained in both clinical studies and well-controlled laboratory settings (Rimsky et al., 1998; Greenberg and Cammack, 2004). A single amino acid substitution in this domain can cause a moderate to high level of resistance, although a combination of two or three mutations is frequently selected to yield >100-fold resistance (Mink et al., 2005). Mutations in HR2 of gp41 are also involved in the development of high-level resistance and/or restoration of proper Env folding and function. These HR2 mutations increase the affinity for the mutated HR1, thereby favouring the HR1–HR2 association over HR1-drug binding (Ray et al., 2009; Tolstrup et al., 2007). We demonstrated the evolution of a peculiar HIV-1 variant with a combination of HR1 and HR2 mutations that caused a T20 drug-dependent phenotype (Baldwin et al., 2004), and the underlying molecular mechanism was subsequently resolved for this individual patient. In particular, we demonstrated that this Env variant is hyperactive, which necessitates the presence of the antiviral drug to restrict Env of "firing" too early. Obviously, the drug has to dissociate eventually to allow the conformational switch to occur (Baldwin and Berkhout, 2007, 2008).

Initial data suggested that the selection of T1249-resistant HIV-1 variants is very difficult (Melby et al., 2007). The first T1249-resistant HIV-1 variants were selected by randomization of the

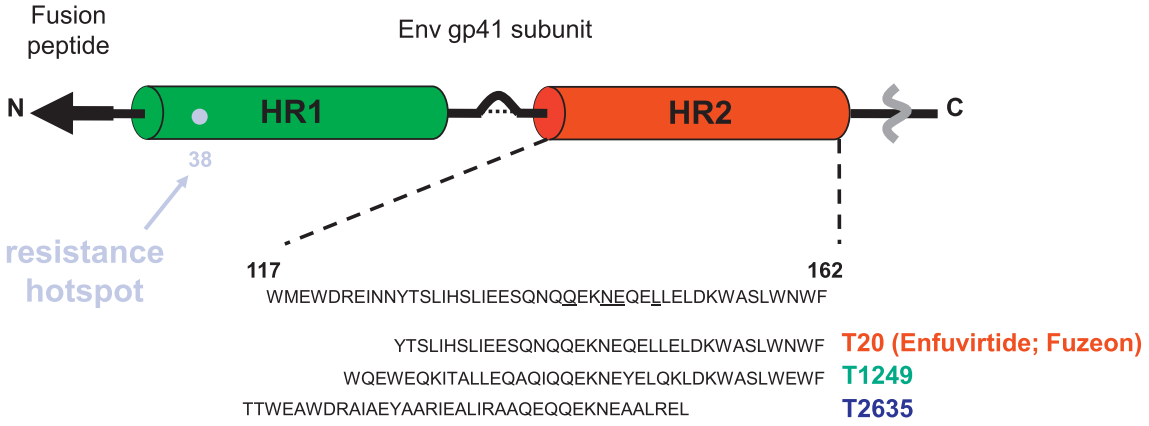

Fig. 1. Three generations of HIV-1 fusion inhibitors. Shown is the extracellular part of the gp41 subunit of the HIV-1 Env protein. The HR1 and HR2 domains are indicated. The HR2 amino acid sequence was copied in the three generations of antiviral peptides: T20, T1249 and T2635. The HR2 domain contains the position 38 residue that forms the hotspot for acquiring resistance against the HR2-based peptides. For further details, see (Eggink et al., 2008).

viral target sequences (Chinnadurai et al., 2007). In general, we now know that the improved potency of the second and third generation peptide inhibitors result in the selection of more dramatic changes in the Env target protein in order for the virus to become resistant. We selected T1249-resistant viruses in multiple parallel evolution cultures and much to our surprise found substitutions at the well-known hotspot position 38, but only charged amino acid residues were selected (Eggink et al., 2008). To better understand the mechanism of action of the fusion inhibitors and the mechanism of viral resistance, we combined virological and biophysical experiments with a comprehensive mutational analysis. For instance, we introduced all 20 possible amino acids at this resistance hotspot position 38. We demonstrated that any substitution causes T20-resistance, but that only a few more dramatic substitutions trigger T1249-resistance (Eggink et al., 2008). In toto, we distinguished at least four molecular mechanisms of drug resistance (Eggink et al., 2009). Reduced contact or steric obstruction by the selection of smaller or more bulky amino acid residues suffices for T20-resistance. However, a larger impact on the peptide drug-gp41 helix–helix interaction as mediated by electrostatic repulsion or electrostatic attraction is required for T1249 resistance.

None of the position 38 variants did cause resistance to the third generation peptide T2635. More recently, we were able to select T2635-resistant HIV-1 variants (Eggink et al., submitted). This viral evolution scheme turned out to be rather tricky as it requires a slow acceleration of drug pressure. It is a very lengthy process that involves the selection of a combination of mutations throughout the Env protein. It took over 6 months to acquire a modest 10-fold level of drug-resistance and this was linked to the acquisition of multiple mutations in the gp41 and gp120 parts of the viral Env protein. Interestingly, these hypermutated Env proteins were found to be seriously hampered in their function, with an associated impact on viral replication fitness. These results indicate that T2635 provides a very high genetic threshold towards the selection of resistant viruses, and the variants that are eventually selected demonstrate a serious loss of replication capacity or viral fitness. These combined results warrant the further clinical development of these peptide drugs (Eggink et al., 2010).

5. Towards escape-proof anti-HIV peptides

Although the three generations of fusion peptide inhibitors share the approximate binding site in the HR1 domain, it struck us that resistance to T2635 is not mediated by a mutation in this domain. This finding correlates with the increased binding energy of the T2635–HR1 interaction, which may necessitate multiple disruptive substitutions to acquire resistance, when compared to the T20 and T1249 complexes (Eggink et al., 2009). Molecular modeling revealed a possible mechanism for the lack of T2635-resistance, even for the more dramatic HR1 substitutions with charged amino acids that cause high-level T1249-resistance. We reasoned that the absence of T2635-resistance is due to masking of charged residues within this third generation peptide because of the introduced salt bridges that were inserted as part of the strategy to stabilize the peptide in a helical conformation. These internal salt bridge interactions between two oppositely charged residues in the peptide "neutralize" their ability to get involved in alternative charge-mediated interactions with other molecules such as the Env protein. To directly test this scenario, we designed a variant T1249 peptide in which the charged residue that is implicated in the resistance mechanism is similarly masked by the introduction of an intra-helical salt bridge (Fig. 2). Indeed, this peptide modification resolved the resistance phenotype (Eggink et al., 2009). These results provide clues on how to design the future generation of antiviral peptide drugs that are less sensitive to viral escape.

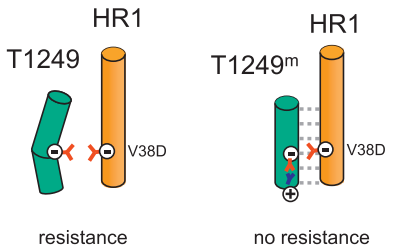

Fig. 2. The charge-masking concept for escape-proof antiviral peptides. The left panel shows the T1249 peptide and the HR1 binding site of the Env subunit gp41. Both peptides adopt an α-helical structure that facilitates the drug–Env interaction. The V38D resistance mutation in HR1 causes charge repulsion with a similarly charged residue in the T1249 peptide. This charged residue was neutralized in the $T1249^{m}$ peptide by introduction of a positively charged residue that forms an intramolecular salt bridge with the negative charge. Peptide $T1249^{m}$ remains active on the V38D virus variant, as illustrated by extensive helix–helix contacts (dashed grey ticks). For further details, see (Eggink et al., 2009).

6. RNA interference

The development of RNAi-based therapies against a wide variety of diseases, including cancer, neurological, autoimmune and infectious diseases was triggered by the discovery of the RNAi mechanism and RNAi-mediated gene silencing in mammalian cells (McCaffrey et al., 2003; Kapadia et al., 2003; Banerjea et al., 2003; Ter Brake et al., 2009; Ding et al., 2003; Takeshita and Ochiya, 2006; Davidson and Paulson, 2004). It is estimated that human cells express more than 500 microRNAs or miRNAs. These miRNAs are important in the process of cell differentiation and development by regulating gene expression at the post-transcriptional level (Carrington and Ambros, 2003; Ambros, 2001; Bartel, 2004; Baehrecke, 2003; McManus, 2004). RNAi-mediated gene silencing occurs mainly by translational repression of the targeted mRNA in mammals (Ambros, 2004). An important determinant of RNAi action is the level of base pair complementarity between the miRNA and the targeted mRNA, leading either to mRNA cleavage with a perfect complementarity or translational repression with a near-perfect complementarity (Brennecke et al., 2003; Doench and Sharp, 2004; Lewis et al., 2003; Kiriakidou et al., 2004; Lai, 2002). Although most mammalian miRNAs base pair with imperfect complementarity to the mRNA to cause translational repression, at least one human case of perfect complementarity and subsequent mRNA cleavage has been reported (Yekta et al., 2004).

In contrast to the action of natural miRNAs, synthetic small interfering RNAs (siRNAs) or short hairpin RNA (shRNA) transcripts (Brummelkamp et al., 2002; Paddison et al., 2002) are usually designed to have full base pairing complementarity, which triggers cleavage of a specific target mRNA. The therapeutic potential includes diseases caused by overexpression of a specific mRNA. RNAi can also be induced to target the RNA genome of invading microbes and pathogenic viruses, including HIV-1.

7. Antiviral RNAi strategies

Potent and sequence-specific HIV-1 inhibition has been reported with RNAi-inducing reagents in cell culture infections, but it soon became apparent that HIV-1 is prone to viral escape when a single shRNA inhibitor is applied (Boden et al., 2003; Das et al., 2004; Nishitsuji et al., 2006; Sabariegos et al., 2006; Ter Brake et al., 2006; Unwalla et al., 2006; Westerhout et al., 2005). The many escape routes available to the virus are illustrated in Fig. 3. The ease of HIV-1 escape mimics what happens in patients treated with

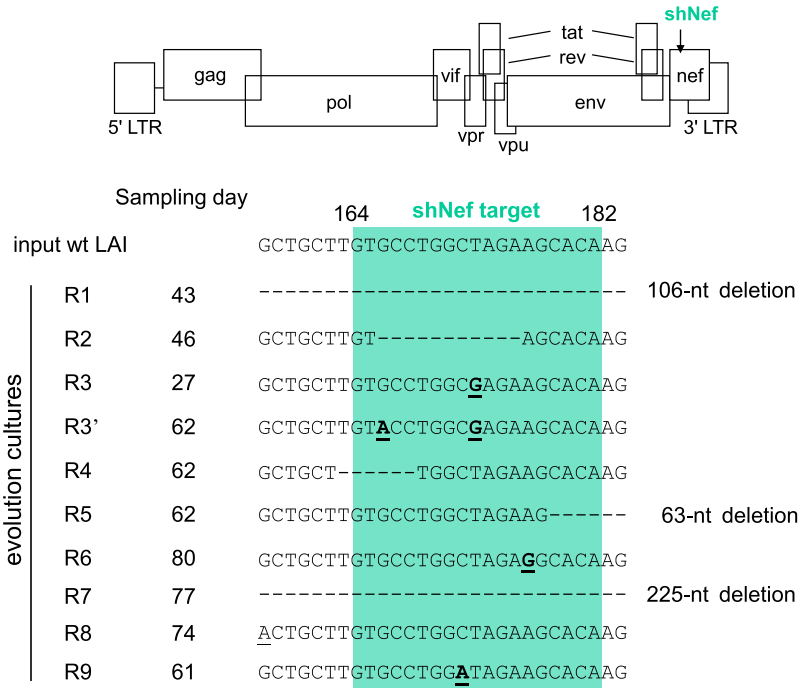

Fig. 3. HIV-1 escape from shRNA attack. Shown on top is the proviral HIV-1 DNA genome and the shRNA that attacks nef gene sequences. The bottom panel shows the actual nef target sequence (green box) and the sequence changes observed in multiple evolution cultures. The input wild-type (wt) virus was the primary LAI isolate. The day of sampling and the mutations are indicated, dashes represent nucleotide deletions. The R7 culture acquires a mutation outside the actual target. This mutation causes a shift in the local RNA structure such that the target becomes inaccessible for RNAi attack (Westerhout et al., 2005). For further details, see (Das et al., 2004).

a single antiretroviral drug, but we know that a combinatorial drug regimen can prevent viral escape and therapy failure. Thus, the therapeutic RNAi vector to be developed for clinical testing should tackle the virus with multiple shRNA inhibitors at the same time. Such a combinatorial RNAi attack can target the virus at multiple genome positions, but one can also add an attack against host-encoded co-factors (Eekels et al., 2011). Another elegant solution to avoid viral escape is the use of the second generation shRNAs that specifically target viral escape variants (Ter Brake and Berkhout, 2005). Although an effective block of popular viral escape routes was obtained (Schopman et al., 2010), the relatively high number of alternative viral escape routes does limit the feasibility of this approach (von Eije et al., 2008; Ter Brake et al., 2008b).

It is important to select viral targets that are highly conserved among virus isolates, simply because one wants to inhibit as many virus strains as possible. Targeting of highly conserved genome regions may also restrict the evolution of viral escape mutants because well-conserved sequences will likely exhibit an important function in HIV-1 biology, such that RNAi-induced sequence variation may be expected to have an impact on the viral replication capacity and HIV-1 fitness (Kafri et al., 1997; Ter Brake et al., 2008b). To optimize the therapeutic effect, one should also consider the direct targeting of viral RNAi suppressor functions (de Vries et al., 2009; Haasnoot et al., 2007; Schnettler et al., 2009; Bennasser et al., 2005).

We performed an extensive shRNA screen against highly conserved sequences of the HIV-1 genome, yielding approximately 20 potent shRNAs (Ter Brake et al., 2006). Stable shRNA-expressing T cell lines were generated that were subsequently infected with HIV-1, which yielded four durable shRNA inhibitors that restricted virus replication for more than 100 days (von Eije et al., 2009). We and others have identified effective shRNAs and siRNAs targeting regulatory HIV-1 sequences, e.g. in the long terminal repeat (LTR) and untranslated leader RNA (Jacque et al., 2002; Ter Brake et al., 2006) and most viral genes (McIntyre et al., 2009a): including *gag* (Chang et al., 2005; Novina et al., 2002; Park et al., 2002; Ter Brake et al., 2006), *pol* (Chang et al., 2005; Berkhout and Ter Brake, 2009; Surabhi and Gaynor, 2002), *vif* (Jacque et al., 2002), *tat* (Ter Brake et al., 2006; Coburn and Cullen, 2002; Lee et al., 2002; Surabhi and Gaynor, 2002), *rev* (Ter Brake et al., 2006; Coburn and Cullen, 2002; Lee et al., 2002), *vpu* (Chang et al., 2005), *env* (Park et al., 2002) and *nef* (Jacque et al., 2002). Prolonged culturing in the presence of HIV-1 should be done to test the likelihood of viral escape. Guidelines for the proper testing and selection of potent and safe shRNA inhibitors against HIV-1 have been formulated (Haasnoot and Berkhout, 2009).

8. Combinatorial RNAi approaches to prevent HIV-1 escape

Combinatorial drug/RNAi approaches are essential to restrict HIV-1 evolution and to prevent viral escape, which will lead to therapeutic failure. A variety of strategies have been described for multiplexing of shRNA cassettes in a single therapeutic vector (Fig. 4). As repeat sequences should be avoided in the lentiviral vector to prevent recombination-mediated deletions (Ter Brake and Berkhout, 2007; McIntyre et al., 2009b; Ter Brake et al., 2008a), the multiple shRNA cassettes generally use separate polymerase III promoters or a combination of polymerase II and III promoters. Multiple shRNAs can also be expressed from a single transcript (Saayman et al., 2010a). We and others developed extended-shRNAs that are processed into two or maximally three functional siRNAs (Liu et al., 2007; Saayman et al., 2010b; Saayman et al., 2008). Another strategy uses truly long hairpin RNAs (lhRNAs) that should encode numerous siRNAs (Barichievy et al., 2007; Sano et al., 2008; Konstantinova et al., 2006). A disadvantage of the lhRNA approach is that it is unknown whether the produced siRNAs will be active inhibitors and it was recently demonstrated that a very low level of siRNAs are produced from such constructs (Liu et al., 2009). Several groups have reported toxicity of shRNAs (Grimm et

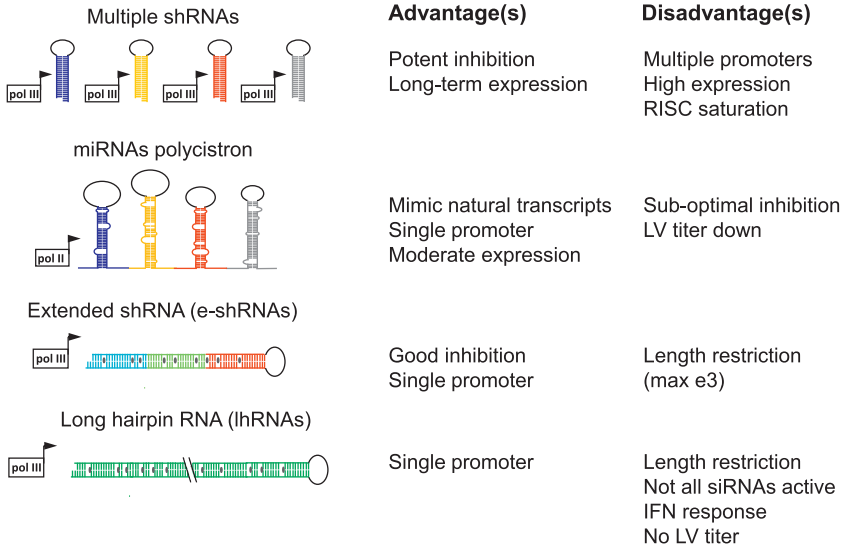

Fig. 4. Combinatorial RNAi approaches. Several methods for combinatorial RNAi are shown. We listed some of the advantages and disadvantages. LV is lentiviral vector. For further details, see (Liu and Berkhout, 2008).

al., 2006; McBride et al., 2008; Castanotto et al., 2007; Vickers et al., 2007), which can perhaps be solved by inserting the inhibitor in a natural miRNA backbone (McBride et al., 2008). Polycistronic miRNA transcripts that trigger a combinatorial attack on the virus have also been developed (Aagaard et al., 2008; Liu et al., 2008).

Comprehensive reviews on combinatorial RNA approaches are available (Grimm and Kay, 2007; Liu and Berkhout, 2008). Other types of inhibitory RNA molecules can be added to the RNAi-inducing antiviral regimen. The currently ongoing phase I clinical trial at the City of Hope uses a lentiviral vector that encodes a TAR-decoy, CCR5-ribozyme and a shRNA targeting the HIV-1 genome in the *tat–rev* region (Li et al., 2005; DiGiusto et al., 2010). The ribozyme cleaves the CCR5-encoding mRNA to cause reduced expression of this important HIV-1 receptor on the cell surface (Sarver et al., 1990). Alternative antiviral RNA molecules include antisense transcripts (Chatterjee et al., 1992; Levine et al., 2006), decoys (Kohn et al., 1999), ribozymes (Sarver et al., 1990) and aptamers (Symensma et al., 1996). A new addition to this arsenal is an antisense molecule that can elicit transcriptional gene silencing of the viral LTR promoter (Weinberg et al., 2006).

9. Future perspective

We presented new drug design and gene therapy strategies with the common goal to achieve durable HIV-1 suppression by limiting the chance of viral escape. We must first acknowledge that the current combination drug therapy that is used in the clinic is very effective in this respect for most HIV-infected individuals, such that HIV-1 infection has become a chronic disease. But it is also apparent that new drug classes with an anti-escape profile would be very welcome, and a gene therapy has the potential advantage of offering a durable effect with a single treatment. We discussed the design of optimized fusion inhibitor peptides based on molecular insight of the drug–gp41 interaction and the drug-resistance mechanisms. Despite promising clinical results, Roche/Trimeris decided to halt the further clinical development of the next generation fusion inhibitors. We recently argued that there remains a future for next-generation peptides with enhanced efficacy, a higher genetic barrier to resistance and improved pharmacological properties (Eggink et al., 2010). A natural inhibitory peptide called VIRIP or analogs thereof are also of much interest as they seem to exhibit a strong genetic barrier for resistance and the gp41-targeting peptides are safe in the first test in patients (Münch et al., 2007; Forssmann et al., 2010). Even if viral resistance develops, there may be a clinical advantage if the mutational burden leads to a serious loss of viral fitness that translates into a reduced viral load and delayed disease progression.

We also presented the promising research on RNAi-based gene therapy approaches, where again viral escape is a serious threat. There is an alternative strategy to prevent viral escape that should be mentioned. One could target host cell co-factors that assist virus replication. Silencing of several co-factors resulted in HIV-1 inhibition: nuclear factor kappa B (Surabhi and Gaynor, 2002), CD4 (Novina et al., 2002; Anderson et al., 2003a), CXCR4 (Martínez et al., 2002; Anderson et al., 2003a,b; Anderson and Akkina, 2005), DDX-3 (Ishaq et al., 2008), LEDGF/p75 (Vandekerckhove et al., 2006) and CCR5 (An et al., 2007; Anderson et al., 2003a; Anderson and Akkina, 2005). CCR5 has received much attention as critical receptor for HIV-1 entry and a promising drug target. Individuals with the delta-32 mutation in CCR5 are resistant to HIV-1 infection. Importantly, these persons are healthy, and only an increased risk for infection with the West Nile virus has been reported (Lim et al., 2006). A potent shRNA targeting this host cell factor has been developed (Anderson et al., 2003a; An et al., 2007). The potential of down regulation of CCR5 is supported by the cure of an HIV-1 infected patient in Berlin who developed leukemia. This patient received a bone marrow transplantation of a matching donor who was homozygous for the 32-bp deletion in the CCR5-gene. Surprisingly, HIV-1 has not been detected in the patient's plasma, suggesting a functional cure (Hutter et al., 2009; Allers et al., 2010).

Of course there is no guarantee that HIV-1 cannot escape from co-factor inhibition. CCR5-tropic viruses are generally responsible for HIV-1 transmission, but the virus can also use the alternative CXCR4 receptor. Down regulation of the CCR5 receptor will potentially set the stage for selection of CXCR4-tropic HIV-1 variants, but this possibility remains to be tested. The same virus escape route was discussed when CCR5-blocking drugs were developed, and such receptor-switch escape routes have indeed been reported in patients treated with the CCR5-antagonist maraviroc (Menéndez-Arias, 2010), although not yet in the Berlin patient. In general, the concept that targeting of a cellular co-factor prevents viral escape should be verified experimentally (Eekels et al., 2011).

Many cellular targets will obviously not be proper candidates for a gene therapy because they are essential for the cell and the

host. Although HIV-1 is a well studied virus, many details of the viral replication cycle remain elusive. Three high-throughput RNAi gene knockdown screens recently reported many new candidate HIV-1 co-factors (Yang et al., 2008; Gaspar and Thrasher, 2005; Parkin et al., 1992). An additional advantage of an attack on cellular co-factors may be that one can restrict multiple other human pathogenic viruses that use the same cellular pathways and co-factors.

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