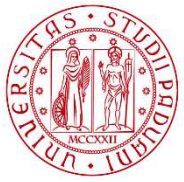

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Department of Molecular Medicine

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CHEMICAL AND GENETICALLY-BASED APPROACHES FOR THE TREATMENT OF HIV-1 INFECTION

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1. SUMMARY

Advances in HIV-1 drug therapy have drastically decreased mortality and significantly improved quality of life for HIV-infected patients, since the early days of epidemic. However, HIV drug-resistance, drug toxicities, therapy adherence, and the need for life-long treatment remain major challenges that continue to contribute to HIV-related global health concerns. In this context, different approaches are currently been investigated to interfere with HIV-1 infection.

In this PhD project, on one side, we are screening natural extracts and bioactive compounds and we are assessing their antiviral activity in appropriate cell models. Our goal is to identify plant-derived molecules to be employed as alternative or complementary compounds to the conventional drug regimen. Among them, proanthocyanidins (PACs), a class of polyphenols, have raised interest in the past years for their antiviral and antimicrobial properties against different pathogens. Oximacro®, a cranberry (*Vaccinium macrocarpon*, Ait.) extract, produced by Biosfered S.r.l. (Turin, Italy), is a reddish powder with a high content of PACs, and it has shown to be effective in preventing urinary tract infections (Occhipinti et al., 2016) and to exert a potent antiviral activity against HSV-1 and HSV-2 replication *in vitro*. Moreover, among the Oximacro®-derived purified fractions, fraction 4, the richest in A-type PACs, has proven to greatly prevent HSV infection, by targeting viral attachment to the cells (Terlizzi et al., 2016). With these promising results, we investigated whether Oximacro® could be effective also against HIV-1 infection. Indeed, the identification of natural antivirals with a broad-spectrum of activity could represent a promising starting point for a reliable co-therapy for HIV-1-positive individuals as well as a potent tool in the prevention of sexually transmitted diseases. Indeed, our results indicate that Oximacro® is able to inhibit HIV-1 infection and replication. In particular, we demonstrated that viral pre-incubation with the compound could greatly reduce HIV-1 infectivity, independently from the co-receptor usage, while continuous administration of the compound was able to protect cells from the infection over time. We are currently investigating the mechanism of inhibition of the viral replication, especially concerning the viral attachment and the HIV-1 Env-co-receptor binding, and to further support the feasibility of Oximacro® as a valid candidate for the development of a broad-spectrum microbicide.

On the other side, the safety and the feasibility of third generation self-inactivating lentiviral vectors (LVs) expressing anti-HIV-1 transgenes have been investigated. Specifically, these vectors express a selected combination of shRNAs (against the viral proteins Tat, Rev and Vif and the cellular co-receptor CCR5) along with the maC46 fusion inhibitor, capable of rendering HIV-1 target cells stably resistant to the viral infection. The ultimate aim is to explore the use of genetic medicines to modify CD4+ T lymphocytes (and their precursor HSCs), in order to treat AIDS-related lymphoma (ARL) patients, who offer a unique opportunity to evaluate gene therapy-based, anti-HIV strategies in an ethically acceptable clinical setting. Our previous work demonstrated that two LVs, one triple cassette vector (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev) and one extended vector (i.e. H1e-shRNA) proved to be particularly successful once challenged against two strains of HIV-1, in human primary CD4+ T lymphocytes, from healthy patients (Spanevello et al., 2016). Thus, we decided to further implement the antiviral potency of our platform by including a powerful fusion inhibitor peptide (maC46), to prevent viral entry of both CCR5- and CXCR4-using strains (Egerer et al., 2015). First, we were able to demonstrate the correct localization of the maC46 peptide on the plasma membrane upon 293T cell transfection with the developed vectors. Then, recombinant lentiviral particles (RLVPs) were produced, concentrated by ultracentrifugation and then titrated, by measuring the Reverse Transcriptase activity and, when possible, by determining the infectious titer by FACS analysis of the eGFP expression, after transduction of 293T cells with the RLVPs. Thus, CD4+ T lymphoblastoid Jurkat cells were transduced with equivalent RT units of recombinant lentiviral particles (700000 cpm). Three days after transduction, Jurkat cells were challenged with the T-tropic laboratory-adapted HXBc2 Vpr+/Vpu+/Nef+ strain of HIV-1, at different M.O.I: 0.1 $TCID_{50}$/mL. The results obtained indicate that three of the optimized vectors (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; U6shCCR5-7SKshVif H1lhTat/Rev EF1maC46*fused*eGFP; H1e-shRNA EF1maC46*fused*eGFP) potently blocked viral replication up to 25 days post infection, in absence of toxicity. Then, to discriminate the best performing RLVP, Jurkat cells were transduced with a lower amount of equivalent RT units (35000 cpm), even though a high efficiency of transduction was maintained. Our results indicate that, when Jurkat cells were infected with an M.O.I. of 0.01 $TCID_{50}$/cell of HIV-1 strain, one vector (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP) was able to protect up to 25 days post infection; on the other hand, in less favorable conditions, at an higher M.O.I. (0.1 $TCID_{50}$/cell), a more pronounced antiviral effect was observed for this lentiviral vector, compared to the others (protection

up to 14 days after infection). To understand whether differences in viral protection could be related to differences in the peptide expression, maC46 mRNA levels were quantified in transduced Jurkat cells at different time points, by qRT-PCR. Overall, we observed no significant changes in the expression of maC46 transcripts among different lentiviral vectors, or significant decrease of the peptide mRNA over time. In parallel, in collaboration with the C. Baum's group at the Department of Experimental Hematology of the Hannover Medical School, we tested the mutagenic potential of our vectors (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; H1e-shRNA EF1maC46; H1*scrambled*RNA EF1maC46), in an *in vitro* immortalization (IVIM) assay. The results showed no vector/supernatant-associated toxicity and a significantly reduced insertional transformation potential in primary murine cells transduced with high M.O.I. of test vector U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46. In conclusion, our data indicate that the U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP and U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 (even though the latter to a less extend), represent the best performing vectors, at least under the experimental conditions we adopted. Overall, the developed combinatorial platforms represent a promising strategy to render HSPCs, and, consequently, all the HIV-1 susceptible cell types, resistant to viral infection.

2. SOMMARIO

I notevoli progressi nella terapia farmacologica per infezione da HIV-1 hanno drasticamente ridotto la mortalità legata al decorso della patologia e significativamente migliorato la qualità della vita dei pazienti affetti. Tuttavia, questo trattamento non è ancora del tutto esenti da limiti. Infatti, la non eradicabilità dell'infezione, correlata alla persistenza del virus in determinati distretti anatomici e cellulari (*reservoir* di infezione), obbliga il paziente a sottostare ad un regime terapeutico a tempo indefinito, sollevando, quindi, nuove problematiche, quali: l'insorgenza di specie virali farmaco-resistenti, la tossicità d'organo, le possibili interazioni fra i farmaci somministrati e, non ultimi, gli elevati costi per la sanità pubblica. Da ciò, deriva una costante ricerca ed ottimizzazione di strategie alternative e/o complementari per la cura dell'infezione da HIV-1.

Questo progetto di dottorato si inserisce in questa ricerca globale di nuove terapie, proponendo due diversi approcci.

Da un lato, il crescente interesse verso l'inclusione di prodotti di origine naturale nell'alimentazione, nella cosmesi e in terapia, ha fatto sì che molecole derivanti da estratti botanici venissero presi come oggetto di studio per il trattamento di alcune infezioni di diversa origine. In questo contesto, sono stati esaminati diversi composti, testandoli in saggi di infezione *in vitro* contro HIV-1, con l'obiettivo di identificare quali tra questi potessero dimostrare un'azione antivirale tale da poter essere implicati come coadiuvanti nella terapia farmacologica convenzionale. Tra questi, Oximacro®, un estratto di mirtillo rosso (*Vaccinium macrocarpon*, Ait.), prodotto da Biosfered S.r.l. (Torino, Italia), possiede un elevato contenuto di proantocianidine, composti appartenenti alla classe dei polifenoli e ben noti per le loro proprietà antivirali e antimicrobiche. Oximacro® è già stato testato contro infezioni delle vie urinarie e contro HSV-1 e HSV-2, dimostrandosi estremamente efficace in entrambi i casi (Occhipinti et al., 2016; Terlizzi et al., 2016). In particolare, tra le frazioni purificate derivanti da Oximacro®, la frazione 4, la più ricca di proantocianine, ha dimostrato una notevole attività antivirale contro l'infezione da HSV, presumibilmente interferendo con il riconoscimento dei recettori cellulari da parte del virus. Pertanto, Oximacro® è stato testato anche contro l'infezione da HIV-1, con lo scopo di identificare un composto ad ampio spettro d'azione, da poter inserire sia in combinatione con le terapie tuttora in uso per i pazienti sieropositivi, sia come principio attivo nella formulazione di un microbicida topico, in prevenzione delle malattie sessualmente trasmissibili.

Analizzando i dati ottenuti da diversi esperimenti, Oximacro® si è dimostrato in grado di inibire l'infezione e la replicazione del virus. In particolar modo, esponendo il virus ad una prolungata incubazione con il composto, prima dell'infezione, è possibile ottenere una maggiore riduzione dell'infettività rispetto alla mancata pre-esposizione. Questo effetto, inoltre, non sembra essere correlato al tropismo o alla preferenza di uno o altro co-recettore cellulare CCR5 o CXCR4. D'altro canto, analizzando il decorso dell'infezione nel tempo, è possibile affermare che una somministrazione continua del composto è in grado di proteggere le cellule dall'infezione più a lungo. Al momento, non è noto il meccanismo di questa inibizione a livello molecolare, oggetto degli studi in corso. In particolare, verrà investigata la possibile interferenza di Oximacro® nel riconoscimento tra le glicoproteine virali dell'Envelope e il recettore cellulare, al fine di supportare ulteriormente l'eleggibilità di Oximacro® come valido candidato microbicida, ad ampio spettro d'azione.

Un possibile approccio alternativo al trattamento farmacologico è, d'altro canto, rappresentato dalla terapia genica, finalizzata all'espressione di geni antivirali non solo nelle cellule bersaglio dell'infezione, ma soprattutto nelle cellule staminali ematopoietiche, in grado di generare un sistema immunitario permanentemente resistente al virus. In particolare, gli inibitori dell'ingresso o delle fasi iniziali della replicazione virale vengono considerati molto vantaggiosi, dal momento che possono prevenire l'instaurarsi di una infezione cronica, dovuta all'integrazione del genoma virale nel DNA cromosomico della cellula ospite. Un obiettivo ulteriore della terapia genica, già adottato nell'approccio farmacologico antiretrovirale, consiste nell'agire contemporaneamente nei confronti di più siti virali e fattori cellulari endogeni, in modo da poter interferire con diverse fasi del ciclo replicativo. L'intento è quello di ridurre al minimo la probabilità di insorgenza di varianti virali resistenti. A tal proposito, sono state sviluppati in laboratorio diversi vettori lentivirali "self inactivating" di terza generazione (SIN), in grado di conferire un'espressione stabile e duratura nel tempo dei transgeni. Tra le diverse combinazioni testate, un vettore a tripla cassetta (cioè U6shCCR5-7SKshVif-H1lhTat/Rev) e un vettore extended (cioè H1e-shRNA) si sono rivelati particolarmente efficaci in bloccare l'infezione di due ceppi di HIV-1, in linfociti T CD4 +, provenienti da pazienti sani (Spanevello et al., 2016). Pertanto, è stato deciso di implementare ulteriormente l'attività antivirale della nostra piattaforma includendo un peptide di inibitore della fusione (maC46), per prevenire l'ingresso virale di ceppi che usano CCR5 e CXCR4 come corecettore (Egerer et al., 2015). In primo luogo, è stata dimostrata la corretta localizzazione del peptide di maC46 sulla membrana plasmatica in cellule 293T, trasfettate con i vettori sviluppati. Quindi, sono state

prodotte particelle lentivirali ricombinanti (RLVP), le quali sino state prima concentrate mediante ultracentrifugazione e quindi titolate, misurando l'attività di trascrittasi inversa e, quando possibile, determinando il titolo infettivo mediante analisi FACS dell'espressione eGFP. Successivamente, le cellule CD4+ Jurkat T-linfoblastoidi CD4 sono state trasdotte con un quantitativo di RLVP pari a 700000 cpm. Tre giorni dopo la trasduzione, le cellule Jurkat sono state infettate con il ceppo HIV-1 T-tropico HXBC2 Vpr+/Vpu+/Nef+, con un valore di molteplicità di infezione (M.O.I.) pari a 0.1 $TCID_{50}$ / mL. I risultati ottenuti indicano che tre dei vettori ottimizzati (cioè U6shCCR5-7SKshVif-H1lhTat / Rev EF1maC46; U6shCCR5-7SKshVif H1lhTat/Rev EF1maC46f*used*eGFP; H1e-shRNA EF1maC46*fused*eGFP) sono in grado di bloccare la replicazione virale fino a 25 giorni dopo l'infezione, in assenza di tossicità. Quindi, per discriminare la RLVP migliore, le cellule Jurkat sono state trasdotte con un quantitativo lentivirale inferiore (35000 cpm), pur mantenendo un'alta efficienza di trasduzione, e successivamente infettate con diverse M.O.I. I risultati ottenuti indicano che, quando le cellule Jurkat sono state infettate con un M.O.I. di 0.01 $TCID_{50}$/cellula di ceppo HIV-1, un vettore (cioè U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP) è stato in grado di proteggere fino a 25 giorni dopo l'infezione; d'altra parte, in condizioni meno favorevoli, ad una M.O.I. più elevata (0.1 $TCID_{50}$/cellula), è stato osservato un effetto antivirale più pronunciato per questa RLVP, rispetto agli altre (protezione fino a 14 giorni dopo l'infezione). Per capire se le differenze nella protezione virale possano essere correlate a differenze nell'espressione del peptide, i livelli di mRNA di maC46 sono stati quantificati in cellule Jurkat dopo 3, 15 o 21 giorni dalla trasduzione, mediante qRT-PCR. Nel complesso, non sono stati osservati cambiamenti significativi nell'espressione dei trascritti di maC46 tra diversi vettori lentivirali, o una significativa diminuzione dell'mRNA del peptide nel tempo. Parallelamente, in collaborazione con C. Baum (Dipartimento di Ematologia Sperimentale della Scuola di Medicina di Hannover), è stato testato il potenziale rischio di mutagenenesi di alcuni vettori (cioè U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; H1e-shRNA EF1maC46; H1scrambledRNA EF1maC46), in un saggio di immortalizzazione *in vitro* (IVIM). I risultati non hanno mostrato tossicità associata alla RLVP in esame ed un potenziale di trasformazione inserzionale significativamente ridotto nelle cellule murine primarie trasdotte con una M.O.I. elevata, specialmente nel caso del vettore U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46.

I risultati ottenuti fino ad ora hanno chiaramente dimostrato che i vettori sviluppati, in cui è stato combinato per la prima volta un potente inibitore di fusione con tre siRNA, sono

estremamente promettenti in termini di attività antivirale nella linea cellulare impiegata. Inoltre, tali vettori si sono dimostrati incapaci di indurre effetti citotossici e genotossici. L'obiettivo ultimo del più ampio progetto di ricerca, in cui si inserisce il presente lavoro, consiste nell'accertare l'efficacia e la sicurezza di tali vettori in modelli animali, per giungere infine alla manipolazione genetica di cellule staminali ematopoietiche, derivanti da pazienti HIV-positivi ed affetti da linfoma, per i quali il trapianto di cellule staminali ematopoietiche geneticamente modificate rappresenterebbe l'unico contesto clinico eticamente accettabile.

3. ABSTRACT

Background: HIV-1 infection still remains a worldwide burden, with more than 35 million people currently affected and approximately 2.1 million of new cases every year. The introduction of HAART, into the clinical practice, has greatly contributed in suppressing viral replication and drastically reducing patients' mortality. However, this treatment is often associated with considerable side effects and organ toxicity, as well as emergence of drug-resistant escape mutants. Moreover, it cannot eradicate the virus, which persists in *reservoir* cell and tissue compartments. In this context, many improvements are made in developing molecules and active inhibitors with a broad-spectrum of activity and a low toxicity, especially from natural sources (**1**). On the other side, advances in the fields of gene-targeting strategies, based on intracellular immunization of autologous T cells or CD34+ HSPCs, have revealed promising results in the repopulation of the immune system. In particular, the genetic modification of target cells with self-inactivating (SIN) lentiviral vectors, encoding a RNAi platform together with fusion inhibitor, can provide AIDS-patients a stable and long suppression of viral replication (**2**).

Materials and Methods: (**1**) The natural compounds (i.e. Oximacro®; Lupeol and Lupeol acetate; 1,5-dicaffeoylquinic acid) were firstly tested for the cytotoxicity and antiviral activity in the early phase of replication, in Jurkat cells, determining CC_{50} and EC_{50} value. Oximacro® activity was also assessed against pseudotyped viruses, harboring different Envelope proteins, and in acutely infected PM1 cells, against two different strains of laboratory-adapted HIV-1.

(**2**) SIN lentiviral vectors were generated in order to express multiple HIV-1 inhibitors. Recombinant particles were produced in 293T cells and titrated by flow cytometry and Reverse Transcriptase (RT) activity assay. To address the antiviral activity of the combinatorial vectors, CD4+ T lymphoblastoid Jurkat cells were transduced with lentiviral particles, then subjected to the cell viability and, finally, infected with the HIV-1 HXBc2 Vpr+/Vpu+/Nef+ laboratory-adapted strain using different M.O.I. The levels of expression of the maC46 peptide was assessed by qRT-PCR. In order to test the mutagenic potential of this SIN lentiviral platform, IVIM assay was also employed.

Results: (**1**) Oximacro®, a cranberry extract, rich in proanthocyanidins, is able to inhibit HIV-1 infection and replication. In particular, viral pre-incubation with the compound can greatly reduce HIV-1 infectivity, independently from the co-receptor usage, while

continuous administration of the compound is able to protect cells from the infection over time.

(**2**) The developed lentiviral particles efficiently transduced Jurkat cells, without cytotoxic effect, and potently blocked HIV-1 replication; in particular, the lentiviral combinatorial platform, expressing the triple cassette sequence along with the peptide fused with the eGFP, conferred the best antiviral activity under all tested experimental conditions. Furthermore, no impact on the viability or on the proliferation of transduced murine cells was observed in the IVIM assay.

Conclusions: (**1**) Preliminary results have assessed the antiviral activity of Oximacro® against HIV-1 infection. Studies are ongoing to investigate the mechanism of inhibition of the viral replication, especially concerning the viral attachment and the HIV-1 Env-co-receptor binding, and sustain the feasibility of Oximacro® as a valid candidate for the development of a broad-spectrum microbicide.

(**2**) The developed vectors, combining three shRNAs with the maC46 fusion inhibitor, appear to be extremely promising as anti-HIV-1 strategy. Current experiments are testing the best performing vector in in human primary CD4+ cells and in CD34+ HSPCs, in order to further validate this approach for clinical application in selected HIV-1-positive-lymphoma patients.

4. INTRODUCTION

4.1 The Human Immunodeficiency Virus type I

HIV-1 belongs to the Retroviridae family and the Lentivirus genus. Human retroviruses originated from a zoonotic transmission of Simian Immunodeficiency Virus (SIV) from non-human primates into humans in West and Central Africa, leading to the HIV and HTLV epidemic (Peeters et al., 2014). Lentiviruses are characterized by the establishment of a persistent infection with a long incubation period, leading to the degeneration of multiple organ systems, cachexia and death (Clapham and McKnight, 2002).

Two correlated forms of HIV have been isolated from AIDS patients, HIV-1 and HIV-2, which are distinguishable by serological and nucleotide sequence differences. Both viruses result in AIDS; however, HIV-2 is characterized by lower transmissibility and reduced probability of progression to AIDS (Nyamweya et al., 2013). One of the major characteristics of HIV-1 worldwide spread is its high genetic variability and extensive heterogeneity among viral isolates, circulating in several individuals or even at different times of the infection in the same individual (the so called “viral quasispecies”). This diversity is due to the high number of mutations by the Reverse Transcriptase enzyme, that lacks proof-reading activity, combined with a significant turnover of the viral population (over 1010 virions produced every day), leading to the development of a viral population adaptable to each dynamic pressure and selection process (Domingo and Perales, 2018). Based on the phylogenetic relationships, HIV-1 strains have been classified into distinct genetic groups, designated Major (M), which is the main cause of worldwide HIV epidemic, Outlier (O), Non-M, Non-O (N) and P, each of which resulted from an independent cross-species transmission event (Santoro and Perno, 2013). Within HIV-1 group M, nine genetically distinct subtypes or clades are recognized: A through D, F through H, J and H. Subtype B dominates in Australia, Americas, and Europe, whereas subtype C predominates in India and Africa (Zulfiqar et al., 2017).

4.1.1 Virion morphology, structure and genome organization

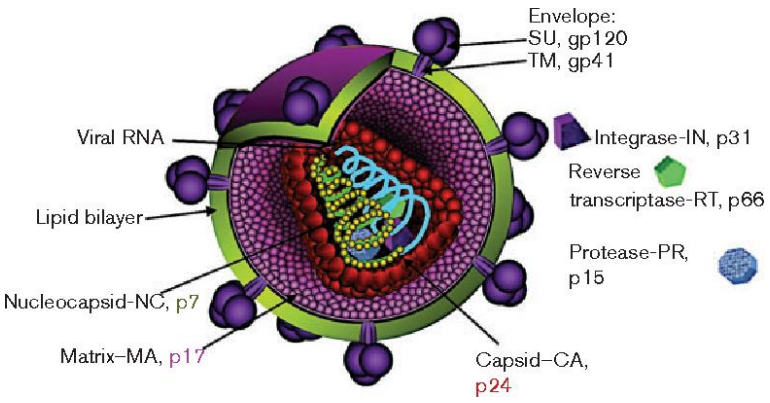

Figure 4.1. The structure of the Human Immunodeficiency Virus (HIV)-1 particle. The viral particle is covered by a lipid bilayer, derived from the host cell, and studded with viral Envelope glycoproteins, composed of a surface domain (gp120) and a transmembrane domain (gp41), which are noncovalently associated and form trimeric spikes. The mature virion contains the Matrix (MA, p17) protein, the conical core composed of the Capsid (CA, p24) protein, the Nucleocapsid (NC, p7) protein which binds the genomic RNA, the p6 protein required for budding, the accessory protein Vpr, the Reverse Transcriptase (RT), the Protease (PR), the Integrase (IN) and two copies of the genomic RNA. The accessory protein Nef is also virion associated (Steckbeck et al., 2013).

Mature HIV-1 virions measure from 100 to 150 nm in diameter and are surrounded by a lipoprotein-rich membrane. Each viral particle membrane includes glycoprotein heterodimer complexes composed of trimers of the external surface gp120 and the transmembrane spanning gp41 glycoproteins bound together (Fanales-Belasio et al., 2010) (**Figure 4.1**). The core contains two long positive sense, single stranded, linear RNA molecules of approximately 9.4 kb in length, which encode the major structural and non-structural proteins, common to all replication-competent retroviruses. The three majors structural and enzymatic proteins are Gag, Pol and Env. The *gag* gene encodes the viral matrix and core proteins. Gag proteins are initially synthesized as a polyprotein precursor, Pr55Gag, which is sufficient to produce noninfectious, virus-like particles in the absence of other viral proteins or packageable viral RNA. During or shortly after virus budding from the host cell, Pr55Gag is cleaved by the HIV-1 Protease (PR) into the mature Gag proteins p17 Matrix (MA), p24 Capsid (CA), p7 Nucleocapsid (NC), and p6. The MA protein is primarily a peripheral membrane protein located along the inner of the viral lipid envelope, where it directs the incorporation of the Envelope glycoprotein (Env) into the forming virion. The CA protein p24 forms the conical core that encloses the viral genome (Freed, 1998).

The *pol* gene, which lacks an initiation codon, partially overlaps and it is in the –1 reading frame with respect to *gag*. As a result, Pol is only synthesized as part of a Gag-Pol fusion protein. Thus, Pol is expressed in the form of a 160 kDa Gag-Pol fusion protein (Pr160-Gag-Pol) and is cleaved to produce MA, CA, p2, NC, the transframe protein (TF), and the viral enzymes PR, Reverse Transcriptase (RT) and Integrase (IN). PR, RT and IN are not functional in their monomeric forms and must come together as either dimers (PR), heterodimers (RT) or tetramers (IN) to be catalytically active (Hill et al., 2005). The enzymatic activity of these proteins will be analyzed in the description of the replication cycle.

The Env glycoproteins are synthesized as a polyprotein precursor, gp160, that is cleaved by cellular proteases to the mature surface glycoprotein gp120 and the transmembrane glycoprotein gp41. During virus assembly the gp120/gp41 complex is incorporated as heterotrimeric spikes into the lipid bilayer of nascent virions. These gp120/gp41 complexes then initiate the infection process by binding receptor and co-receptor on the surface of target cells (Checkley et al., 2011).

In addition to these major proteins, the HIV-1 genome encodes the Tat and Rev regulatory proteins. Tat is a small basic protein coded by two exons whose length varies between 99 and 103 amino acids. Tat recognizes a short-stem loop structure, known as the transactivation response element (TAR), located at the 5' terminus of the viral transcript. This binding activates the transcription complex that assembles onto the viral promoter, leading to a strong increase in viral transcripts. In addition to promoting viral transcription, Tat regulates the expression of cellular genes, modulating key pathways and mechanisms to generate an environment that favors the production and spread of HIV (Clark et al., 2017). On the other side, Rev is a 116-residue protein, which targets partially spliced and unspliced viral mRNA for transport from cell nuclei to the cytoplasm, by binding to an mRNA segment in the *env* gene known as the Rev responsive element (RRE).

Vif, Vpr, Vpu and Nef are accessory proteins, that are essential for viral infectivity and spreading during *in vivo* infection. Vif is crucial for the productive infection and dissemination of HIV-1 in non-permissive cells, containing the cellular anti-HIV defense cytosine deaminases APOBEC3 (A3G and A3F). Vif neutralizes the antiviral activities of the APOBEC3G/F by diverse mechanisms, including their degradation through the ubiquitin/proteasome pathway and their translational inhibition. In addition, Vif appears to be an active partner of the late steps of viral replication by interacting with Pr55Gag, RT and genomic RNA (Sleiman et al., 2014). Vpr is a 96-amino acid protein, which increases

the rate of viral replication and accelerates the cytopathic effect of the virus on T cells and, more importantly, it is essential for viral replication in macrophages (González, 2017). Vpu is a small membrane protein composed of a transmembrane helical domain and two α-helices in an amphipathic cytoplasmic domain, that down modulates several cellular proteins, including CD4, BST-2/CD317/tetherin, NTB-A, and CCR7. The interactions of Vpu with these proteins interfere with the immune system and enhance the release of newly synthesized virus particles (Zhang et al., 2015). Finally, Nef is a 27–35 kDa protein that is N-terminally myristoylated, a modification that allows its association with the cytosolic face of cellular membranes. Nef controls expression levels of various cell surface molecules that play important roles in immunity and virus lifecycle, by directly interfering with the itinerary of these proteins within the endocytic and late secretory pathways (Pereira and daSilva, 2016).

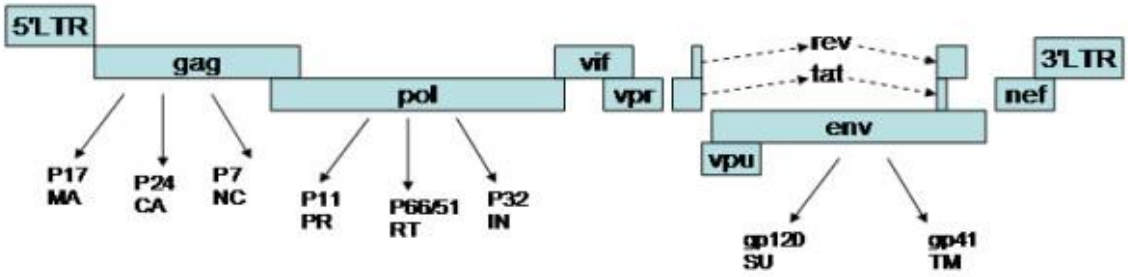

Figure 4.2. HIV-1 genome, transcripts and proteins. The viral genome of 9,6 kb is flanked by two long terminal repeats (LTR), which drive the transcription of the integrated provirus. This gives rise to the unspliced full length mRNA that will serve as genomic RNA to be packaged into virions or used as a template for translation of structural, functional and accessory proteins (Costin, 2007).

Besides the protein-encoding regions, cis-acting elements are interspersed throughout the HIV-1 genome, such as regulatory sequences, consisting of repeat elements (R) followed by 5'-unique elements (U5) at the 5'-terminus of the RNA genome, and of 3'-unique elements (U3) followed by repeat elements (R) at the 3'-terminus of the RNA genome. During the reverse transcription, these sequences undergo duplication giving rise to identical 5'- and 3'- long terminal repeat (LTR) consisting of U3-R-U5 sequences at the DNA genome termini. The 5'-LTR contains the enhancer-promoter sequences for viral transcription and the 3'-LTR contains the polyadenylation signal.

Other cis-acting sequences within the HIV-1 genome include the primer binding site (PBS), from which the reverse transcription starts, the viral RNA packaging signals (Ψ), the central polypurine tract (cPPT) and the central termination sequence (CTS), which lead

to the formation of a DNA structure called the central DNA Flap during reverse transcription (Pluta and Kacprzak, 2009) (**Figure 4.2**).

4.1.2 The HIV-1 life cycle

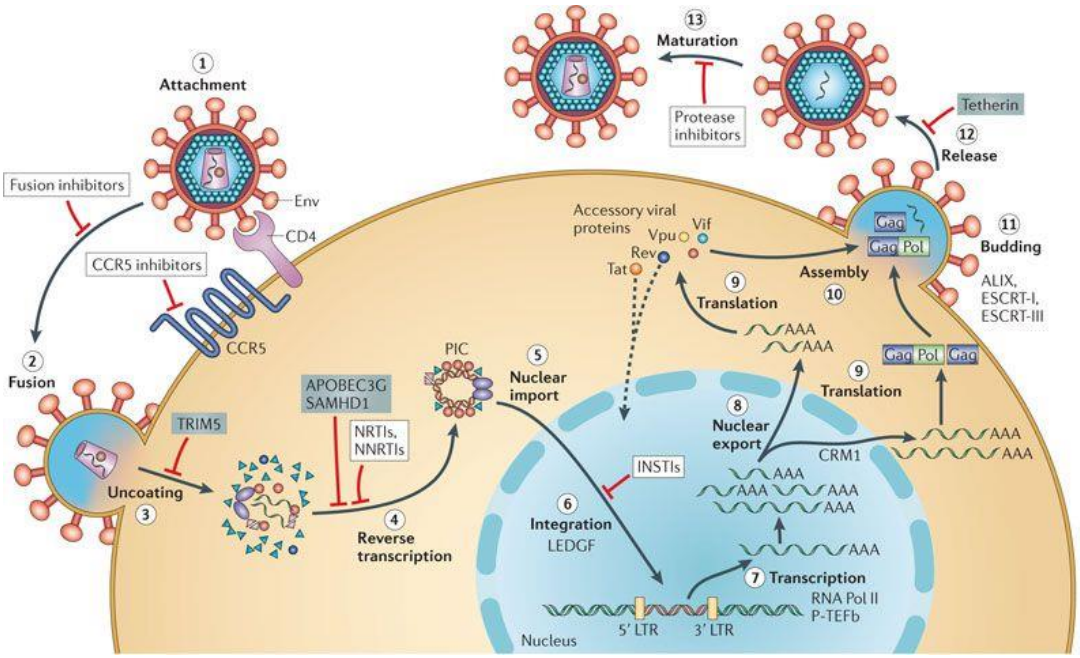

Figure 4.3 Schematic diagram of the HIV-1 life cycle. After the interaction between viral glycoproteins and specific receptors (CD4 primary receptor and CCR5 or CXCR4 co-receptors; step 1), the viral replication cycle proceeds through fusion of viral and cellular membranes and entry into the host cells (step 2). The viral core is released into the cytosol, where the uncoating takes place (step 3), while the RNA genome is reverse transcribed into double-stranded DNA (step 4), which remains associated with several viral proteins, in the pre-integration complex (PIC). After translocation into the cell nucleus (step 5), the dsDNA is integrated into the host genome (step 6). Exploiting host RNA Polymerase II, different viral transcripts are transcribed (step 7). The viral mRNAs are exported from the nucleus into the cytoplasm (step 8), where they are translated into proteins (step 9). Genome-length RNA and protein components move to the cell surface and assemble into an immature (non-infectious) particles (step 10). In the late phase of the viral replication, the budding viral-particle is formed (step 11) and release (step 12) from the cell. Following the protease-mediated maturation (step 13), the infectious viral particle is produced. Antiviral molecules can interfere with different step of the viral cycle; the sites of action of clinical inhibitors (white boxes) and cellular restriction factors (blue boxes) are reported (Engelman and Cherepanov, 2012).

The entire replication cycle of HIV-1, from the attachment of virions to target cells to the release of infectious progeny, is completed in approximately 24 hours, both *in vitro* and *in vivo* (Kim et al., 1989). The natural HIV-1 infection cycle is initiated by the attachment of the Envelope glycoprotein gp120 to the CD4 primary receptor exposed on the surface of the host cells (**Figure 4.3**), in particular T-lymphocytes, monocytes/macrophages and dendritic cells. After binding to CD4, activated gp120 undergoes a conformational change which produces a binding site for a secondary host cell receptor. The most common strains

of HIV-1 utilize a β-chemokine receptor, CCR5, or an α-chemokine receptor, CXCR4, as co-receptors. CCR5 is expressed on monocytes, macrophages and dendritic cells, while CXCR4 is mainly expressed on T lymphocytes. Viral populations are classified as R5-tropic, X4-tropic or dual tropic according to the co-receptor usage (CCR5, CXCR4 or both, respectively) (Naif, 2013). Tropism of HIV-1 has been considered associated with disease progression. The CCR5 coreceptor is used during the primary or early, asymptomatic stage of HIV-1 infection and R5 viruses are largely responsible for person-to-person transmission. Probably this is due to the fact that dendritic cells in the mucous epithelium express CCR5 but not CXCR4 and are therefore more susceptible to the infection of M-related viruses (Verhofstede et al., 2012). On the other side, the emergence of X4-using viruses generally occurs at later stages. Variation in R5 Env proteins can also influence the ability of a virus to utilize various levels of CD4 and CCR5 found in different cell types, such as macrophages and T-cells. The presence of X4 viruses is consistently associated with low CD4+ T-cell counts and accelerated disease progression, although it is still unclear whether it is the cause or consequence of disease progression (Sede et al., 2014).

Following the formation of the gp120/CD4/co-receptor complexes, gp41 adopts a conformation that allows the N-terminal hydrophobic fusion peptide to insert into the target cell membrane. The fusion is enhanced by the formation of a six-helix bundle, a rearrangement of the gp41 structure, which couples the viral and cellular membranes (Wilen et al., 2012). Once the fusion has initiated, the viral core is released into the cell cytosol. The core consists of a conical viral capsid composed of a polymer of CA subunits encasing the viral RNA genome and associated proteins, including NC, RT and IN (Ambrose and Aiken, 2014). After cell entry, the viral ssRNA is reverse transcribed into double-stranded DNA, while the core disassembles in a process termed uncoating, which is strictly dependent on reverse transcription (Rankovic et al., 2017). Different studies support the pivotal role of CA proteins in coordinating uncoating, reverse transcription, nuclear import of the pre-integration complex (PIC) and integration of the dsDNA into the host genome (Le Sage et al., 2014).

Although there are viral and cellular factors that assist in the process of reverse transcription, the two enzymatic activities that are necessary and sufficient to carry out reverse transcription are present in RT. These ones are associated to the two RT enzymatic functions: the DNA polymerase, that copies either a RNA or a DNA template, and the Ribonuclease H (RNase H), that degrades RNA if it is part of a RNA–DNA duplex. To initiate the process, RT requires a primer for the synthesis of the first DNA strand (the

minus strand), which is the cellular Lys3 tRNA, whose 3'-end is base paired to a complementary sequence near the 5'-end of the viral RNA: the PBS. Once the DNA-RNA duplex is formed, the RNase H degradation removes the viral RNA, exposing the newly synthesized minus-strand DNA. The ends of the viral RNA are direct repeats, called R. These repeats act as a bridge that allows the newly synthesized minus-strand DNA to be transferred to the 3'-end of the viral RNA. After this transfer, minus-strand synthesis can continue along the length of the genome. As DNA synthesis proceeds, so does RNase H degradation. However, there is a purine-rich sequence in the RNA genome, called the polypurine tract, or ppt, that is resistant to RNase H cleavage and serves as the primer for the initiation of the second (or plus) strand DNA (Hu and Hughes, 2012).

The nascent viral DNA with associated virion proteins form the PIC, which is transported into the nucleus. Evidence shows that MA, IN and Vpr have a relevant role in nuclear entry (Lee et al., 2010). The HIV-1 DNA is then integrated into the host-cell genome by the IN, in combination with the cell repair system, preferentially into transcriptionally active regions of the host cell genome (Marini et al., 2015). The integrated form of HIV is known as the provirus and it is characterized by the presence of identical LTR copies flanking the coding regions. Once the proviral DNA is integrated, it depends on the host RNA polymerase II (Pol II) for transcription. The HIV-1-encoded Tat protein is absolutely essential for activating transcriptional elongation from the viral LTR. The early viral transcripts are extensively spliced to form a group of mRNAs that are 1.8- to 2-kb in size and encode the proteins Tat, Rev and Nef. After Tat becomes available, it dramatically enhances the efficiency of Pol II elongation to produce the full-length viral transcripts, by directing the cellular transcriptional elongation factor P-TEFb to nascent RNAs. Tat recruits viral RNA by interacting with the transactivation response (TAR) RNA element, which is a stem-loop structure located at the 5′-end of all nascent viral transcripts (Liu et al., 2014). To produce the full range of mRNAs needed to encode the viral proteins, HIV-1 primary transcripts undergo extensive and complex alternative splicing in the nucleus of infected cells. HIV-1 mRNAs are grouped into three size classes: the unspliced 9-kb primary transcript, which can be cleaved to generate the Gag and Gag-Pol precursors or be packaged into virions to serve as the genomic RNA; the incompletely spliced RNA includes heterogeneous mRNAs of 4- to 5-kb; and the completely spliced 1.8-kb transcripts, which encode Tat, Rev, and Nef. Unspliced and incompletely spliced transcripts require the expression of Rev for the nuclear export, which interacts with the highly structure RNA element RRE, in the *env* gene. On the other hand, the proteins

encoded by the fully spliced mRNAs, Nef, Tat, and Rev, can be exported to the cytoplasm in the absence of Rev by an endogenous cellular pathway used by cellular mRNAs (Karn and Stoltzfus, 2012).

During its trafficking to the plasma membrane, or after its arrival there, Gag interacts with dimeric viral RNA. Virus assembly proceeds at the plasma membrane, and viral Env glycoproteins accumulate at the site. Gag then recruits the cellular ESCRT machinery, which drives the membrane scission reaction required for particle release (Freed, 2015). On reaching the plasma membrane, Gag induces the recruitment and coalescence of cholesterol- and sphingolipid-enriched membrane microdomains, often referred to as lipid rafts (Hogue et al., 2011). Lipid rafts probably serve as platforms for virus assembly, and the targeting of Gag to these microdomains may facilitate the incorporation of Env glycoproteins into virions. Formation of the budded particle and final scission from the cell is associated with the proteolytic processing of the Gag polyprotein into its component fragments, morphogenesis of the spherical particle into the MA-enveloped virion with the conical core and maturation of the loosely associated paired RNA genomes into a mature tightly linked dimer (Meng and Lever, 2013)

4.1.3 Natural history and pathogenesis of HIV-1 infection

HIV-1 is the etiologic agent of the Acquired Immunodeficiency Syndrome (AIDS), a pathologic condition that leads to a slow and progressive failure of the host immune system. Initial cases of HIV were reported in 1981 to Centre for Disease Control, and the virus was first isolated from patients in 1983 (Barré-Sinoussi et al., 1983). Nowadays HIV/AIDS pandemic represents one of the most important global health challenges. More than 35 million people are currently living with HIV and each year approximately 2.1 million of individuals became newly infected (UNAIDS Report on the Global AIDS epidemic 2016).

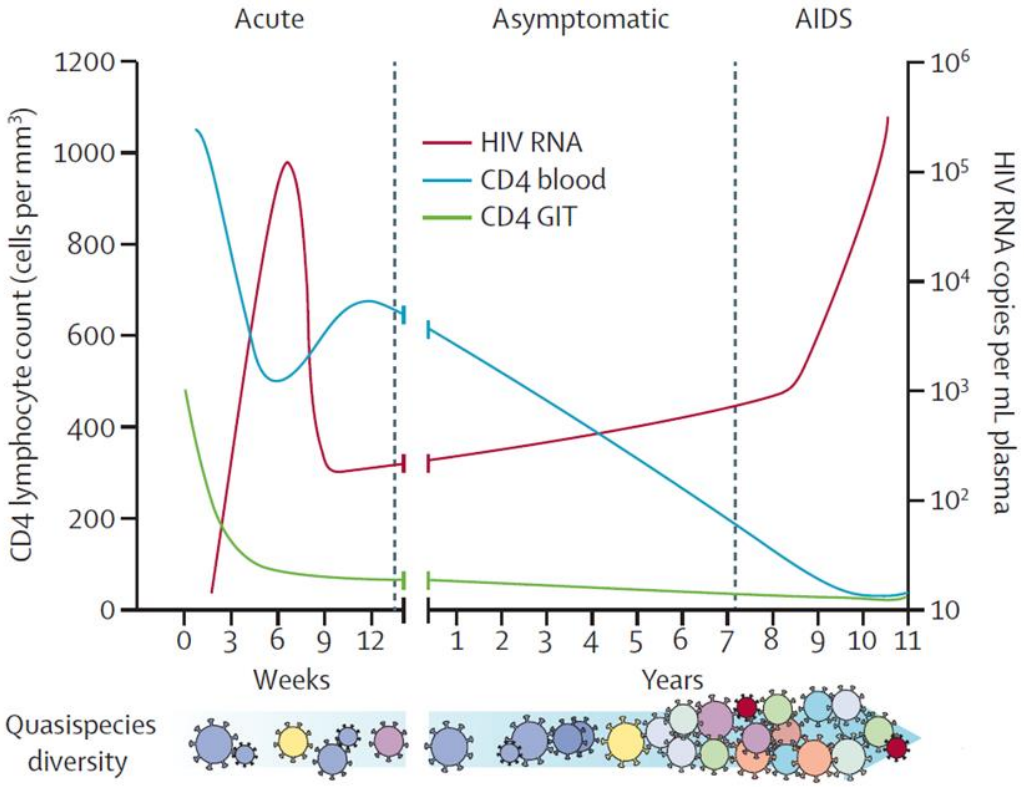

Figure 4.4 Time course of an untreated infection. The natural history of HIV-1 infection can be divided into three phases. The higher the viral load (HIV-1 RNA) in plasma, the more rapidly CD4+ T cells decreases leading to AIDS. The viral load and the CD4 count represent the markers for deciding when to begin therapeutic treatment (Maartens et al., 2014)

HIV-1 is transmitted by sexual contact across mucosal surfaces, by maternal-infant exposure, and by percutaneous inoculation. Independently from the route of infection, viral replication occurs primarily in the lymphoid organs and subsequently in the blood stream. Once infection with HIV-1 has become established in the lymphoid tissues, there is extensive virus replication, which is soon reflected in very high levels of plasma viraemia. The peak viraemia occurs an average of 6–15 days after the onset of symptoms, with viral load levels of 1–10 million/copies/mL, at which time the donor is probably highly infectious (Hansasuta and Rowland-Jones, 2001). As the immune response to HIV-1 develops, there is a dramatic reduction in the level of viremia, which falls to a lower plateau level (the set point) ranging from 10^3 to 10^5 copies/mL. The acute phase is usually accompanied by a dramatic reduction of the CD4+ T cell count reflecting both the virus-induced CD4+ T-cell depletion (Streeck and Nixon, 2010). Different mechanisms allow HIV-1 to evade host immune responses leading to a chronic immunodeficiency and making impossible the eradication of the infection: among these, the establishment of persistent resting memory CD4+ T cell *resorvoirs* occur very early in the course of infection (Chomont et al., 2009).

The second phase of HIV-1 infection is the long asymptomatic period between primary infection and the development of clinical immunodeficiency and it is characterized by the gradual loss of CD4+ T cells. Although the asymptomatic period may represent a phase of clinical latency, the virus replicates continuously, especially in lymphatic tissues, leading in some cases to a generalized lymphadenopathy. When CD4 cell count drops to lower than 200 cells/mmc, it leads to several AIDS associated events. Some HIV-1 positive patients progress to AIDS within 2 years of infection, whereas others, referred to as long-term non progressors, have lived with HIV-1 infection for over 10 years without significant CD4 depletion and with low level of viral load, in the absence of treatment. The final phase is characterized by a significant impairment of host defenses, a dramatic increase in viral load and the appearance of the disease. The risk of serious, potentially life threatening opportunistic infections (such as: *Pneumocystis jiroveci* pneumonia, esophageal candidiasis, and disseminated *Mycobacterium avium* complex or *Mycobacterium kansasii* infection), as well as secondary neoplasms or neurological manifestations (including Kaposi's sarcoma, non-Hodgkin's lymphomas, primitive central nervous system lymphoma) increase significantly, up to culminate with death if the patient is not treated with appropriate pharmacological therapy (Mokaya and Maurer, 2017).

4.2 Treatment strategies in chemotherapy of AIDS

A few years after the identification of HIV-1 to be the cause of AIDS, FDA approved a first enzyme-linked immunosorbent assay (ELISA) test kit to screen for antibodies against the virus, as primary tool for the detection of infected patient (Kühnl et al., 1985). In this period, the clinical management of HIV-1 largely consisted of prophylaxis against common opportunistic pathogens and managing AIDS-related illnesses.

In 1987, zidovudine (AZT) was the first drug to be licensed for the treatment of HIV infection (Ezzell, 1987). AZT is classified as nucleoside RT inhibitor (NRTI), and it was administered as monotherapy and, as such, it would prolong the lives of patients for 6 to 18 months. The introduction of a second class of antiretroviral drugs, i.e. the PR inhibitors (PIs), with the approval of Saquinavir in 1995, changed the picture of treatment of HIV infection. This opened the era of Highly Active Antiretroviral Therapy (HAART) (de Béthune, 2010).

4.2.1 Highly Active Antiretroviral Therapy (HAART)

The advent of HAART regimen in the treatment of patients was the most significant improvement in the medical management of HIV-1. This combined therapy is characterized by a cocktail of drugs that target different viral proteins and phases of the virus life cycle, in order to reduce the possibility of onset of resistant viral variants, due to the high mutational rate of HIV-1. Indeed, HAART has been seminal in reducing the morbidity and the mortality associated with HIV-1 infection and AIDS (Arts et al., 2012). The U.S. Food and Drug Administration (FDA) approved 35 antiretroviral drugs for the treatment of HIV-1 infection, up to April 2018 (www.fda.gov). These drugs are distributed into six distinct classes, based on their chemical nature, the molecular target and the resistance profiles: nucleoside-analog RT inhibitors (NRTIs), non-nucleoside RT inhibitors (NNRTIs), PR inhibitors (PIs), fusion inhibitors, entry inhibitors – CCR5 co-receptor antagonist and IN strand transfer inhibitors. Alongside to these classes, a seventh class comprehends those drugs which are a combination of multi-class products. Typical HAART regimens include a backbone of two NRTIs and a base of either PIs or NNRTIs. An NRTI is converted to a dNTP analog by a phosphorylation cascade performed by cellular kinases, which is incorporated by the viral RT as an NRTI monophosphate (or a nucleotide analog) at the 3'-end of the growing viral DNA primer. Upon incorporation, an NRTI inhibits the elongation of DNA primer because it lacks a 3′-OH group and/or it contains a modified sugar moiety that prevents addition of the next nucleotide (Das and Arnold, 2013). On the other side, NNRTIs are a group of small hydrophobic compounds, that interact with HIV-1 RT by binding to a single site on the p66 subunit of the p66/p51 heterodimeric enzyme, termed the NNRTI-binding pocket (NNRTI-BP). This binding interaction results in both short-range and long-range distortions of RT structure, blocking its enzymatic activity (Sluis-Cremer et al., 2004). The PIs mimic the cleavage sites recognized by the virus protease to process the viral Gag-Pol polyprotein. They bind to the enzyme and block its function (Lv et al., 2015). Concerning viral fusion inhibitors, T20 (enfuvirtide) is currently the only approved drug used in combination therapy of HIV-1 infections. It prevents membrane fusion by competitively binding to gp41 and blocking the formation of the post-fusion structure (Eggink and Sanders, 2010). However, T20 has several defects that limit its clinical use, including a low genetic barrier for drug resistance and a short *in vivo* half-life (McGillick et al., 2010). Up to date, only one entry inhibitor has passed FDA approval: Maraviroc, a CCR5 antagonist, which has been shown to be

effective at inhibiting HIV-1 entry into cells and is well tolerated (Woollard and Kanmogne, 2015). The newest class of antiviral drugs is represented by IN inhibitors: they are able to interfere with the transfer of viral dsDNA into the host genome. These drugs have demonstrated efficacy against HIV-1 strains that are resistant to other drug classes (Gu, 2014).

Overall, the introduction of HAART has prolonged lifespan and improved quality of life of HIV-1-infected patients, and transformed HIV infection from a fatal disease into a chronic infectious condition. However, individuals on HAART require lifelong adherence, and withdrawal of the therapeutic regimens inevitably leads to rebound of HIV replication. In addition, long-term medication may increase the risk of adverse reactions and drug-related toxicity, such as immune system disorders, gastrointestinal and nervous system sicknesses (Xu et al., 2017). Finally, this therapeutic regimen cannot eradicate the disease, due to the persistence of integrated viral DNA (that is replication competent but transcriptionally silent) in particular cell types (such as cells of the monocyte-macrophage lineage or long lived memory CD4+ T cells and, to a lesser degree, naïve CD4+ T cells) and anatomic districts (renal and central nervous system, lymph nodes, cervical epithelium, bone marrow), not easily accessible by current antiretroviral drugs (Hodel et al., 2016).

In this scenario, the search for an innovative strategy to overcome the limits of HAART is now a key priority for the HIV-1 community.

4.2.2 New approaches to antiviral drug discovery: natural compounds with multi-target mode of action and low cytotoxicity

Several studies are exploring alternative strategies, mainly aimed at eradicating the virus from the *reservoirs*, potentiating the immune responses to either control or eliminate HIV infected cells and/or finding new molecules with a broad-spectrum of activity and low toxicity for the patients (Passaes and Sáez-Cirión, 2014).

The main *reservoir* resides in latently infected resting CD4+ memory T cells. These cells carry stably integrated and transcriptionally silent but replication-competent proviruses, and do not produce virus particles while in the resting state, but can give rise to infectious virions following activation by various stimuli, leading to viral rebound when HART is interrupted. A widely proposed approach to reduce HIV-1 *reservoir* size, the so-called ‘shock and kill’ strategy, involves reversing latency in patients on HAART. Cells harboring induced proviruses could then be lysed by viral cytopathic effects or host

cytolytic effector mechanisms. However, major hurdles have emerged, including the heterogeneous nature of HIV-1 latency, from one patient to the other, and even from one cell to another in a single patient (Darcis et al., 2017). A promising solution to this issue could be represented by the employment of single-cell omics technologies, to find signature patterns of HIV-1 integration sites, and link these patterns with the various omics profiles at single-cell resolution. These results would be fundamental for identifying key players affecting treatment outcomes, thus contributing towards curative efforts (Kok et al., 2017). Alternatively, specific molecules targeting Tat-dependent transcription of HIV products could represent an opposite strategy aimed at reinforcing latency, repressing viral reactivation from *reservoirs* and reducing residual viremia during HAART (Mousseau et al., 2012). On the other side, many researchers are focusing their attention in enhancing immune responses in the context of combinatory approaches, to either control or eliminate HIV infected cells (Shan et al., 2012).

Another viable approach will be to revert to "nature" as sources of new molecules, since it has worked for drug discovery in the past. Anticancer drugs such as Taxol (*Taxus brevifolia*), Vinblastine (*Catharanthus roseus*) and antimalarial drugs such as quinine (*Cinchona* spp.) and Artemisinin (*Artemisia annua*) were all discovered from natural products and are effective in treating these diseases (Thomford et al., 2018). Natural products from plants, marine organisms, bacteria, and elsewhere are a rich source of structurally novel chemical compounds including antivirals. Several are potent inhibitors of HIV and could therefore be useful leads for future antivirals to supplement existing regimens of HAART. Indeed, natural extracts and purified products could represent a more sustainable process compared to the designing and synthetizing *de novo* drug leads (Cordell and Colvard, 2012). Identification of the antiviral mechanisms from these natural agents has shed light on where they interact with the viral life cycle, such as viral entry, replication, assembly, and release, as well as on the targeting of virus–host-specific interactions (Li and Vederas, 2009; Lin et al., 2014) or activating and eliminating HIV *reservoirs* through "shock-and kill" (Andersen et al., 2018). Although no natural compound has yet been developed up to clinical approval for HIV treatment, several classes of plant-derived agents are under investigation, and have been structurally and biologically characterized for their antiviral activity, either in biochemical assays and *in vitro* studies. In particular, anti-HIV-1 activity of natural extracts in *in vitro* has been reported for a range of botanicals, including green and black teas, Echinacea, ginseng and cinnamon, as well as red and green algae, and many others (De Clercq, 2000; See et al.,

1997; Singh and Bodiwala, 2010; Turville et al., 2008; Xu et al., 2015). Recently, in collaboration with E. Tramontano, our group demonstrated the antiviral activity *in vitro* of Sennoside A, derived from Rhubarb, against RT-associated and IN activities (Esposito et al., 2016), as well as the properties of Lupeol, a triterpenes derived from *Hemidesmus Indicus* decoction, to inhibit RT-associated RNase H functions and α-glucosidase activity in biochemical assays (Esposito et al., 2017). Moreover, with the same collaborators, we tested some purified molecules from *Onopordum illyricum* L., a Mediterranean plant, identifying a few compounds effective on RT-associated RNase H function and IN function in biochemical assays and, among them, one (1,5-Dicaffeoylquinic acid) was able to inhibit the early stages of HIV-1 replication in cell-based assays (Sanna et al., 2018)

Polyphenols and flavonoids comprise a huge number of compounds, capable of inhibiting a wide variety of enzymes, including several critical enzymes involved in the HIV life cycle, such as RT, PR, and IN (Cos et al., 2008). Among these compounds, proanthocyanidins (PACs) have raised interest for their ability to prevent HIV-1 virion binding and entry into the host cells (Fink et al., 2009; Nance et al., 2009; Neurath et al., 2005; Suedee et al., 2013). Viral entry inhibitors offer a new and promising means to address HIV-1 infections from a therapeutic and from a topical microbicide prospective because they address a new therapeutic target.

Oximacro®, a cranberry (*Vaccinium macrocarpon*, Ait.) extract, produced by Biosfered S.r.l. (Turin, Italy), is a reddish powder with a total proanthocyanidins content > 360 mg/g, and it has recently shown to be effective in preventing urinary tract infections (Occhipinti et al., 2016) and to exert a potent antiviral activity against HSV-1 and HSV-2 replication *in vitro* (Terlizzi et al., 2016). Moreover, among the Oximacro®-derived purified fractions, fraction 4, the richest in A-type PACs (420 mg/g), has proven to greatly prevent HSV infection, as well as human Influenza virus type A and type B, by targeting viral attachment to the cells (Luganini et al., 2018; Terlizzi et al., 2016). These studies also determined that this mechanism of inhibition is related to unspecific interactions between the compound and the glycosylated Env proteins, suggesting that a similar effect could be achieved also for different enveloped viruses and leading the way for investigating it against HIV-1 infection. Indeed, in the absence of a prophylactic vaccine against HIV, health measures, designed to limit the diffusion of HIV infection through sexual transmission, have included abstinence and condom use. Unfortunately, these methods can be impractical in certain countries and social situations where women don't have the power to influence decisions regarding sexual intercourse (Grown et al., 2005). A strong effort is

made in improving and discovering new pre-exposure prophylactic agents, such as topical microbicides, capable of preventing vaginal and rectal transmission of HIV-1 (Friend and Kiser, 2013). Thus, the development of a broad-spectrum microbicide could play a key role in this preventing transmission of HIV-1 and other major sexually transmitted infections.

4.3 Gene therapy of AIDS: towards intracellular immunization

With advances in understanding the molecular basis for HIV-1 replication and mechanisms for host control, several investigators are focusing on cell-based and gene therapy (GT) either as stand-alone approaches or as adjuvants to HAART regimen. These approaches can contribute to the treatment of HIV-1 infection in numerous ways: by rendering the target cells resistant to infection or viral replication (intracellular immunization); by targeting either viral proteins or RNAs; by inducing the selective activation of suicide or antiviral genes upon HIV-1 infection; by activating the immune system to recognize and destroy the infected cells. Indeed, the ultimate goal of GT for HIV-1/AIDS is to achieve a sustained inhibition of viral replication after a single therapeutic intervention and, in doing so, provide a pool of immune cells that would be resistant to HIV-1 infection and would reconstitute the immune system. The recovered immune function would then simultaneously control infection and destroy the endogenous viral *reservoir* (Stan and Zaia, 2014).

A single proof of concept was provided by the so-called “Berlin patient”, who received a heterologous stem cell transplant for AIDS-related lymphoma from a CCR5 homozygous null HLA-matched donor (CCR5-/-), and remained free of detectable HIV-1 even after discontinuation of conventional therapy (Allers et al., 2011; Hutter et al., 2009). This was a unique case of sustained virological remission, and it is considered the “Holy Grail” for the cure of HIV-1. The concept of virological remission was used to defined those cases where people living with HIV-1 have temporarily stopped HAART and yet maintained low or undetectable levels of the virus for an extended period of time without adverse impact on their health or disease status (Chun et al., 2015). Actually, a rare population of HIV-infected individuals, termed long-term non progressors or “elite controllers”, present a plasma viremia below the limit of detection and a CD4+ T cell counts within the normal range, without the requirement for HAART. These patients may represent a model for achieving sustained virologic remission, and researchers are looking for molecular markers associated to their status. Indeed, in many of these patients, highly-potent HIV-1-specific

CD8+ T cells seem to be the players of their highly activated antiviral immune defense and they have functional and phenotypic characteristics that distinguish them from HIV-1-specific T cells from individuals with progressive HIV-1 infection (Walker and Yu, 2013).
On the other hand, many patients who seemed to be "cured" from the infection, relapsed after some time. For example, an HIV-1 infected patient suffering from anaplastic large cell lymphoma also received a stem cell transplant from a homozygous CCR5-null donor. Unfortunately, in that case, X4-tropic HIV-1 strains emerged that necessitated the re-initiation of HAART (Kordelas et al., 2014). A similar situation of two patients (also known as "the Boston patients") was reported in 2009. They had undergone bone marrow transplants for non-AIDS malignancies and were HIV-negative by well-accepted laboratory tests. However, in both patients, despite the initial success, when HAART was stopped, the virus rebounded in plasma and tissue compartments in less than a year (Henrich et al., 2014).
Overall, autologous or allogeneic hematopoietic stem and progenitor cells (HSPCs) transplantation can be applied only to selected HIV-1 patients affected by leukemia and/or lymphomas, thus necessitating a bone marrow engraftment. Moreover, due to risky and expensive procedures, these are recommended only for those who developed cancer. On the other side, the identification of human leukocyte antigen (HLA)-matched CCR5Δ32 homozygous donors presents significant logistic barrier to the general application of this approach, thus increasing the interest in the development of strategies that could recreate this phenotype using a patient's own cells (Cannon and June, 2011).
As alternative approach to CCR5 Δ32 bone marrow engraftment, different studies are employing the genetic modification of HSPCs, taken *ex vivo* from the patients, by introducing protective genes against HIV-1 (**Figure 4.5**). Once successfully re-engrafted, genetically engineered HSPCs could proliferate and differentiate, continuously producing the antiviral genes in all differentiated cells, including HIV-1 target cells, such as T lymphocytes, macrophages and dendritic cells (**Figure 4.6**). In this way, this treatment could provide the patient a continuous, long-term production of HIV-1-resistant cells that have enhanced antiviral activity and can reconstitute the patient's immune system. If the host can be repopulated with a HIV-1-resistant hematopoietic system and eliminate all viral *reservoirs*, then a lifelong cure could be achieved (Herrera-Carrillo and Berkhout, 2015; Zhen and Kitchen, 2013).

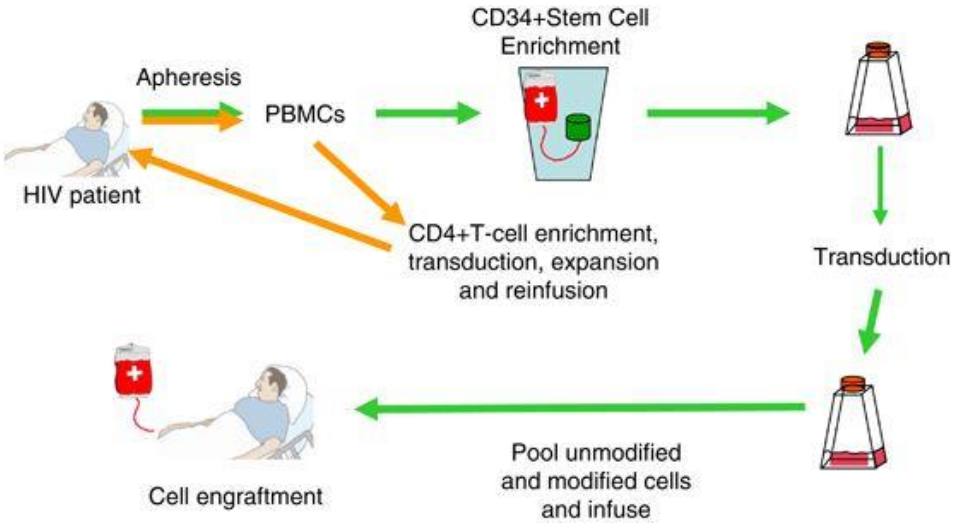

Figure 4.5 Representation of autologous stem and T-cell gene therapy for treatment of HIV-1 infection. CD34+ progenitor cells are mobilized, collected, transduced and infused into patients. This process requires some myeloablation to create space for stem cell engraftment. The T-cell approach requires isolation of peripheral blood mononuclear cells, removal of CD8+ cells and transduction of CD4+ cells followed by *ex vivo* expansion and infusion into the patients (Zhou and Rossi, 2011).

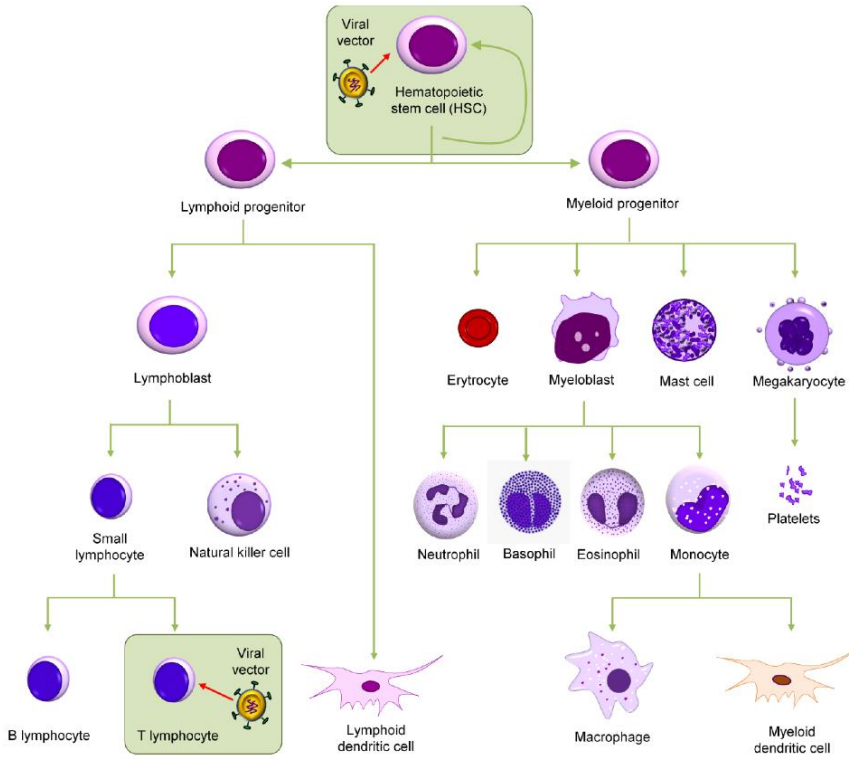

Figure 4.6 Target cells for an anti-HIV-1 gene therapy. Either hematopoietic stem and cells (HSCs) from bone marrow or the mature CD4+ T cells from peripheral blood can be genetically modified using different anti-HIV-1 gene therapy techniques, such as lentiviral vectors, expressing multiple transgenes. In particular, engineered HSCs can self-renew and proliferate to provide a pool of differentiated HIV-1 resistant mature immune cells (including T lymphocytes, macrophages and dendritic cells) over time (Herrera-Carrillo and Berkhout, 2015).

4.3.1 Gene therapy strategies to prevent HIV-1 infection

A first HIV-1 gene therapy approach was proposed by D. Baltimore, who laid the foundation for the intracellular immunization (Baltimore, 1988). Over the past 30 years, researchers have developed numerous gene-based reagents capable of inhibiting HIV-1 infection by intracellular immunization, including the expression of protein-based or nucleic acid-based inhibitors against cellular or viral targets (**Figure 4.7**) (Hoxie and June, 2012).

In the first group are included: dominant negative inhibitors (mutant HIV-1 structural or regulatory genes, with dominant repressor activity) (Hashimoto et al., 2014; Podsakoff et al., 2005), intracellular antibodies (Lo et al., 2008; Zhang et al., 2009) and fusion inhibitors. The latter have raised the attention of many scientists for their elevated efficacy in preventing HIV-1 entry and some of them entered clinical trials (Wolstein et al., 2014; Yang et al., 2017; Zhang et al., 2016).

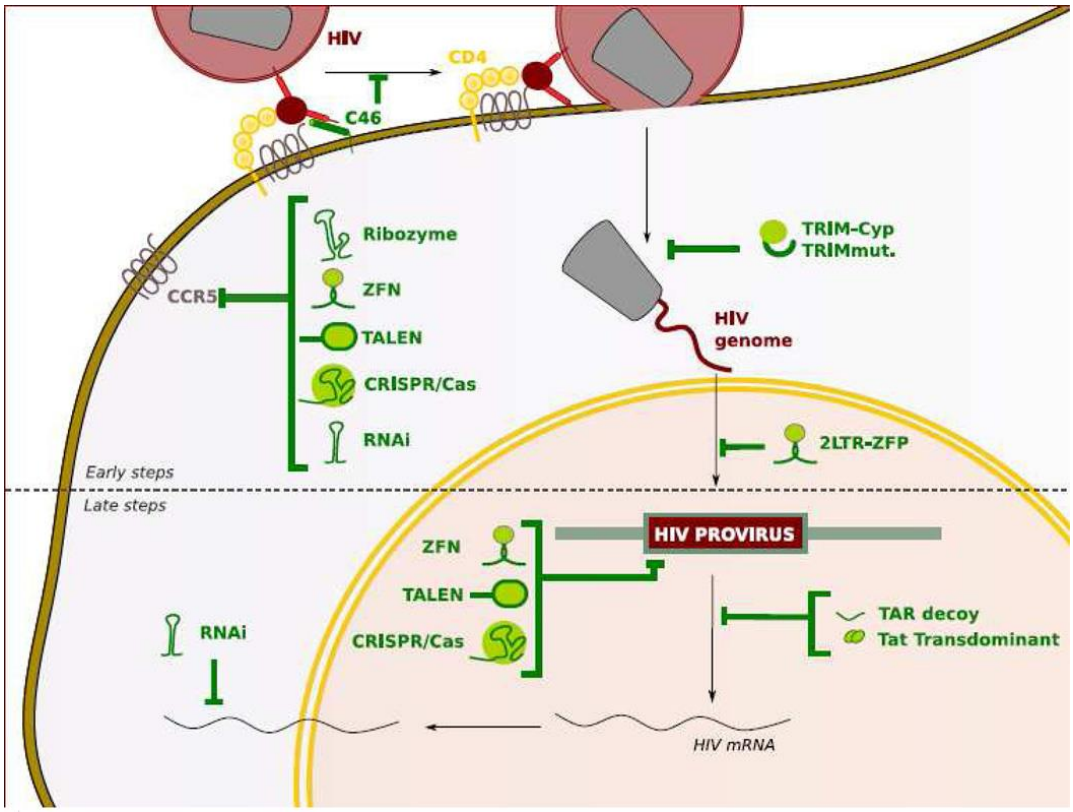

Figure 4.7 Inhibitory agents used in HIV hematopoietic cell gene therapy trials. Different steps of HIV-1 replication cycle can be inhibited by anti-HIV-1 genes, either before (early step) or after (late step) integration into host genome. Blocking viral entry may be achieved through the knock out/down of CCR5 co-receptor (by ribozyme, ZFN, TALEN, CRISPR/Cas9 and RNAi), or employing a peptide fusion inhibitor, C46. HIV-1 provirus can be excised from the host DNA of latently infected cells, by editing technologies (ZFN, TALEN, CRISPR/Cas9). Gene expression at the post transcription step can be inhibited by RNAi, TAR decoy or Tat negative transdominant (Pernet et al., 2016).

As already mentioned, upon binding to CD4, structural changes within gp120 leads to uncover the binding sites for one of the two coreceptors, CCR5 or CXCR4. Receptor engagement results in a major conformational change in the gp41 subunit inducing transformation into a fusion-active state. The hydrophobic fusion peptide at the N-terminus of gp41 is exposed and penetrates into the plasma membrane of the target cell (Chan and Kim, 1998; Hildinger et al., 2001). Subsequently, the N-terminal (HR1) and the C-terminal heptad repeat domains (HR2) of gp41 assemble into a six-helix bundle. The six-helix bundle formation brings the viral and cellular membranes in close proximity, enabling the fusion pore formation. As the fusion pore widens, the nucleocapsid of HIV-1 is introduced into the cytoplasm (Egerer et al., 2015; Melikyan et al., 2000). Indeed, many studies have proven the ability of synthetic peptides derived from the heptad repeats to efficiently inhibit HIV-1 entry, as they prevent the formation of the six helix-bundle of the gp41 subunit. In particular, C-peptides derived from the highly conserved amino acid sequence of the C-terminal are able to compete with HR1 for the binding site in HR2, hampering the structure stability of the six helix-bundle (Hildinger et al., 2001) (**Figure 4.8a**). Currently, Enfuvirtide (T-20) is the only C-peptide fusion inhibitor approved for treatment of HIV-1 infection (Cervia and Smith, 2003). Despite its proven efficacy in lowering viral infectivity, many drawbacks hamper its diffusion in the clinical practice: T-20 is not orally bioavailable and its administration requires frequent subcutaneous injections at high doses, due to a very short half-life (only 2-4 hours), thus inducing possible local reactions at the injection sites. Moreover, a rapid emergence of resistant viral species has been recorded (Egerer et al., 2015). However, several peptides have been developed and employed in GT, either as secreted antiviral entry inhibitors (iSAVE), which are released into the extracellular space (Egerer et al., 2011), or as membrane-anchored C-peptides (maC), which contains an N-terminal signal peptide, to mediate transport of the peptide through the endoplasmic reticulum to the cell surface. Among these, a 46 amino acids version of the maC (maC46) has been developed by D. Von Laer (Egelhofer et al., 2004; Hildinger et al., 2001). This elongated version of T-20 (**Figure 4.8b**) can interact with the highly conserved hydrophobic pocket at the C-terminus of the HR1 coiled-coil core, providing a robust antiviral effect, independently from the co-receptor usage. Moreover, maC46 was proven to have a selective advantage in protecting cells from HIV-1 entry in comparison with RNA antisense and RNA interference approach (Kimpel et al., 2010). Concerning the activity against patients' isolates, maC46 was found to be highly active also against virus strains resistant to T-20 (Lohrengel et al., 2005) and to induce a relatively weak resistance

in patients. Indeed, a complex combination of mutations in gp120 and gp41 associated with different resistance mechanisms must be acquired by HIV-1 to evade entry inhibition by maC46 (Hermann et al., 2009), thus making it a very attractive tool for intracellular immunization.

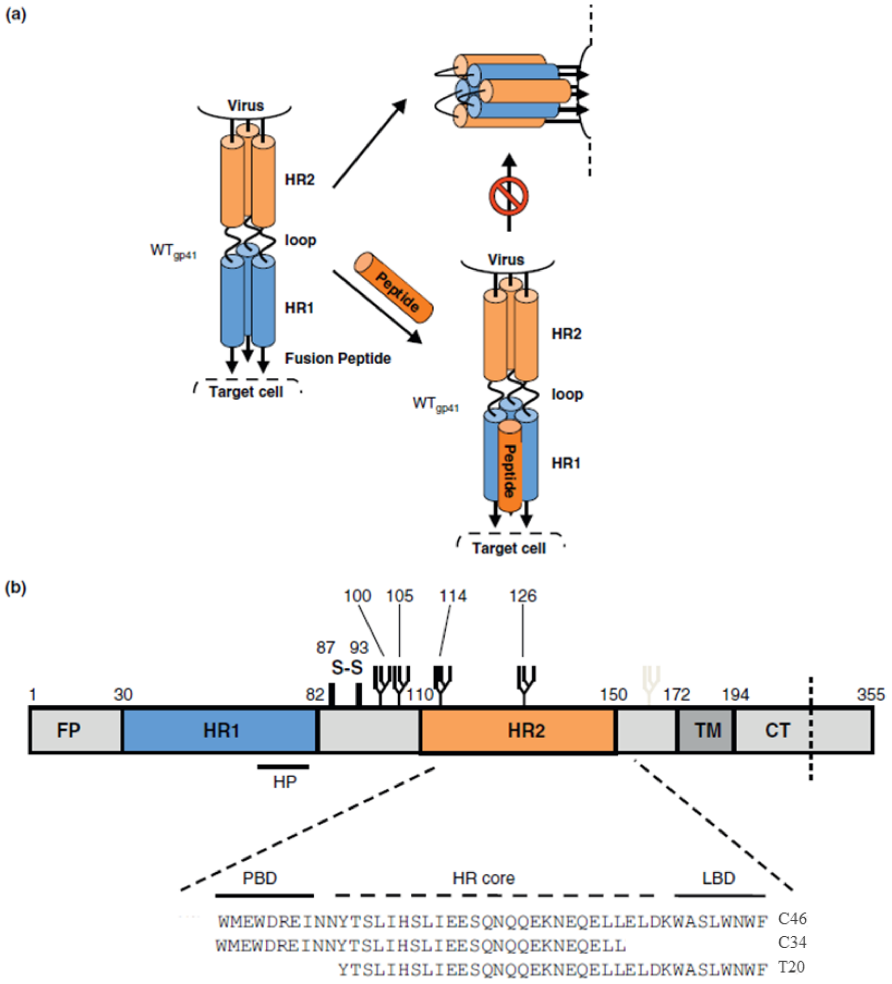

Figure 4.8 Mechanism of HR2-based fusion inhibitors. (a) The key conformational change in gp41 can be blocked by HR1-based and HR2-based fusion inhibitors. Upon binding of gp120 to CD4 and the chemokine receptor, gp41 folds into the pre-hairpin intermediate structure that spans both viral and target membranes, which subsequently collapses in the six-helix bundle structure. This folding can be inhibited by competitive binding of HR1 and HR2-based fusion inhibitors to their respective targets. (b) The various gp41 subdomains (FP: fusion peptide; HR1 and HR2: heptad repeats 1 and 2; TM: transmembrane domain; CT: cytoplasmic tail) are indicated. HR1 contains the hydrophobic pocket (HP) and the subdomains of HR2 are shown in more detail: pocket-binding domain (PBD), heptad repeat core and the lipid-binding domain (LBD). The sequences of T20, C34 and C46 (maC46) fusion inhibitors are given (Berkhout et al., 2012, *adapted*).

On the other side, a number of RNA-based inhibitors, including antisense RNAs (able to inhibit reverse transcription) (Levine et al., 2006), aptamers (Duclair et al., 2015; Zhou et al., 2015), decoys (against HIV-1 transcription and processing) (Michienzi et al., 2002), ribozymes (which catalytically cleave and inactivate the various HIV-1 RNA species)

(Michienzi et al., 2000; Scarborough et al., 2014) and si/shRNAs, which will discussed in detail in the following chapter, have been developed. An advantage of these inhibitors is that, in contrast to protein-based inhibitors, they do not elicit adaptive T-cell responses and are unlikely to be immunogenic. However, RNA approaches can potentially have off-target toxicity because of activation of innate immune responses and competition with endogenous RNA function.

Within the past few years, different novel technologies have emerged, able to recognize specific DNA target sequences to enable site-specific gene editing (Manjunath et al., 2013). All the currently available gene-editing techniques (such as: zinc finger nucleases (ZFNs), Transcription activator-like effectors nuclease (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9)) are based on a specific genomic DNA recognition site, where a nuclease activity induce double-stranded breaks (DSBs). These double stranded breaks are then repaired by cellular repair pathways which depend largely on the cell cycle: error-free homologous recombination occurs particularly during S and G2 phases of the cell cycle, and results in incorporation of the externally provided DNA at the cleavage site; while, in resting cells, DSBs are commonly repaired by error-prone non-homologous ends joining (NHEJ) pathway, which is known to induce small nucleotide additions or deletions (indels), resulting in disruption of the reading frame and gene expression. In most applications, these DNA-editing enzymes are exploited to disrupt HIV coreceptors sequences (especially CCR5) or pro-viral LTR sequences, in order to excise the viral genome from the host DNA (Jerome, 2016). ZFNs are comprised of zinc finger DNA binding domains fused to an endonuclease domain, that generates a DSB at a specific DNA target site. ZFN are often employed either to disrupt CCR5 co-receptor (Didigu et al., 2014; Hofer et al., 2013; Holt et al., 2010; Li et al., 2013) or to act on integrated provirus leading to viral excision from the host genome (Ji et al., 2018; Qu et al., 2014). CCR5 gene editing was tested in a clinical trial using modified T cells to treat HIV-1-positive patients and demonstrated prolonged reduction of viremia, while HAART was suspended (DiGiusto, 2015). TALEN are naturally occurring DNA binding proteins, and contain DNA-binding domains composed of a series of 33–35 amino acid repeat domains, each recognizing a single base pair. TALEN specificity is determined by two hypervariable amino acids that are known as the repeat-variable di-residues, and like zinc fingers, modular TALE repeats are linked together to recognize contiguous DNA sequences (Gaj et al., 2013). In different study, TALEN disruption of CCR5 demonstrated much lower cytotoxicity, compared to ZFN activity, giving promising results to pursue this path

(Mussolino et al., 2011; Shi et al., 2017). Finally, CRISPRs, together with CRISPR-associated genes (Cas), are an efficient defense system developed in nature by bacteria and archaea against viruses. In comparison to ZFN and TALEN, which usually requires labor-intensive design and screening, CRISPR/Cas9 has more advantages, among which a simpler design and a single cloning step. This system has been engineered to target CCR5 (Qi et al., 2018), as well as CXCR4 co-receptor gene, without significant impairment of cell viability (Hou et al., 2015). Recent progress in this technology has proven the potential to excise out the whole proviral genome/or parts of it from the host genome, as a consequence would deplete the viral reservoir from the system (Dey and Pillai, 2015; Liao et al., 2015; Zhu et al., 2015). On the other side, additional implementation is required. Indeed, genome-editing strategies typically comprise site-specific allelic disruption in an attempt to minimize collateral genomic damage, associated with random insertion-related oncogene activation. Nonetheless, these tools can also generate unintended, permanent, deleterious changes in the genome including genomic instability, chromosomal translocation, chromosome loss, and aneuploidy. Moreover, NHEJ can also occur at off-target sites, often at loci homologous to the intended nuclease target. If either type of off-target mutation occurs in long-term repopulating HSPCs and alters genes or genomic loci important for cell survival, self-renewal, or proliferation, either cell death or aberrant cell expansion can arise, followed by the acquisition of secondary or tertiary mutations. Thus, the specificity and off-target effects of gene-editing systems must be key considerations in their development, particularly in terms of the potential clinical applications of these methods in human HSPCs (Yu et al., 2016).

4.3.2 RNA-interference as antiviral therapeutics

RNAi (**Figure 4.9**) is an evolutionary conserved mechanism that triggers sequence-specific inhibition of complementary mRNA (Fire et al., 1998). In mammals, RNAi is a post-transcriptional gene silencing mechanism that functions to regulate gene expression via small hairpin-like dsRNA, called miRNAs. miRNAs are expressed from Polymerase II promoters as primary miRNA transcripts (pri-miRNAs), forming distinctive imperfect hairpin structures. pri-miRNAs are first processed by a Drosha complex cleaving ~22 bp back form the stem-loop junction, to release 60-80 nucleotide hairpin (pre-miRNA) and, then, exported from the nucleus to the cytoplasm by the nuclear Exportin-5 protein. In the cytoplasm, Dicer next cleaves from the opposite end, removing the loop to release a small

RNA duplex of ~21 bp with characteristic 2-nt 3'-overhangs (the mature miRNA). The duplex is then loaded into the RNA-induced silencing complex (RISC), consisting of Argonaute family (Ago1-4) and other proteins (Meister, 2013), and unwound into the effector guide strand and the passenger strand. The guide strand is selected according to thermodynamic stability at the ends of the duplex. This strand directs RISC to bind target RNA within the 3'-untranslated region, resulting in translational repression, mRNA destabilization or a combination of both. Near-perfect base pairing of the miRNA with the mRNA results in cleavage-mediated inactivation of the target mRNA. The targeted mRNA is translocated to cellular processing (P)-bodies where storage, de-adenylation, de-capping and degradation take place (Jonas and Izaurralde, 2015; Liu and Berkhout, 2011).

The RNAi pathway can be artificially engaged at any point in the process, typically either through delivering synthetic siRNAs to the RISC or by expressing short hairpin RNAs (shRNAs) to be processed by Dicer and possibly Drosha, to mediate post-transcriptional silencing of target genes (Berkhout and Eekels, 2013; Wittrup and Lieberman, 2015).

Studies have shown that the stable expression of shRNAs within target cells can lead to a very efficient silencing effect of the target genes, making them the election effectors in anti-HIV-1 RNAi gene therapy approaches (McIntyre et al., 2011). shRNAs consist of the sense and antisense sequences of a siRNA connected by a loop of unpaired nucleotide. The shRNAs are transcribed in the nucleus as stem loop RNA and transported to the cytoplasm, where are cleaved by the same machinery employed to process miRNA. However, when the siRNA is formed, it perfectly pairs to complementary target mRNA. The mRNA is then rapidly degraded by exonucleases, leading to gene silencing. Expression of shRNAs is mostly driven by RNA Polymerase III promoters, including the small nucleolar RNA U6 promoter, the RNase P RNA H1 promoter and the tRNA promoters, because of their natural function in the production of small cellular transcripts (Liu and Berkhout, 2011; Ter Brake et al., 2006). These promoters are compact, active in many tissues and they usually generate a huge amount of transcription products. On the other side, if a choice of combinatorial approach is undertaken, different promoter should be used, as the presence of repeated sequences might cause recombination within the vector genome during the transduction process.

One of the potential limitations of using multiple shRNAs can rise from competition with endogenous microRNAs for nuclear-to-cytoplasmic export and incorporation into the RNA-silencing machinery. Moreover, toxicity of RNAi may occur, due to improper target recognition, overloading of the silencing machinery (Grimm et al., 2006), toll-like receptor

(TLR) activation and interferon production (Hornung et al., 2005). Thus, in order to reduce off-target effects of siRNAs, a careful design of the RNA platform is necessary. Moreover, the levels of expression for each type of anti-HIV-1 siRNA are to be considered, to obtain a sufficient therapeutic effect without cytotoxicity.

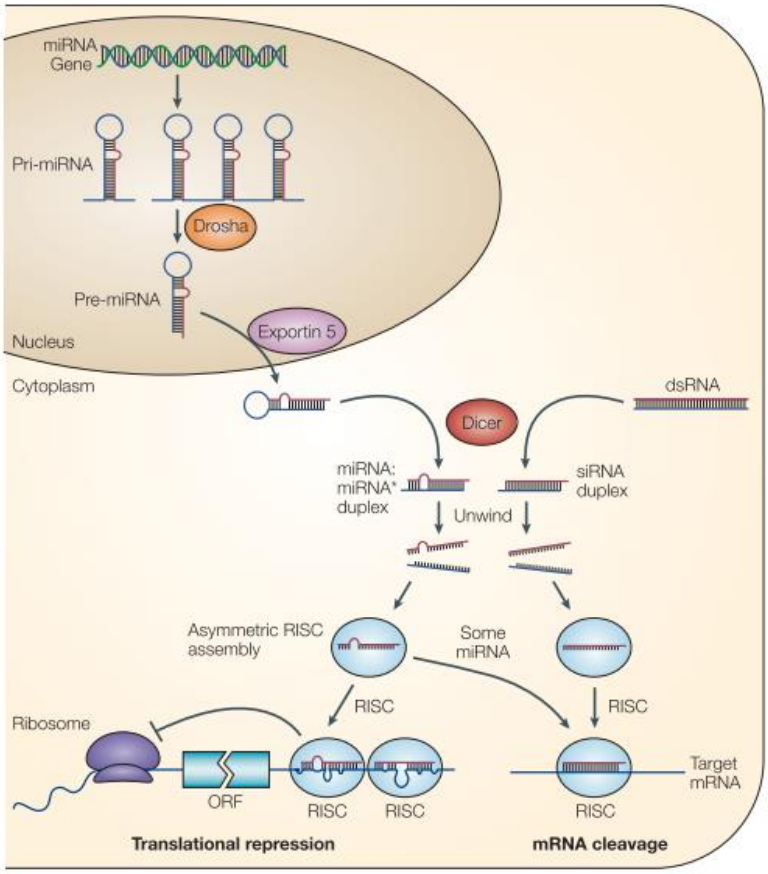

Figure 4.9 The miRNA and siRNA pathways of RNAi in mammals. The nascent pri-microRNA (pri-miRNA) transcripts are firstly processed into ~70-nucleotide pre-miRNAs by Drosha inside the nucleus. Pre-miRNas are transported to the cytoplasm by Exportin 5 and are processed into miRNA:miRNA duplexes by Dicer. Dicer also processes long dsRNA molecules into small interfering RNA (siRNA) duplexes. Only one strand of the miRNA:miRNA or the siRNA duplex is preferentially assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target (He and Hannon, 2004).

Almost all HIV-encoded mRNAs could be identified as target of an RNAi approach (Bobbin et al., 2015).

A number of criteria have been proposed for developing genetic inhibitors of HIV-1 for human clinical trials. Firstly, it is important to target sequence that are conserved among different virus strains, to reduce the chance of mutant escape. Secondly, anti-HIV-1 therapeutics that block entry and replication before virus integration are considered the best inhibitors, in comparison with the ones inhibiting later steps of the viral replication cycle

(Zhou and Rossi, 2011). Moreover, it may also be beneficial to select target sequences in the early spliced mRNAs encoding the early HIV-1 proteins, such as Tat and Rev. Indeed, an early block of viral gene expression will seriously hamper the expression of the late structural proteins and virion assembly (Sano et al., 2008).

On the other side, some regulatory factors, such as Vif, were proven to have a crucial role in promoting viral infectivity, replication and pathogenesis *in vivo*. Indeed, Vif counteracts the restriction factors of the cellular APOBEC3 cytosine deaminases family (i.e. APOBEC3G and APOBEC3F) by inducing their proteasomal degradation (Wissing et al., 2010). Downregulating Vif, the APOBEC3 proteins are incorporated into the newly synthetized virus particles, leading to hypermutation in the viral DNA during reverse transcription in the following round of infection, generating poorly infective viruses.

Targeting cellular factors that are essential for HIV-1 replication cycle can be a useful approach, since the mutation rate of the cellular DNA replication machinery is significantly lower than the one of the lentiviral RT enzyme. Among these, the CCR5 co-receptor has shown to be an intriguing target for HIV-1 therapy. As already mentioned, heterozygous or homozygous individuals for a 32-base-pair deletion in the CCR5 gene (CCR5Δ32), that prevents CCR5 expression on the cell surface, have shown a slower progression or a resistance to HIV-1 infection, respectively, and are otherwise healthy.

The use of a single anti-HIV-1 factor may not be sufficient to achieve a long-term protection from the infection, due to the high mutational rate of HIV-1. Indeed, it is necessary to have multiple effective anti-viral inhibitors to achieve a durable block of HIV-1 replication and prevent the generation of viral escape mutants (Centlivre et al., 2013; Ter Brake et al., 2006). The expression of multiple shRNAs-expression cassettes from a single vector has shown to be very promising in inhibiting HIV-1 replication (McIntyre et al., 2011; Ter Brake et al., 2006). However, as already mentioned, different promoters should be used, to avoid recombination within the vector genome. Alternatively, long hairpin RNAs (lhRNAs) or extended shRNAs (e-shRNA) expressing multiple effective siRNAs from a single promoter can mediate a durable HIV-1 inhibition (Liu et al., 2009; Saayman et al., 2008).

In the contest of a combinatorial platform, the success obtained with either RNAi and fusion peptides as inhibitors of HIV-1 replication and infectivity has pushed researchers to combine the two strategies to achieve an even higher and longer protection from viral infections. Among these, O. Wolstein and colleagues have engineered a novel SIN lentiviral vector, LVsh5/C46, that carries both a CCR5-targeted shRNA (sh5) and the

maC46 peptide (C46). Indeed, the stable expression of these HIV-1 inhibitors in the target cells (such as peripheral blood mononuclear cells, CD4+ T lymphocytes and CD34+ HSPC) have shown to effectively protect gene-modified cells against infection with CCR5- and CXCR4-tropic strains of HIV-1 (Wolstein et al., 2014), as well as in humanized Bone marrow Liver Thymic (BLT) mice model *in vivo* (Burke et al., 2015). A phase 1/2 of clinical trial is ongoing to evaluate the antiviral activity of this construct administered *ex vivo* using both autologous CD4+ T lymphocytes and CD34+ HSC in HIV-1+ subjects, without malignancy (ClinicalTrials.gov identifier: NCT01734850).
This evidence has shown promising elements to pursue this strategy as an actual and successful anti-HIV-1 therapy.

4.3.3 Development of lentiviral vectors as gene delivery system

Lentiviral vectors are being successfully employed with increasing frequency in human clinical trials, since they have distinct characteristics that favor their use in delivery and long-term gene expression. These include the ability to accommodate large gene inserts and to transduce both dividing and non-dividing cells and the high levels and the prolonged duration of transgene expression (McGarrity et al., 2013). On the contrary, other retroviral-derived vectors, such as Moloney Murine Leukemia Virus (Mo-MLV)-based gammaretroviral vectors, are known to be prone to silencing of expression by DNA methylation, that specifically targets the LTR sequences. Moreover, for integration to occur, gammaretroviral vectors require cells to enter division shortly after infection.
Insertional genotoxicity is another important issue to consider when using an integrating vector for gene therapy purpose. Indeed, insertions could give rise to dominant gain-of-function mutations, such as the activation of proto-oncogenes flanking an insertion site, or loss-of-function mutations in tumor suppressor genes. These events are more likely in gammaretroviral vectors, which have caused several cases of leukemia in SCID-X1 gene therapy (Hacein-Bey-Abina et al., 2008). As opposed to MLV, lentiviruses tend to integrate into intronic regions of genes, without any bias towards insertion near promoters. In addition, the generation of replication-competent species (RCS), during vector production, is proven to be higher for MLV-based vectors rather than lentiviral vectors.
The first systems using replication-defective HIV-1 vectors were described in the early 1990s and it was based on two plasmids: one containing an HIV-1 provirus with a deletion on the *env* gene and a chloramphenicol acetyltransferase (CAT) gene replacing the *nef*

gene, and the other carrying the *rev* and *env* genes, either mutated or left intact, under the control of the HIV-1 LTR (Helseth et al., 1990). The further developments of lentiviral vectors system (**Figure 4.10**) were based on the concept of separating the cis-acting sequences that are essential for vector RNA synthesis, packaging, reverse transcription and dsDNA integration, from the trans elements that encode viral enzymes as well as structural and accessory proteins. Hence, such a system typically consists of a packaging expression cassette(s) (helper), an Envelope expression cassette and a vector cassette (transfer vector) (Pluta and Kacprzak, 2009).

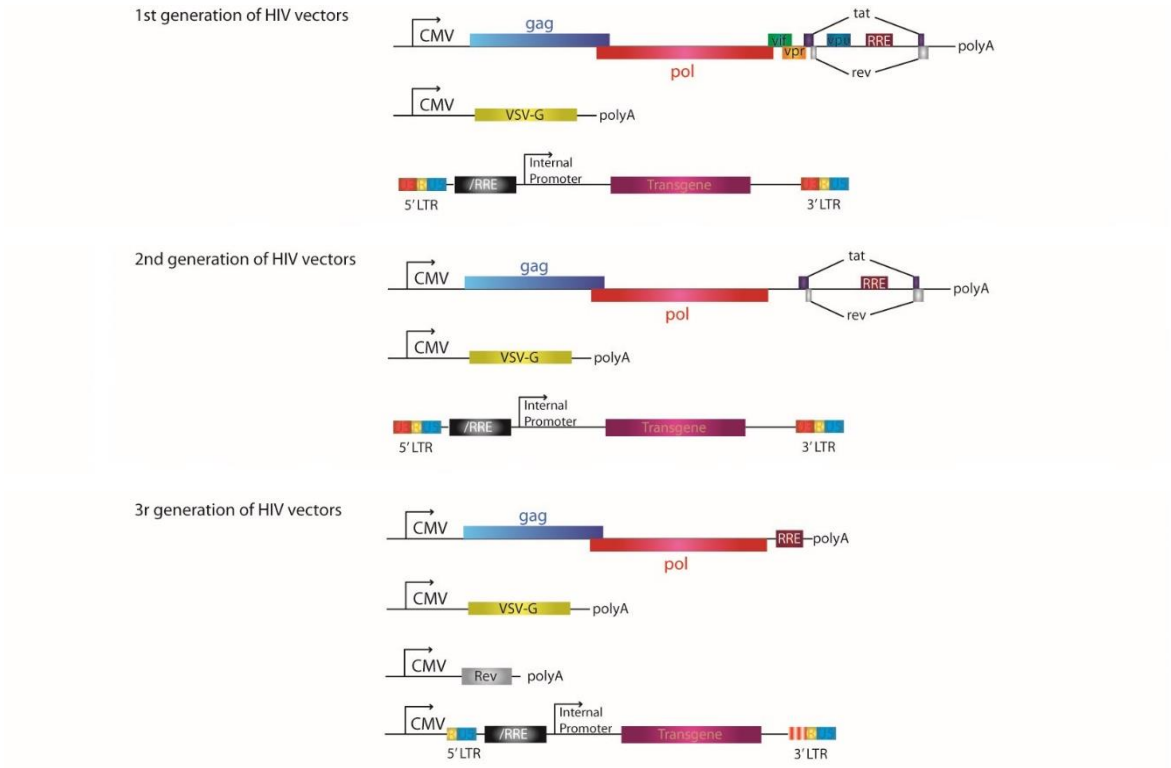

Figure 4.10 Schematic representation of HIV vectors. The first generation of HIV vectors includes all the viral proteins, except the Env protein, in a packaging plasmid. VSV-G is provided by a different plasmid. The HIV vector plasmid contains LTRs and the transgene is expressed under a strong viral promoter such as the CMV promoter. For the second generation of HIV vectors, all the accessory proteins are excluded from the packaging plasmid. Similar to the first generation of HIV vectors, expression of glycoprotein and transgene are provided by different plasmids. The third generation of HIV vectors requires four different plasmids. In addition to the three plasmids (i.e. a packaging plasmid, an Env-encoding plasmid and a vector plasmid), Rev protein is provided by a different plasmid. The vector plasmid is also modified by deleting the U3 region from 5'-LTR and partially deleting 3'-LTR to reduce the possible production of replication-competent viruses, and a strong viral promoter such as RSV or CMV is inserted for expression of the vector (Sakuma et al., 2012).

In the first generation of lentiviral vectors, the packaging construct expresses HIV-1 Gag, Pol and regulatory/accessory proteins from a strong mammalian promoter, while the Env plasmid expresses a viral glycoprotein, such as the Vescicular Stomatitis Virus

Glycoprotein (VSV-G). These two plasmids have been specifically engineered without either packaging signal (Ψ) or LTRs to avoid their integration into vector particles and to reduce the production of RCS during vector preparation. Moreover, the replacement of the HIV-1 Env glycoprotein with VSV-G has shown several advantages, such as a higher stability during manipulation and a broader tropism of transducible cells, allowing a wider set of cells for *in vitro* and *in vivo* applications. Finally, the transfer vector contains the transgene(s), and all the essential cis-acting elements (LTRs, Ψ and RRE) for packaging, reverse transcription and integration, but expresses no HIV-1 proteins (Sakuma et al., 2012). However, first generation of lentiviral vectors can give origin to RCS, following homologous recombination.

In the second generation of lentiviral vectors, the packaging constructs were refined by eliminating all accessory proteins that are associated with virulence and cytotoxicity and are not required for virus replication *in vitro*. In this system, only Rev and Tat proteins were expressed along with the Gag and Gag-Pol polyproteins (Pluta and Kacprzak, 2009).

A higher level of biosafety was obtained with the third generation of lentiviral vectors, by placing the *rev* gene on a second plasmid and completely removing the *tat* gene. Thus, a system of four plasmids was employed: the packaging construct containing the *gag* and *pol* gene, under the control of CMV promoter, and the RRE sequence; a rev-encoding construct; the VSV-G construct and the transfer vector.

Conventional lentiviral vectors integrate transgene cassettes flanked by two LTRs into the host genome. The presence of wild type LTRs can be very dangerous if some RCS are produced, since they will be able to replicate in a similar manner to that of wild-type viruses. On the other hand, a similar problem could arise if vector-transduced cells are subsequently infected by a wild-type lentivirus, which can act as helper virus and generate new RCS. Another important issue is the undesired activation of cellular genes by integrated vectors, since LTRs have an enhancer and promoter regions which can activate adjacent cellular genes. If integration occurs near a proto-oncogene, these enhancers/promoters have the potential to activate transcription of these genes, moving the cells towards oncogenesis (Sakuma et al., 2012). On the other side, the self-inactivating (SIN) lentiviral vectors, indeed, carry a 3'-LTR with a deletion in the U3 region which prevents unwanted activation of the flanking genes after integration. Moreover, an hybrid 5'-LTR was designed by substituting the U3 region with either the CMV or the Rous Sarcoma Virus (RSV) promoter.

Recently, different modifications have been introduced into the transfer vector, such as the substitution of the CMV promoter with the human Polymerase II promoter Elongation Factor 1 (EF1), to achieve an increased expression of the transgene and ensure a higher safety during therapeutic approaches. Furthermore, the addition of the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) into the 3'-untranslated region (3'-UTR) of lentiviral vectors has proven to enhance both titer and transgene expression. However, cases of development of hepatocellular carcinomas associated with the use of WPRE-inserted vectors, led researcher to develop a mutant form of WPRE (WPRE*), optimized to guarantee an increased safety and feasibility in clinical applications (Schambach et al., 2006).

5. AIM OF THE STUDY

In the continuous search for new antivirals and alternative therapeutic strategies against HIV-1 infection and AIDS, this PhD project explores two different approaches to fight this worldwide burden.

On one side, given the need of identifying new and improved HIV-1 inhibitors, natural extracts and bioactive compounds are constantly under investigation. Among these, Oximacro®, a cranberry extract produced by Biosfered (Turin, Italy), possesses a high content of proanthocyanidins, well known for their antimicrobial and antiviral properties. Recently, Oximacro® resulted effective in preventing urinary tract infections and was proven to be a promising natural candidate for the development of novel drug formulation for the prevention of HSV-1 and HSV-2 infection, leading the way for the investigating it also against HIV-1 infection. Moreover, in collaboration with E. Tramontano's group (University of Cagliari), some plant-derived molecules, mainly active *in vitro* against the RT-associated RNase H (one of the major targets of the anti-HIV-1 drug therapy), have been screened for their antiviral activity in appropriate cellular models.

On the other side, in the context of an international consortium for the development of an innovative gene therapy protocols for AIDS, our research group generated combinatorial vectors based on a SIN lentiviral HIV-1 platform, simultaneously expressing multiple siRNAs, targeting both cellular (CCR5) and viral factors (Tat, Rev and Vif) along with the membrane-anchored form of C-peptide (maC46) fusion inhibitor to inhibit simultaneously different steps of HIV-1 entry and replication and to confer protection against a broad range of isolates, reducing the occurrence of resistant viral species. Furthermore, a careful analysis of the potential genotoxicity of these new developed vectors is necessary before moving to clinical application. Once discriminated the best performing vector, the efficacy and safety of in animal models would be assessed, before moving to the clinical practice. Indeed, the final aim of this research project would be to provide a valid tool for the genetic manipulation of autologous HSPCs from selected AIDS-lymphoma patients, which would represent the ideal population in a clinical setting ethically acceptable, since they are often subjected to bone marrow transplantation.

6. MATERIALS AND METHODS

6.1 Cell lines and culture conditions

Human embryonic kidney 293T (ATCC® CRL-11268™) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen).
Human T lymphoblastoid Jurkat (Clone E6-1; ATCC® TIB-152™), C8166 (Sigma-Aldrich) and PM1 (M. Zazzi, University of Siena, Italy) cell lines were maintained in Roswell Park Memorial Institute's 1640 medium (RPMI) (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen). These cells were cultured at 37°C in a controlled atmosphere with 5% of carbon dioxide.

6.2 Compounds

Herein a list of the different compounds is reported.

Oximacro®: a cranberry (*Vaccinium macrocarpon*, Ait.) extract, produced by Biosfered S.r.l. (Turin, Italy), is a reddish powder with a high content of A-type proanthocyanidins (PACs-A). The content of PAC-A and PAC-B of Oximacro® was determined by HPLC-ESI-MS/MS (Occhipinti et al., 2016) and fractionation of Oximacro® by gel filtration chromatography was performed as described (Terlizzi et al., 2016). These experiments were performed by G. Gribaudo and collaborators (University of Torino). The experiments described here were performed with the Oximacro®-derived purified fraction 4, the richest in A-type PACs (420 mg/g), thus, herein we will refer to Oximacro®-derived purified fraction 4 as Oximacro®. The Oximacro® powder was dissolved in RPMI to generate a final concentration of 10 mg/mL.

Lupeol – Lupeol Acetate: extracts from the Indian plant *Hemidesmus indicus*, kindly provided by F. Poli (University of Bologna) (Esposito et al., 2017).

1,5-dicaffeoylquinic acid: compound purified among others from *Onopordum illyricum* extract and provided by D. Rigano and colleagues (University of Naples) (Sanna et al., 2018).

The reference compound Efavirenz were purchased from Sigma-Aldrich.

6.3 Plasmids

Some vector plasmids were already available in the laboratory. Herein a brief description is reported.

pSVC2.1 Vpr$^+$Vpu$^+$Nef$^+$: derivative of the pSVC2.1 plasmid and contains the HXBc2 molecular clone (Ratner et al., 1985), where the *vpu*, *vpr* and *nef* sequences were substituted with those derived from the pNL4-3 (*vpu/vpr*) (Adachi et al., 1986) and pLAI (*nef*) (Peden et al., 1991) molecular clones, in order to introduce functional *vpu*, *vpr* and *nef* genes.

pNL4-3-ADA: derivative of the HIV-1 pNL4-3, where the *env* sequence was replaced by the CCR5 coreceptor-using HIV-1 ADA Env (Theodore et al., 1996), kindly provided by H. Göttlinger (University of Massachusetts Medical School, USA).

pSVC2.1 Vpr$^+$Vpu$^+$Nef$^+$ Δenv CAT: obtained from the pSVC2.1 which contains the complete proviral genome of the HIV-1 HXBc2 (vif$^+$-vpu$^-$-vpr$^-$-nef$^-$) (Ratner et al., 1985), by inserting the Vpu, Vpr and Nef encoding sequences and deleting the BglII-BglII *env* region. The chloramphenicol acetyltransferase (CAT) reporter gene (Parolin et al., 1996) was inserted in the pSVC2.1 Vpr$^+$Vpu$^+$Nef$^+$ Δenv vector with resulting inactivation of the *rev* gene.

pSVIIIenv: encodes the HIV-1 Rev protein along with the Envelope glycoproteins derived from different HIV-1 strains. In particular **pSVIII-HXBc2** carries the encoding sequences of the laboratory-adapted T-cell-tropic HXBc2 Env; **pSVIII-ADA** and **pSVIII-JFRL** carry the encoding sequences of the laboratory-adapted macrophage-tropic ADA and JFR-L Env, respectively; **pSVIII-89.6** carries the encoding sequences of the primary dualtropic 89.6 Env. These HIV-1 isolates can use CXCR4 (Feng et al., 1996), CCR5 (Choe et al.,

1996) or either one (Collman et al., 1992) respectively, as a co-receptor. Kindly provided by J. Sodroski (Harvard University, Boston, USA).

The packaging system (**Figure 6.1**):

pMDLg/pRRE (Addgene #12251): contains the *gag* and *pol* genes as well as the RRE sequence from HIV-1 HXBC2 molecular clone under the control of the CMV promoter and the polyadenylation signal (pA) from the human β-globin gene (Dull et al., 1998).

pMD2.G (Addgene #12259)**:** contains the Vescicular Stomatitis virus glycoprotein (VSV-G) encoding sequence under the control of the CMV promoter and the pA signal from the human β-globin gene. Lentiviral particles, indeed, can be efficiently "pseudotyped" by substituting the HIV-1 glycoprotein with the one derived from the VSV. This substitution allows the lentiviral particle to transduce a wider range of cell types (Dull et al., 1998; Finkelshtein et al., 2013).

pRSV-Rev (Addgene #12253): contains the Rev encoding sequence under the control of the Rous Sarcoma Virus (RSV) promoter and the pA signal from the human β-globin gene (Dull et al., 1998).

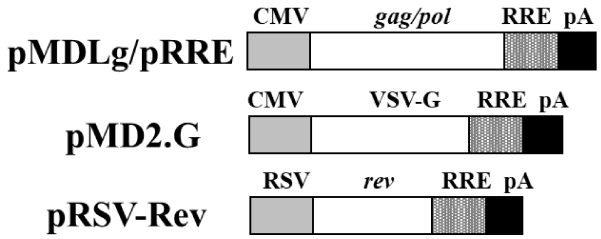

Figure 6.1. The packaging system. From the top: pMDLg/pRRE endoces the *gag* and *pol* genes and the RRE sequence from HIV-1 under the control of the CMV promoter. pMD2.G contains the VSV-G glycoprotein encoding gene under the control of the CMV promoter. pRSV-Rev contains the Rev coding sequence driven by the RSV promoter. pA indicates the polyadenylation signal from the human β-globin gene.

pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev: third-generation, SIN lentiviral vector derived by the pLL3.7 plasmid (Rubinson et al., 2003). This vector encodes for two short hairpin RNAs (shRNA) against the cellular co-receptor CCR5 (i.e. shCCR5) and the viral gene *vif* (i.e. shVif) and a long hairpin RNA (lhRNA), which generates two siRNA against

the first common exon of the genes *tat/rev* (i.e. lhTat/Rev). These siRNA are under the control of three independent Polymerase III human promoters, respectively: the human U6 small nuclear RNA promoter (U6), the 7SK small nuclear RNA promoter (7SK) and the human RNase P RNA H1 promoter (H1). The transgene cassette was subcloned into the pLL3.7 plasmid between the *XbaI* and *XhoI* sites, in place of the murine U6 promoter. (**Figure 6.2A**).

pLL3.7 H1e-shRNA: third-generation, SIN lentiviral vector derived by the pLL3.7 plasmid. This vector encodes a single hairpin encompassing 64 bp in the stem under the control of the Polymerase III promoter, H1. The e-shRNA gives rise to three distinct siRNAs targeting the CCR5, the *tat/rev* common transcript and the *vif* transcript In order to attenuate the innate immune response to long dsRNAs and to facilitate the propagation of this plasmid in *E. coli*, G:U wobble parings were included at regular intervals in the sense strand of the lhtat/rev (Saayman et al., 2008). The transgene cassette was subcloned into pLL3.7 between the *XbaI* and *XhoI* sites, in place of the murine U6 promoter. (**Figure 6.2B**).

pLL3.7 H1*scrambled*CCR5 (H1scrCCR5): third-generation, SIN lentiviral vector, derived from the pLL3.7 plasmid. This vector carries a sequence expressing a shRNA, under the control of either the H1 promoter, in place of the murine U6 promoter. The shRNA generates a siRNA that is three nucleotides different from the one produced by the shCCR5 (5′-GAGCAAGCTCTCGTTACACC-3′), preventing, in this way, an efficient pairing with the CCR5 sequence. This vector is used as control (Liang et al., 2010). The transgene cassette was subcloned into pLL3.7 between the *XbaI* and *XhoI* sites, in place of the murine U6 promoter (**Figure 6.2C**).

The following lentiviral vectors (i.e. T385, M809 and T392) were kindly provided by D. von Laer's laboratory (Innsbruck Medical University) (**Figure 6.2D**):

T385: third-generation, SIN lentiviral vector, that carries a gene conferring the resistance to the antibiotic Ampicillin, the SV40 origin of replication, the hybrid 5'-LTR in which the U3 region is replaced with the Rous Sarcoma Virus (RSV) promoter and enhancer sequences and a self-inactivating 3'-LTR. Moreover, this vector carries the membrane-anchored form of C-peptide (maC46) encoding sequence fused with the eGFP reporter

gene, driven by the human Elongation Factor 1 (EF1) Polymerase II promoter and an optimized version of the WPRE (WPRE*) between the *NotI-HindIII* restriction sites.

M809: third-generation, SIN lentiviral vector, which backbone is identical to the one reported for the T385 vector and it carries the maC46 gene driven by the EF1 promoter.

T392: third-generation, SIN lentiviral vector, which backbone is identical to the one reported for the T385 and M809 vectors and it carries the eGFP reporter gene, driven by the EF1 promoter.

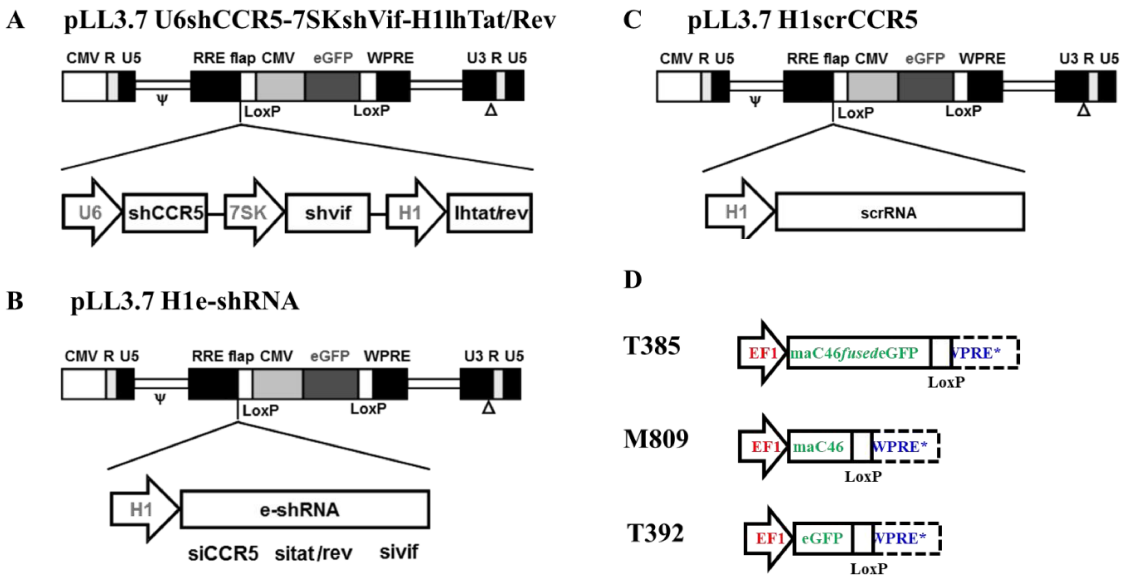

Figure 6.2. Schematic diagram of the combinatorial vectors and the maC46 cassettes. A. The triple vector contains one shRNA against the cellular CCR5 gene under the control of Polymerase III U6 promoter, one shRNA against the viral *vif* gene under the control of Polymerase III 7SK promoter, one lhRNA generating two siRNAs against the first common exon of the genes *tat/rev*, under the control of Polymerase III H1 promoter. B. The H1e-shRNA vector encodes for a single hairpin under the control of Polymerase III H1 promoter. This e-shRNA gives rise to three distinct siRNAs targeting the CCR5, the *tat/rev* and the *vif* transcripts. C. The *scrambled* vector generates a siRNA that is three nucleotides different from the one produced by the shCCR5, thus preventing the pairing. It's used as control. D. maC46 cassettes: T385 carries the maC46 gene fused with eGFP; M809 carries the maC46 peptide alone; T392 carries the eGFP. The expression of these genes is under the control of human Polymerase II EF1 promoter. The optimized version of WPRE (WPRE*) enhances the expression of the genes with a reduced risk of carcinogenesis.

Optimized vectors:

The following vectors were obtained by subcloning either the EF1maC46*fused*eGFP or EF1maC46 or EF1eGFP into the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev and the pLL3.7 H1e-shRNA lentiviral vectors, in place of the CMVeGFP region. Moreover, these new recombinant vectors carry an optimized version of WPRE (WPRE*), able to reduce the risk of oncogenesis and ensure an increased level of biosafety of the platform (**Figure 6.3**).

pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP and **pLL3.7 H1e-shRNA EF1maC46*fused*eGFP:** lentiviral vectors obtained by subcloning the *NotI-HindIII* EF1maC46*fused*eGFP region into either the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev or the pLL3.7 H1e-shRNA vector.

pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and **pLL3.7 H1e-shRNA EF1maC46:** lentiviral vectors obtained by subcloning the *NotI-HindIII* EF1maC46 region into either the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev or the pLL3.7 H1e-shRNA vector.

pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP and **pLL3.7 H1e-shRNA EF1eGFP:** lentiviral vectors obtained by subcloning the *ClaI-HindIII* EF1eGFP region into either the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev or the pLL3.7 H1e-shRNA vector.

pLL3.7 H1scrCCR5 EF1maC46*fused*eGFP, pLL3.7 H1scrCCR5 EF1maC46 and **pLL3.7 H1scrCCR5 EF1eGFP:** lentiviral vectors obtained by replacing the *XbaI-XhoI* H1e-shRNA region into pLL3.7 H1e-shRNA EF1maC46*fused*eGFP, H1e-shRNA EF1maC46 and pLL3.7 H1e-shRNA EF1eGFP, respectively.

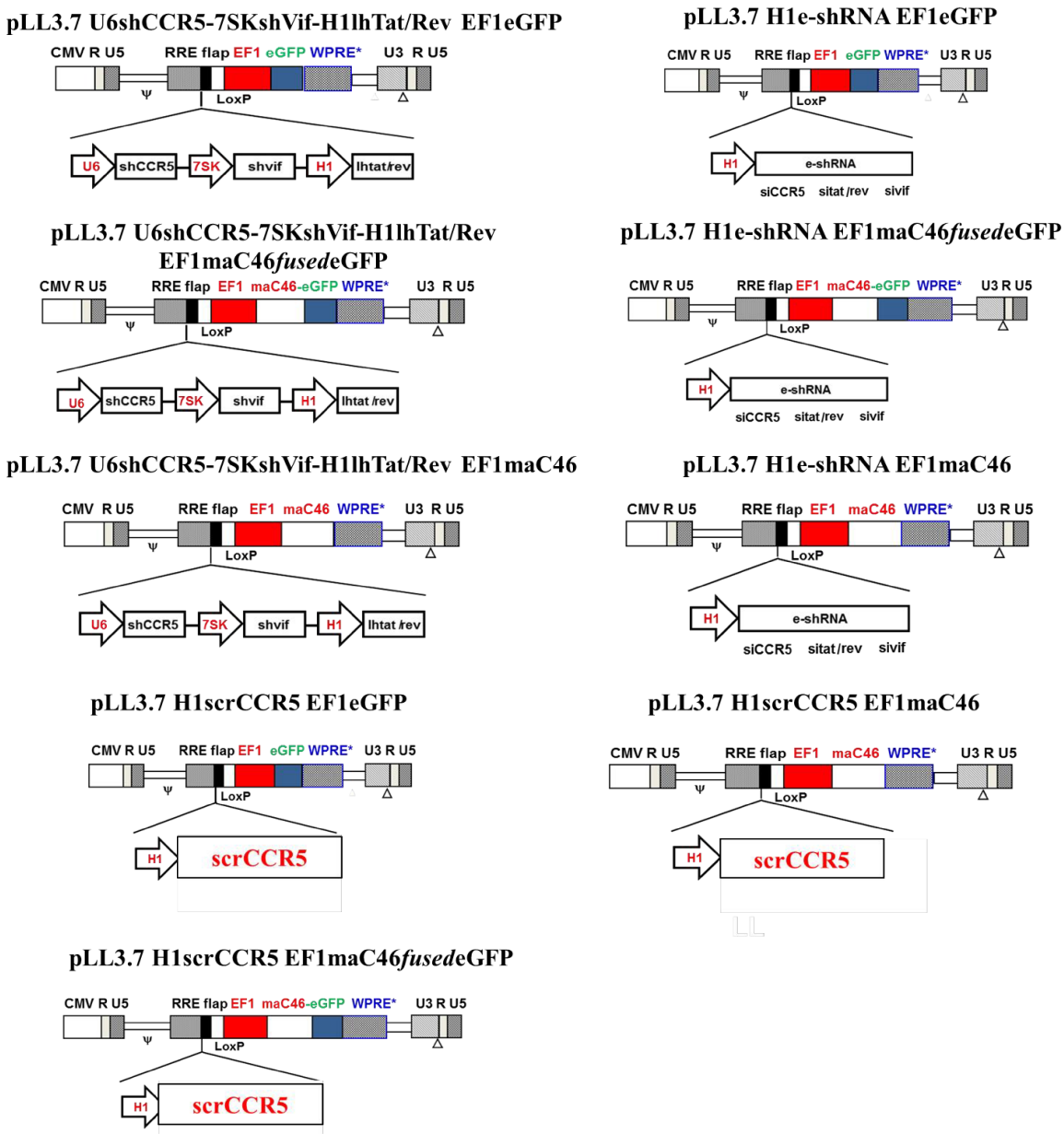

Figure 6.3. Schematic diagram of the optimized plasmid vectors. Self-inactivating, third-generation, lentiviral vectors, derived by either the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev or the pLL3.7 H1eshRNA vectors. The maC46 fusion inhibitor was inserted either fused with eGFP reporter gene or alone, under the control of the EF1 promoter. Moreover, controls vectors were made by inserting eGFP under the control of the EF1 promoter. Scrambled vectors were obtained by inserting the H1scrCCR5 region in place of the H1e-shRNA region into either pLL3.7 H1e-shRNA EF1maC46*fused*eGFP, pLL3.7 H1e-shRNA EF1maC46 or pLL3.7 H1e-shRNA EF1eGFP.

These plasmid vectors were amplified in competent *E. coli* cells by means of bacterial transformation and the positively selected colonies were grown in 3 mL of Luria-Bertani medium under Ampicillin selection (100 µg/mL) for 16 hours at 37°C, with continuous shaking at 118 rpm. Large-scale preparation of plasmid DNA was obtained by using the *QIAprep Maxiprep Kit* (QIAGEN), from 200 mL of transformed *E. coli* culture.

6.4 Production and titration of recombinant lentiviral particles

In order to obtain a suitable high titer for the following experiments, conditions of recombinant lentiviral particles (RLVPs) production by calcium phosphate were optimized. Briefly, cells were seeded at 12×10^6 per T150 tissue culture flask 24 hours before transfection, and, once the 80% confluence was reached, cells were co-transfected with 20 µg of the appropriate gene transfer vector, 10 µg of pMDLg/pRRE, 10 µg of pMD2. G and 10 µg of pRSV-Rev, previously diluited in TE 1:10 (TE: Tris-HCl 1 mM pH 8, EDTA 0.5 mM pH 8) up to a final volume of 1800 µL. Subsequently, 200 µL of 2.5 M $CaCl_2$ was added to the DNA and then 2 mL of 2X HPB buffer pH 7.1 (5M NaCl, 0.5 M HEPES pH 7.1, 0.15 M Na_2HPO4 $7H_2O$) was added dropwise into the precipitate. Finally, the mixture was added dropwise to the cell culture, that was incubated 3 to 4 hours at 37°C. Then, cells were gently washed twice with 10 mL of phosphate buffer saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO4, 1.8 mM KH_2PO4, pH 7.4) and fresh growth medium was replaced. The culture supernatants were collected on day 2 post-transfection, filtered with a 0.45-µm-pore-size membrane (Millipore), concentrated 100 times by ultracentrifugation (27000 rpm, 2 hours, 4°C in a Beckman SW28 rotor) and stored at -80° C until use.

The infectious titer of RLVPs that carried the reporter gene was determined by flow cytometry analysis of the eGFP expression, after transduction of HEK 293T cells with the RLVPs. Briefly, 1×10^5 cells were seeded per well in 12-well plates in 1 mL of DMEM 10% FBS, 24 hours prior to transduction. The next day, medium was replaced with 0.5 mL of DMEM 10% FBS containing serial dilutions of the lentiviral stocks. 0.5 mL of fresh culture medium was added to the cells approximately 8 hours later. Three days after transduction, cells were detached from the tissue culture dish and transferred into 15 mL Falcon tubes, washed twice with PBS and by centrifugation at 1200 rpm at 4°C for 7 minutes and resuspended in 500 µL of cold PBS to evaluate the eGFP expression by flow cytometry. Samples were acquired on FACSCalibur (Berton Dickinson) and the data analysis was performed with CellQuest (Berton Dickinson).

The infectious titer was expressed as transducing units/mL (TU/mL) and was calculated according to the following formula:

$$\frac{\left(\frac{\%\ \text{eGFP positive cells}}{100}\times \text{transduced cell number}\times \text{dilution factor}\right.}{\text{lentiviral particle dilution valume (mL)}}$$

When required, RLVPs titers were also determined using INNOTEST HIV Antigen mAb (Fujirebio), according to manufacturer's instructions.

6.5 Viruses and virus production

Recombinant HIV-1 CAT viruses were produced by co-transfection of 293T cells with the pSVC2.1 $Vpr^{+}Vpu^{+}Nef^{+}$ Δenv CAT plasmid along with the pSVIIIenv plasmids, or with the pMD2.G plasmind and the pRSV-Rev plasmid, with the calcium phosphate method. Different.

Calcium phosphate transfection was carried out as following: 24 hours before transfection, 2.5×10^{6} cells were seeded on a $10cm^{2}$ Petri dish, in 10 mL of DMEM medium, supplemented with 10% heat-inactivated FBS. The day after, cells were co-transfected with 20 µg of pSVC2.1 $Vpr^{+}Vpu^{+}Nef^{+}$ Δenv CAT and 5 µg of pSVIIIenv plasmids expressing the HIV-1 HXBc2, JRF-L, ADA, or 89.6 Env or 20 µg of pSVC2.1 $Vpr^{+}Vpu^{+}Nef^{+}$ Δenv CAT, 5 µg of pMD2.G and 1 µg of pRSV-Rev. The DNA was diluted in TE 1:10 up to a final volume of 450 µL. Then, 50 µL of 2.5 M $CaCl_2$ and 500 µL of 2X HPB buffer pH 7.1 (5M NaCl, 0.5 M HEPES pH 7.1, 0.15 M $Na_2HPO4 \cdot 7H_2O$) were added. The transfection mixture was incubated for 30 minutes at room temperature and then added to the cell culture. The culture medium was changed 6 h later. The culture supernatants were collected 48 hours after transfection, passed through a 0.45-µm-pore-size filter (Millipore), and the viral titer was measured by the RT activity assay, as previously described (Rho et al., 1981).

HIV-1 HXBC2 $Vpr^{+}Vpu^{+}Nef^{+}$ stock was produced by transient transfection of 5×10^{6} T lymphoblastoid Jurkat cells with 10 µg of pSVC21 $Vpr^{+}Vpu^{+}Nef^{+}$ plasmid by the DEAE-dextran method (Smale, 2010). Cells supernatants were harvested at approximately 48 hours post-transfection and filtered (0.45-µm-pore-size). Viral titer was determined as 50% Tissue Culture Infective Doses ($TCID_{50}$)/mL on C8166 cells by Reed and Muench end point dilution method (Reed and Muench, 1938) and by measuring the RT activity.

HIV-1 NL4-3-ADA stocks were produced by calcium phosphate transfection of 2.5×10^{6} 293T cells with 15 µg of the infectious proviral plasmid pNL4-3-ADA. The virus was collected from the culture supernatants on day 2 post-transfection, filtered (0.45-µm-pore-size) and stored at −80°C. The HIV-1 NL4-3-ADA viral titer was determined by the RT assay.

6.6 Reverse Transcriptase (RT) activity assay

The RT activity was measured as previously described (Rho et al., 1981). Briefly, viral particles were precipitated from 500 µL of the filtered culture supernatants (or from 500 µL of 1:20 dilution of concentrated RLVPs) by centrifugation at 13000 rpm for 2 hours at 4°C. The precipitate was resuspended in 10 µL of a Suspension buffer (containing 50 mM Tris-HCl pH 7.5, 1 mM dithiothreitol (DTT), 20% glycerol, 250 mM KCl and 0.25% Triton X-100), transferred in dry ice and lysed through three cycles of freezing and thawing. The sample was added to a reaction mixture containing 10 µL of 5X RT assay buffer (250 mM Tris-HCl pH 7.5, 37.5 mM $MgCl_2$, 0.25% Triton X-100), 1.2 µL of 200 mM DTT, 5 µL of 100 µg/mL oligo-dT-polyA (Roche), 1 µL of 84 Ci/mmol ^{3}H-dTTP (Perkin Elmer) and water in a final volume of 50 µL. The reaction was incubated for 1 hour at 37°C and transferred on Whatman filters. Filters were immediately washed three times in Saline Sodium Citrate (SSC) buffer (0.3 M NaCl, 0.03 M sodium citrate pH 7.2) for 10 minutes each, twice in absolute ethanol for 10 seconds each and, then, dried. Then, filters were soaked into 4 mL of liquid scintillation cocktail (ULTIMA Gold, Perking Elmer). The radioactivity was measures by using a scintillator (TRI-carb 2810 TR, Perkin Elmer) and expressed in counts per minute/500 µL (cpm/500 µL) or counts per minute/25 µL (cpm/25 µL), for concentrated RLVPs. Each experiment was performed in duplicate for each sample and the average value was calculated.

6.7 MTT cell viability assay

Compound cytotoxicity was screened in Jurkat or PM1 cells, incubated at 37°C in the absence or presence of different concentrations of tested compounds. Three days after exposure, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay was performed, according to the manufacturer's instructions (Roche). RLVPs cytotoxicity was tested transducing 1×10^6 Jurkat cells with 700000 cpm. Three days after transduction, 5×10^4 transduced cells were plated in 96-well plate in 100 µL of RPMI 10% FBS. Then, MTT assay was performed, according to the manufacturer instructions (Roche).
Optical density was measured at 620 nm and the value obtained for control cells (untreated cells) was set to 100%. Relative cell viability for the other samples was calculated accordingly. Each concentration of the compounds was tested eight times in a single experiment.

6.8 HIV-1 env complementation assay

Jurkat cells (1×10^6) were incubated with 30000 cpm of recombinant HIV-1 HXBc2 CAT virus at 37°C in the absence or presence of different amounts of compounds. Similarly, PM1 cells (1×10^6) were incubated with 30000 cpm of recombinant HIV-1 HXBc2 CAT, JRF-L CAT, ADA CAT, 89.6 CAT or VSV-G CAT viruses, at 37°C in the absence or presence of different concentration of Oximacro®. In addition, to test whether Oximacro® could perturb the functionality of HIV-1 Envelope, viral preparation was pre-incubated with Oximacro® for 2 hours or 1 hour without target cells prior to assaying its infectivity. Cells were lysed 72 h after infection in 150 µL of Tris-HCl 250 mM pH 7.4 and protein concentration in the lysates was determined with the Micro BCA™ Protein Assay Kit (Thermo Scientific) using BSA as standard, according to the munufacturer's instruction. Equivalent amounts of proteins were used for determination of CAT activity as previously described (Sodroski et al., 1984). Briefly, the cell lysates were incubated with 5 µL of 5 mg/mL Acetyl CoA and 0.5 µL of 57 mCi/mmol D-Threo-[Dichloroacetyl-1,2-14C]-Chloramphenicol (Perkin Elmer) and 250 mM Tris HCl, pH 7.4 in a final volume of 110 µL, at 37°C for 1 hour. Then, the acetylated products and the unmodified reactants were separated from the aqueous solution by organic extraction with ethyl acetate. The different forms of acetylated chloramphenicol were separated by thin layer chromatography (TLC) and visualized with an autoradiographic exposure of 12 h (Kodak Biomax films). The quantitative evaluation was obtained by cutting the TLC paper at the level of the corresponding spots, and by performing a quantification of the spots at the scintillation. The percentage of conversion in the acetylated forms was calculated as follows:

$$\%\ \text{of conversion} = \frac{\text{mono} - + \text{diacetylated forms}}{\text{non acetylated} + \text{mono} - + \text{di} - \text{acetylated forms}}$$

Calculated with the above formula, the percentage of conversion is linear for values up to 50%.

6.9 HIV-1 *de novo* infection

PM1 cells (1×10^6) were infected with 30,000 cpm of HIV-1 HXBc2 $Vpu^+Vpr^+Nef^+$ virus in the absence or presence of 10 µg/mL of Oximacro®. After one hour of incubation at 37°C, the cultures were washed three times and cultured in RPMI medium, supplemented with 10% heat-inactivated FBS. Moreover, viral preparation was pre-incubated with

Oximacro® for 2 hours without target cells prior to assaying its infectivity. When required, Oximacro® was continuously added to the cell culture, to a final concentration of 10 μg/mL. Virus replication was monitored by RT activity in cell-free culture supernatants at different days post-infection.

6.10 Immunofluorescence cell staining

To ascertain the correct expression of the maC46 fusion inhibitor, immunofluorescence cell staining on 293T cells transfected with the developed vectors was performed. Briefly, 24 hours before transfection, 293T cells were seeded in 6-well plate, previously covered by a glass coverslip treated with a solution of Poly-L-Lysine (0.1 mg/mL, Sigma), at a density of 3.5×10^5 cells per well (to achieve a 90-95% confluence at the time of transfection). The next day, cells were transfected with 10 μL of Lipofectamine 2000 (1 mg/mL Invitrogen, Life Technologies), diluted in 250 μL of DMEM Serum free, and 4 μg of DNA, diluted in 250 μL of DMEM Serum free, and incubated for 20 minutes at room temperature. The medium was replaced 6 hours after transfection in order to reduce the cytotoxic effect of the liposomes. 24 hours after transfection, cells were washed with room temperature-PBS and fixed in a solution of 4% Paraformaldehyde (PFA) in PBS for 10 minutes at room temperature. After two washing in PBS, cells were first incubated for 30 minutes at room temperature with a solution of 0.5% Bovine Serum Albumine (BSA) in PBS and subsequently for 2 hours at 4°C with 40 μL of the phycoerythrin-labeled recombinant human monoclonal antibody to HIV-1 gp41 epitope ELDKWA (2F5-PE, Polymun Scientific), diluted either 1:50 or 1:100. After a washing in PBS, cells were incubated for ten minutes at room temperature with 40 μL of nuclear fluorescent staining DRAQ5 (5 mM, Life Technologies), diluted 1:1000 in MQ water, followed by another wash in PBS. To preserve fluorescence, 8 μL of Mounting Medium (VECTASHIELD H-1000, Vector Laboratories) were dispensed on the glass slide before applying the coverslip.

6.11 HIV-1 infection

For transduction of Jurkat cells to be used in the following challenge experiments, 1×10^6 cells were incubated in 12-well plates with equivalent RT units of lentiviral supernatant (700000 or 35000 cpm) in a total volume of 1 mL. After three days of culture, the transduction efficiency was ascertained, when possible, by FACS analysis on the basis of

eGFP expression and cells were then used for HIV-1 challenge. Jurkat cells were prepared for FACS analysis using the same protocol employed for transduced 293T cells.
Then, Jurkat cells were infected with HXBc2 $Vpr^{+}Vpu^{+}Nef^{+}$ at a MOI of 0.1 $TCID_{50}$/cell or 0.01 $TCID_{50}$/cell,in a total volume of 300 µL. After 1 hour of incubation at 37°C, the cultures were washed three times and cultured in RPMI with 10% FBS medium. Virus replication was monitored by RT activity in cell-free culture supernatants at different days post-infection.

6.12 Quantification of maC46 transcript expression in transduced cells

T lymphoblastoid Jurkat cells were seeded in a 12-well plate at the density of 1×10^{6} cells/well and were transduced with equivalents RT units of lentiviral supernatants (35000 cpm) in RPMI 10% FBS. Total RNA was isolated after 3, 15 and 21 days post transduction from cells using RNeasy-kit (Qiagen, Netherlands) according to the manufacturer's instructions. Total RNA underwent contaminant DNA digestion using DNase I recombinant, RNase-free kit (Roche), according to the manufacturer's instructions. maC46 mRNA levels were quantified relative to transcript levels of the GAPDH housekeeping gene by qPCR. For this purpose, qPCR was performed with AgPath-ID™ One-Step RT-PCR kit (Life Technologies) according to the manufacturer's instructions and the ABI Prism®7900 Sequence Detection System (Applied Biosystems) instrument. Taqman primer and probe sequences for this assay are as follows: C46 forward primer: 5′ CAC AGC CTG ATC GAG GAG AG; C46 reverse primer: 3′ GTC CTG CCA CTG GTG GTG; C46 probe: CAC TCC ACG CAG CAC TTC CGC TCG (5′ 6-FAM; 3′ MGB) (Wolstein et al., 2014). GAPDH forward primer 5′-CCA CTC CTC CAC CTT TGA CG-3′, GAPDH reverse primer 5′-CAT GAG GTC CAC CAC CCT GT-3′, GAPDH probe: 5′-TTG CCC TCA ACG ACC ACT TT-3′ (5′ TET; 3′ TAMRA) (Barbaro et al., 2016). The data was analyzed with the second derivative maximum method and relative expression ratios were calculated by the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), with ΔC_T representing [C_T(maC46) - C_T(GAPDH reference]. $\Delta\Delta C_T$ signifies ΔC_T lentivirus-exposed samples minus ΔC_T of background samples (untransduced cells).

6.13 *In vitro* immortalization (IVIM) assay

To assess the mutagenic potential of the developed SIN lentiviral platform, the IVIM assay was performed (Modlich et al., 2009), in collaboration with C. Baum' s group at the Department of Experimental Hematology of the Hannover Medical School. Only the LVs lacking the eGFP have been selected (U6shCCR5-7SKshVif-H1lhTat/RevEF1maC46 and H1e-shRNAEF1maC46 referred to as U6-EFS-C46 and H1-EFS-C46, respectively) to be tested along with the scrambled vector (H1scrCCR5 referred to as H1-scrambled). A schematic illustration of this assay is shown in the **Figure 6.4**.

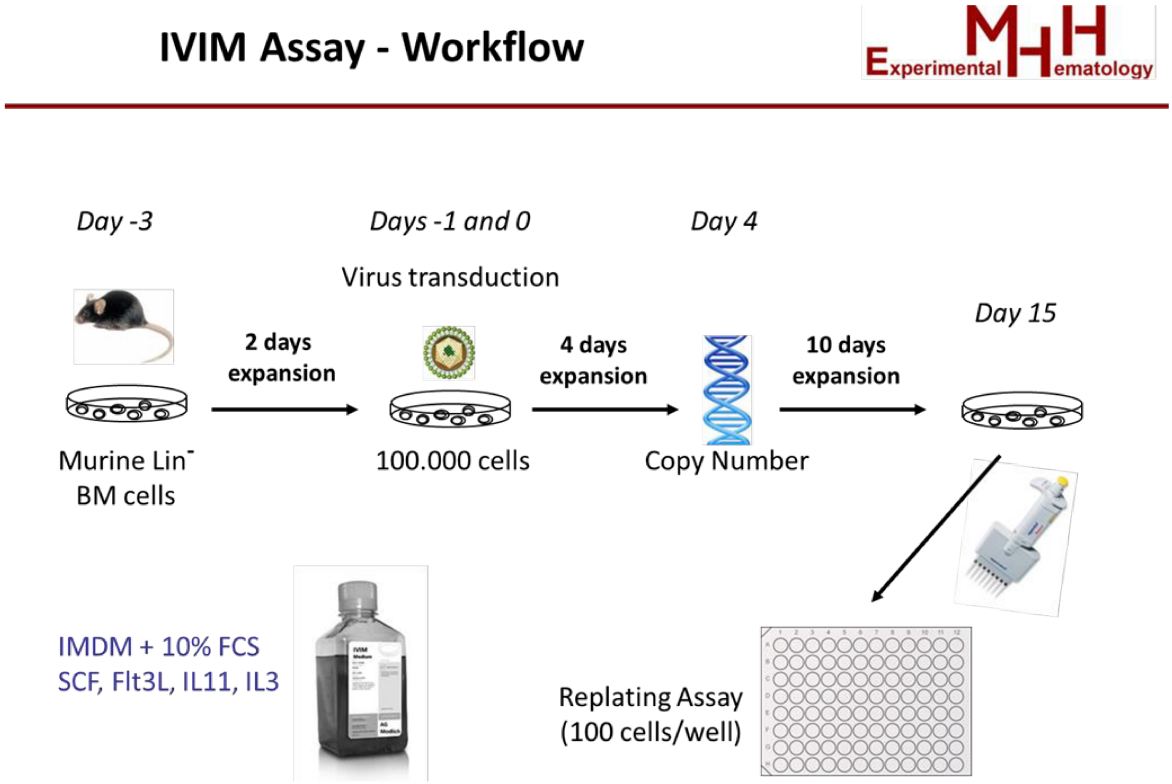

Figure 6.4. Workflow of the IVIM-assay. Lineage-negative (Lin-) bone marrow (BM) cells of untreated C57B16/J mice were isolated, pre-stimulated for 2 days before retroviral transduction, using a protocol that allows efficient and dose-controlled retroviral gene transfer, cytokine-supplemented expansion cultures. Two rounds of transduction were performed (on days -1 and 0) at a defined M.O.I. and the transgene expression was monitored by flow cytometry (on day 4 and 15). The mean vector copy number per cell (VCN) was determined on day 4. The cells were grown in cytokine-supplemented media for another 2 weeks, before being replated in 96-well plates (on day 15) at a very low density of 100 cells/well. Under these conditions, mock cells usually cannot proliferate anymore, apart from cases of spontaneous cell growth. Two weeks later the positive wells were counted and the frequency of replating cells was calculated. The ratio of replating frequency (determined by limiting dilution cloning) per VCN (detected by real-time PCR) 4 days after transduction, is a measure of the degree of transformation.

To examine whether the potential insertional activation of cellular proto-oncogenes represented the driving force of the enhanced fitness detected in the replating assay, primary murine hematopoietic cells (Lin-) were transduced at a cumulative (c) M.O.I. of 60 and 140 and expanded for two weeks under particular myeloid culture conditions, before being replaced on 96-well plates (100 cells/well). A mock infected sample cultured without a viral vector served as a measure of spontaneous immortalization and routinely scored negative. Test vectors were compared to a positive control vector shown previously to induce *in vitro* immortalization by insertional mutagenesis, pRSF91.GFPgPRE (RSF91), a gammaretroviral vector, with internal long terminal repeat – contained spleen focus forming virus promoter sequences. Under these conditions of very low cell concentrations, mock-treated cells hardly survived, whereas insertional mutants could still grow. Thus, after additional two weeks of cell culture, the positive wells were counted to quantify the incidence of cell transformation as replating frequency (RF, the number of positive wells on a plate as a measurement of clonal fitness) per vector copy number (VCN). As an example, in case of 17 positive wells (100 cells seeded), the RF based on Poisson distribution is 1.95×10^{-3}. Thus, the VCN normalization will be:

$$\frac{1.95 \times 10^{-3}}{3} = 6.5 \times 10^{-4}$$

7. RESULTS

7.1 Natural extracts and purified bioactive molecules in the chemotherapy of AIDS

7.1.1 Background

Despite the large and effective arsenal available to fight HIV-1, resistant variants of the virus eventually evolve during therapy; moreover, some antiretroviral drugs can exhibit long term toxicity. Thus, it is essential to identify additional new inhibitors and alternative strategies with low toxicity and broad-range activity against diverse HIV-1 strains, for future success in treating HIV-1 infection.

A variety of natural products have emerged as promising source of new therapeutic agents for the developing of complementary and alternative medicines to conventional drug regimes. Oximacro®, a cranberry extract produced by Biosfered (Turin, Italy), possesses a high content of proanthocyanidins (PACs), well known for their antimicrobial and antiviral properties (Shmuely et al., 2012). Recently, Oximacro® and the Oximacro®-derived purified fraction 4 (the richest in PACs), resulted effective in preventing urinary tract infections (Occhipinti et al., 2016) and active against HSV-1 and HSV-2 infection *in vitro* as well as human Influenza virus type A and type B infection *in vitro*, by targeting viral attachment to the cells (Luganini et al., 2018; Terlizzi et al., 2016). Given these interesting results, we investigated whether Oximacro® could be effective also against HIV-1 infection. Indeed, the identification of natural antivirals with a broad-spectrum of activity could represent a promising starting point for a reliable co-therapy for HIV-1-positive individuals as well as a potent tool in the prevention of sexually transmitted diseases. The experiments described here were performed with the Oximacro®-derived purified fraction 4, named Oximacro® in the rest of this report.

Finally, in collaboration with E. Tramontano (University of Cagliari), our group demonstrated the properties of Lupeol, a triterpene derived from *Hemidesmus indicus* decoction, to inhibit RT-associated RNAse H functions and α-glucosidase activity in biochemical assays (Esposito et al., 2017). Moreover, with the same collaborators, we tested some purified molecules from *Onopordum illyricum* L., a Mediterranean plant, identifying a few compounds effective on RT-associated RNase H function and IN

function in biochemical assays and, among them, one (1,5-Dicaffeoylquinic acid) was able to inhibit the early stages of HIV-1 replication in cell-based assays (Sanna et al., 2018).

7.1.1 Antiviral activity of Oximacro® on the early phases of HIV-1 infection in a single cycle of replication

First, Oximacro® cytotoxicity was evaluated in T lymphoblastoid Jurkat cells, which are highly permissive for T-tropic HIV-1 replication, and the calculated cytotoxic concentration 50 (CC_{50}) was of 19.34 ± 0.97 µg/mL. Next, with the aim of identifying the antiviral potency of the compound, the effect on the early phases of HIV-1 infection was evaluated. To this end, the trans-complementation assay was used, in order to assess the replicative potential of HIV-1 in a single round of infection, using Jurkat cells as a target (Helseth et al., 1990; Parolin et al., 2003). In this assay, an env-defective HIV-1 HXBc2 $Vpr^{+}Vpu^{+}Nef^{+}$ Δenv provirus encoding the bacterial CAT gene was complemented by the Env glycoprotein from the HXBc2 laboratory-adapted T-tropic virus. The level of CAT expression in the cell lysates allowed us to examine the early events in the infection process (**Figure 7.1**). Results showed that Oximacro® was able to inhibit HIV-1 replication in a dose-dependent manner, with an effective concentration 50 (EC_{50}), i.e. the concentration of the compound required to reduce the viral infectivity by 50%, of 11.20 ± 0.35 µg/mL.

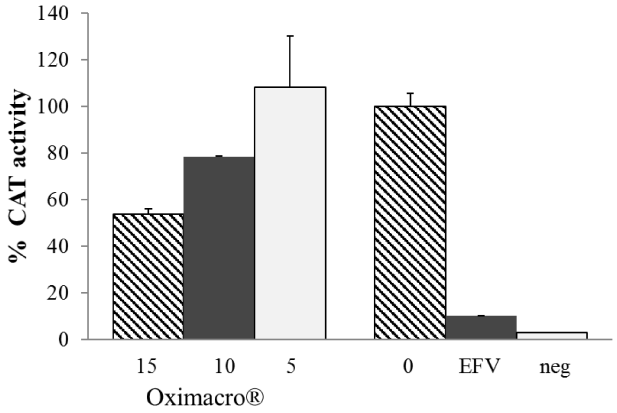

Figure 7.1. Effects of Oximacro® on HIV-1 replication. CAT activity was determined in T lymphoblastoid Jurkat cells infected with HIV-1 recombinant CAT virus pseudotyped with the Env glycoprotein from the HXBc2 laboratory-adapted T-tropic virus, in the absence (0) or presence of 15 µg/mL, 10 µg/mL and 5 µg/mL of the compound, 72 hours post infection. The results are reported as percent of conversion of $^{[14C]}$chloramphenicol to acetylated forms relative to the positive control neg. negative control; EFV, Efavirenz. The means of three independent experiments and standard deviations are presented.

It has been reported that proanthocyanidins are able to interfere with the early phases of the HIV-1 replicative cycle and/or with viral particles directly, in particular through the inhibition of virus attachment to the target cells (Fink et al., 2009; Nance et al., 2009; Neurath et al., 2005).

Similarly, in this work, to understand whether Oximacro® could perturb the functionality of HIV-1 Env, viral preparation was pre-incubated with Oximacro® for 2 hours or 1 hour without target cells prior to assaying its infectivity (**Figure 7.2**). To this end, 1×10^6 Jurkat cells were infected with 30000 cpm of HIV-1 recombinant CAT virus pseudotyped with the Env from the HXBc2 laboratory-adapted T-tropic virus in the absence (0) or presence of 10 μg/mL of the compound. CAT activity was determined in cell lysates 72 hours post infection. Results showed that HIV-1 pre-incubation with Oximacro® greatly reduced the viral infectivity, possibly due to interactions between the compound and the viral envelope glycoproteins.

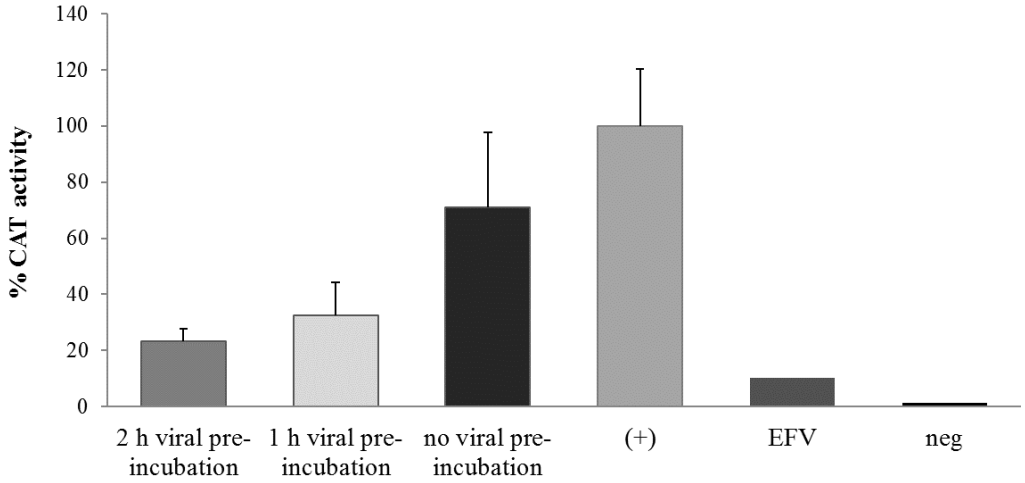

Figure 7.2. Effects of pre-incubation of virus preparations with Oximacro® on the early phase of HIV-1 replication. Inhibition of HIV-1 infection by Oximacro® was enhanced by pre-incubation of virus preparations with the compound, compared to inhibition of infection without virus pre-incubation. HIV-1 recombinant CAT virus pseudotyped with the envelope glycoproteins from the HXBc2 laboratory-adapted T-tropic virus was pre-incubated with 10 μg/mL Oximacro® for 2 hours or 1 hour without target cells prior to assaying its infectivity. CAT activity was determined in treated and untreated Jurkat cells 72 hours post infection. The results are reported as percent of conversion of $^{[14C]}$chloramphenicol to acetylated forms relative to the positive control (+). neg, negative control; EFV, Efavirenz. The means of two independent experiments and standard deviations are presented.

7.1.2 The antiviral activity of Oximacro® is independent from the viral tropism

Next, we investigated the influence of viral envelope proteins on the antiviral activity of Oximacro® by comparing inhibitory effects of the compound on infection by HIV-1 particles pseudotyped with different envelope proteins. For this purpose, we selected the PM1 cell line, which is highly susceptible to infection with both macrophage-tropic and T-tropic viruses (Lusso et al., 1995). Thus, the trans-complementation assay was used, in order to assess the replicative potential of HIV-1 in a single round of infection. The env-defective HIV-1 HXBc2 $Vpr^{+}Vpu^{+}Nef^{+}$ Δenv provirus encoding the bacterial CAT gene was complemented by different envelope glycoproteins: from the HXBc2 laboratory-adapted T-tropic virus, from the JRF-L and ADA macrophage-tropic viruses, from the 89.6 primary dualtropic virus and from the Vescicular Stomatitis Virus (VSV).

First, we tested whether Oximacro® was able to inhibit viral replication in a dose-dependent manner, and whether viral pre-incubation with the compound could alter significantly viral infectivity (**Figure 7.3**). Indeed, results showed that Oximacro® inhibits viral replication with an EC_{50} of 10.22 ± 0.04 μg/mL, with no viral pre-incubation, while an EC_{50} of 2.11 ± 0.07 μg/mL was calculated with the viral pre-treatment, with a CC_{50} value of roughly 43.12 μg/mL.

Next, we adopted the same experimental procedure, using recombinant viruses pseudotyped with different envelope glycoproteins, pre-incubated with 10 μg/mL of Oximacro® (**Figure 7.4**).

Results showed that pre-incubation with Oximacro® similarly affects the infectivity of virus particles with different HIV-1 Env proteins, i.e. X4 (HXBc2), R5 (JRF-L and ADA) and dual (89.6). An efficient inhibition of replication was achieved also with no viral pre-incubation. Interestingly, virions carrying the VSV-G envelope protein on their surface were less sensitive to inhibition by Oximacro® than HIV-1 with native envelope proteins, probably due to a higher efficiency of the pseudotyped-virus in infecting target cells. These results demonstrate that Oximacro® inhibits HIV-1 entry by interfering with the function of the envelope proteins independently from their coreceptor tropism.

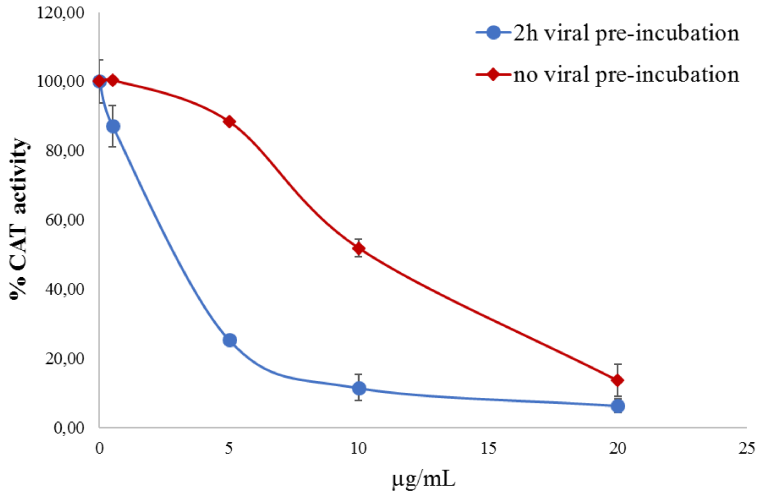

Figure 7.3. Evaluation of antiviral activity of Oximacro® in PM1 cells employing HIV-1 recombinant particles, pseudotyped by HXBc2 Envelope glycoproteins. Inhibition of HIV-1 infection by Oximacro® was enhanced by pre-incubation of virus preparations with the compound (blue line), compared to inhibition of infection without virus pre-incubation (red line). In both cases, CAT activity was determined in PM1 cells infected with HIV-1 recombinant CAT virus pseudotyped with the envelope glycoprotein from the HXBc2 laboratory-adapted T-tropic virus, in presence of different concentration of the compound, 72 hours post infection. The results are reported as relative conversion of $^{[14C]}$chloramphenicol to acetylated forms compared to the positive control (0). The means of two independent experiments and standard deviations are reported.

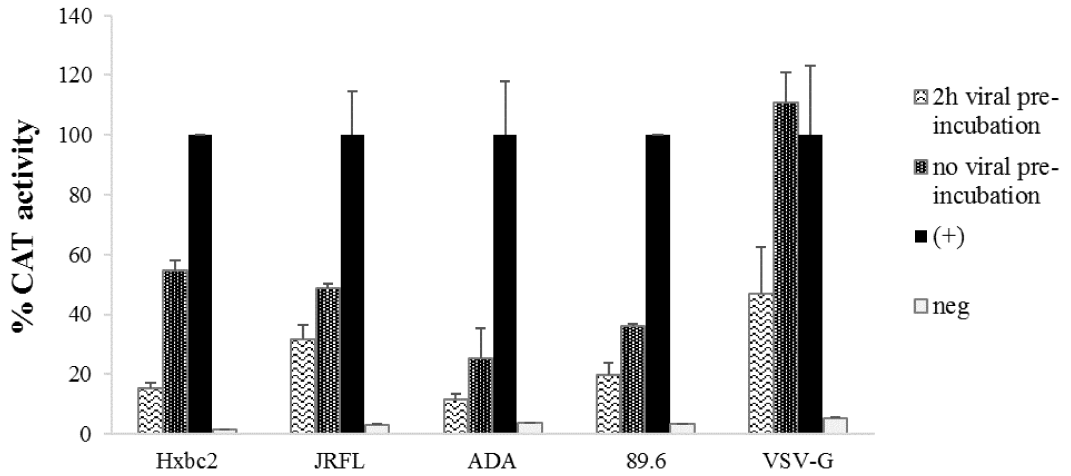

Figure 7.4. Evaluation of antiviral activity of Oximacro® in PM1 cells employing HIV-1 recombinant particles, pseudotyped by different envelope glycoproteins. HIV-1 recombinant CAT virus, pseudotyped with different Envelope glycoproteins (from the HXBc2 laboratory-adapted T-tropic virus, from the JRF-L and ADA macrophage-tropic viruses, from the 89.6 primary dualtropic virus and from the Vescicular Stomatitis Virus), was pre-incubated with 10 µg/mL Oximacro® for 2 hours without target cells prior to assaying its infectivity. CAT activity was determined in treated and untreated PM1 cells 72 hours post infection. The results are reported as percent of conversion of $^{[14C]}$chloramphenicol to acetylated forms relative to the positive control (+). neg, negative control. The means of two independent experiments and standard deviations are presented.

7.1.3 Continuous exposure to Oximacro® inhibits HIV-1 replication in *de novo* infected cells

To determine the efficacy of Oximacro® against acute HIV-1 infection, PM1 cells were infected either with T-tropic HIV-1 HXBc2 $Vpu^{+}Vpr^{+}Nef^{+}$ virus or M-tropic NL4-3-ADA virus, in presence of 10 µg/mL of the compound. After one hour of incubation at 37°C, the cultures were washed and cultured in RPMI medium, supplemented with 10% heat-inactivated FBS. Moreover, viral preparation was pre-incubated with Oximacro® for 2 hours without target cells prior to assaying its infectivity. When required, Oximacro® was continuously added to the cell culture, to a final concentration of 10 µg/mL. Virus replication was monitored by RT activity in cell-free culture supernatants at different days post-infection (**Figure 7.5**).

Results shows that viral pre-incubation and continuous exposure with Oximacro® lead to a higher inhibitory activity on the infection and block viral replication for longer time.

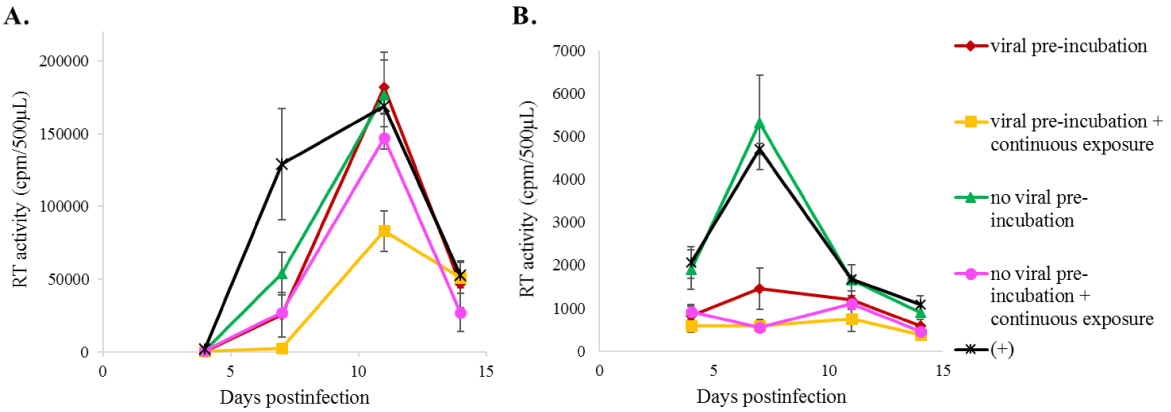

Figure 7.5. Effect of Oximacro® on HIV-1 replication in acutely infected cells.
PM1 cells were infected with the T-tropic HXBc2 Vpu+Vpr+Nef+ (**A.**) or M-tropic NL4-3-ADA (**B.**) viral isolates, following 2 h viral pre-incubation in presence or absence of 10 µg/mL of Oximacro, for 1 h, washed, and maintained in the absence or presence of the compound. Virus replication was monitored by RT production in cell-free supernatants at different days post-infection.

7.1.3 Multi-target activity of *Hemidesmus indicus* decoction against innovative HIV-1 drug targets and characterization of Lupeol mode of action

Among the bioactive compounds purified from *Hemidesmus indicus*, the pentacyclic triterpenes Lupeol and Lupeol acetate have shown to inhibit the RNAse H functions in biochemical assays (Esposito et al., 2017). Once tested in a cell-based system, however, they showed no efficacy against HIV-1 replication. The values of IC_{50}, CC_{50} and EC_{50} are reported in **Table 1**.

E. Tramontano and colleagues (University of Cagliari) tested also the *H. indicus* decoction against HIV-1 RNAse H, RDDP and cellular α-glucosidase, obtaining IC_{50} values of 2.9 ± 0.1 μM, 7.5 ± 2.5 μM and 47 ± 2 μM, respectively. Overall, these results represent a promising starting point for the development of innovative anti-HIV-1 drug candidate with a multitarget mode of action.

Compound	HIV-1 RNAse H [a]IC_{50} (μM)	HIV-1 RDDP [b]IC_{50} (μM)	α-glucosidase [c]IC_{50} (μM)	[d]CC_{50} (μM)	[e]EC_{50} (μM)
Lupeol	11.6 ± 0.5	>100	>100	>50	>20
Lupeol Acetate	63 ± 5	>100	>100	>50	>20
RDS1643	8.1 ± 2.2	-	-		
Efavirenz	-	0.025 ± 0.005	-		
Luteolin	-	-	13.2 ± 0.2		

Table 1. Lupeol – Lupeol Acetate exert an antiviral activity against HIV-1 RNase H.
[a]Compound concentration required to reduce HIV-1 RT-associated RNase H activity by 50%.
[b]Compound concentration required to reduce HIV-1 RNA-dependent RNA Polymerase activity by 50
[c]Compound concentration required to reduce cellular α-glucosidase activity by 50
[d]Compound concentration required to inhibit T Lymphoblastoid Jurkat cells viability by 50%.
[e]Compound concentration required to inhibit early phases HIV-1 replication in T lymphoblastoid Jurkat cells by 50%.

7.1.4 *Onopordum illyricum L.*, a Mediterranean plant, as a source of anti HIV-1 compounds

Different fractions were isolated from the *Onopordum illiricum* extract, belong to different classes: flavonoids, dicaffeoylquinic acids, sesquiterpenes lactones, and lignans, compounds characterized by a broad range of biological properties. Among them, 1,5-dicaffeoylquinic acid and was capable to inhibit both RT and IN enzymes in a low concentration, and the early phases of HIV-1 replication in absence of cytotoxicity (**Table 2**) (Sanna et al., 2018).

This finding represents a very good starting point for further development of novel dual acting anti-HIV compounds. In addition, considering that this plant is traditionally used for medicinal and food purposes, these results make *O. illyricum L.* a good, natural food supplement exploitable as preventive or co-adjuvant agent in the prevention and treatment of HIV-1 infection.

Compound	HIV-1 RNAse H [a]CC_{50} (µM)	IN LEDGF-dependent integration [b]IC50 (µM)	[c]CC_{50} (µM)	[d]EC_{50} (µM)
1,5-dicaffeoylquinic acid	16.9 ± 0.2	0.50 ± 0.1	>50	12.62 ± 2.67
RDS1643	7.5 ± 0.9	--		
Raltegravir	--	0.05± 0.0007		

Table 2. 1,5-dicaffeoylquinic acid from *H. indicus* present a duel-target anti-HIV-1 activity.
[a]Compound concentration required to reduce HIV-1 RT-associated RNase H activity by 50%.
[b]Compound concentration required to inhibit the HIV-1 IN catalytic activities by 50% in the presence of LEDGF.
[c]Compound concentration required to inhibit T Lymphoblastoid Jurkat cells viability by 50%.
[d]Compound concentration required to inhibit early phases HIV-1 replication in T lymphoblastoid Jurkat cells by 50%.

7.2 Gene therapy of AIDS: a novel approach combining anti-HIV-1 siRNAs and a fusion inhibitor

7.2.1 Background

Recently, cell and gene therapy approaches to treat HIV-1 infection have received increased attention because they offer the possibility of simultaneously targeting multiple sites in the HIV replication cycle, thereby minimizing the production of resistant virus.
In this scenario, our group generated combinatorial lentiviral vectors (LVs) based on SIN lentiviral HIV-1 platform, simultaneously expressing shRNA molecules against viral entry (shCCR5), infectivity (shVif) and gene expression (lhTat/Rev). The shRNAs were either expressed as single transcriptional unit under the control of different human RNA Polymerase III promoters (U6, 7SK, H1), or simultaneously, as an extended shRNA (e-shRNA), under the transcriptional control of a single Pol III promoter: once introduced into the cells, it gives rise to more than one siRNA and it was optimized to reduce the activation of the cellular interferon response. Among the different combinations tested, one triple cassette vector (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev) and one extended vector (i.e. H1e-shRNA) proved to be particularly successful once challenged against two strains of HIV-1, in human primary CD4+ T lymphocytes, from healthy patients (Spanevello et al., 2016).
With the promising results obtained with these LVs, the antiviral potency of the combinatorial platform was further implemented by introducing a fusion inhibitor peptide, which is able to efficiently block the entry of both CXCR4 and CCR5 using viruses. Thus, the maC46 fusion inhibitor encoding sequence, kindly provided by D. Von Laer (Innsbruck Medical University, Germany), was inserted in the two previously selected LVs, under the transcriptional control of the Elongation Factor 1 promoter (EF1) which has been shown to confer high level of transgene expression in human hematopoietic stem and progenitor cells (HSPCs) as well as in differentiated blood lineages after transduction with LVs (Salmon et al., 2000). Furthermore, control vectors were generated by inserting the eGFP encoding sequence under the control of the EF1 promoter, either alone or fused with the maC46 fusion peptide, and, in parallel, by replacing the RNA interference cassette with a *scrambled* sequence (i.e. H1scrCCR5) (see *Materials and Methods*, Figure 6.3).

7.2.2 Localization of the maC46 peptide on the cellular membrane

The maC46 amino acidic sequence contains a transmembrane domain which leads to the localization of the peptide at level of the cellular membrane (Egerer et al., 2015).

To verify if the maC46 peptide was correctly processed and delivered to the cell membrane, 293T cells were transfected with the developed LVs (i.e. pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP and pLL3.7 H1e-shRNA EF1maC46*fused*eGFP; pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and pLL3.7 H1e-shRNA EF1maC46; pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP and pLL3.7 H1e-shRNA EF1eGFP). 24 hours after transfection, cells were analyzed by confocal microscopy (**Figure 7.6**).

The results show the effective change in the localization at the level of the cellular membrane, as expected, not only when expressed as a fusion protein (with eGFP as reporter gene, i.e. EF1maC46*fused*eGFP), but also when expressed alone (i.e. EF1maC46). The vectors expressing only eGFP were used as controls.

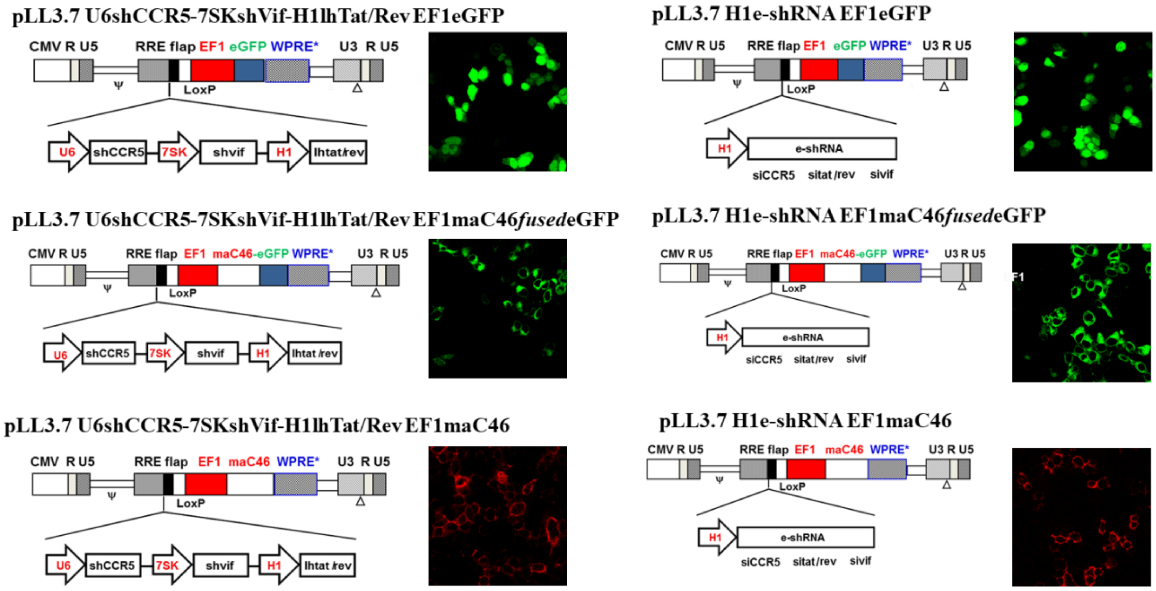

Figure 7.6. The developed lentiviral vectors lead to the expression of the maC46 which correctly localizes at the plasma membrane. 293T cells were transfected with the developed LVs and 24 hours later they were fixed and analyzed by confocal microscopy. In the case of the pLL3.7 U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and of the pLL3.7 H1e-shRNA EF1maC46 (lower panel of A and B, respectively), the expression of the maC46 peptide was evaluated by staining with 2F5-PE antibody (Polymun, Scientific). Immunofluorescence analysis was performed by confocal microscopy with an immersion objective (LEICA DM Irbe).

7.2.3 Production and titration of recombinant lentiviral particles

Vesicular stomatitis virus (VSV)-G pseudotyped vector stocks were produced by calcium phosphate transfection, as already described. Recombinant LV particles (RLVPs) were concentrated by ultracentrifugation and then the infectious titer was determined by flow cytometry in 293T cells, transduced with serial dilutions of lentiviral supernatants, and expressed in TU/mL, considering reliable only the percentages ranging from 1 to 20 (Salmon and Trono, 2007). A titer ranging from 1.6×10^8 to 3.3×10^8 TU/mL for the concentrated one was achieved (**Table 3**). Furthermore, since three LVs (i.e. H1e-shRNA EF1maC46, U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and H1scrRNA EF1maC46) do not encode the eGFP, in order to provide a valid and comparable indication regarding the presence of RLVPs in all samples, the Reverse Transcriptase (RT) activity was measured in the lentiviral lysates; equivalent RT units were obtained for all stocks, ranging from 3.03×10^5 to 8.48×10^5 cpm/25 μL (**Table 4**), and they were employed in the following challenge experiments.

Lentiviral vector	**TU/mL**
H1e-shRNA EF1eGFP	**3.30×10^8**
H1e-shRNA EF1maC46*fused*eGFP	**2.03×10^8**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP	**1.60×10^8**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP	**1.85×10^8**
H1scrCCR5 EF1eGFP	**3.08×10^8**
H1scrCCR5 EF1maC46*fused*eGFP	**1.65×10^8**

Table 3. Transduction efficiency of RLVPs. 293T cells were transduced with the RLVPs shown above as previously described. The titers were obtained with FACS analysis and expressed as the number of eGFP-positive cells transduced with 0.5 mL of viral supernatant. The values are averages from independent experiments performed in duplicate.

Lentiviral vector	cpm/25uL
H1e-shRNA EF1eGFP	**497620**
H1e-shRNA EF1maC46*fused*eGFP	**408820**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP	**425930**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP	**303940**
H1scrCCR5 EF1eGFP	**848040**
H1scrCCR5 EF1maC46*fused*eGFP	**575950**
H1e-shRNA EF1maC46	**348480**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46	**421790**
H1scrCCR5 EF1eGFP	**588890**

Table 4. Results of the RT activity assay. Viral particles were precipitated from cell-free culture supernatants and underwent to RT assay as previously described. The amount of recorded RT activity was measured using a scintillator counter and expressed in count per minutes (cpm/25 μL). Each experiment was performed in duplicate and the shown data represent the average values.

7.2.4 Evaluation of cytotoxicity and transduction efficiency of recombinant lentiviral particles

CD4+ T lymphoblastoid Jurkat cells were transduced with a high equivalent RT unit (700000 cpm) of RLVPs to firstly evaluate the influence of lentiviral transduction on cell viability. Comparable results were observed in untransduced and transduced cells by MTT cell viability assay, performed 72 hours post-transduction (**Figure 7.7**).

Next, CD4+ T lymphoblastoid Jurkat cells were transduced with different amount of equivalent RT unit (700000 and 350000). Three days later, the transduction efficiency was evaluated by flow cytometry, by measuring the percentage of eGFP positive cells, for those vectors carrying the reporter gene. Even considering the transduction variability and the different amount of equivalent RT units employed, the developed lentiviral particles were clearly able to efficiently transduce Jurkat cells, obtaining a percentage of eGFP positive cells, ranging from 91% to 99% and 49% to 73%, when equivalent RT units (700000 and 35000 cpm, respectively) of lentiviral particles were respectively employed, as reported in **Table 5**.

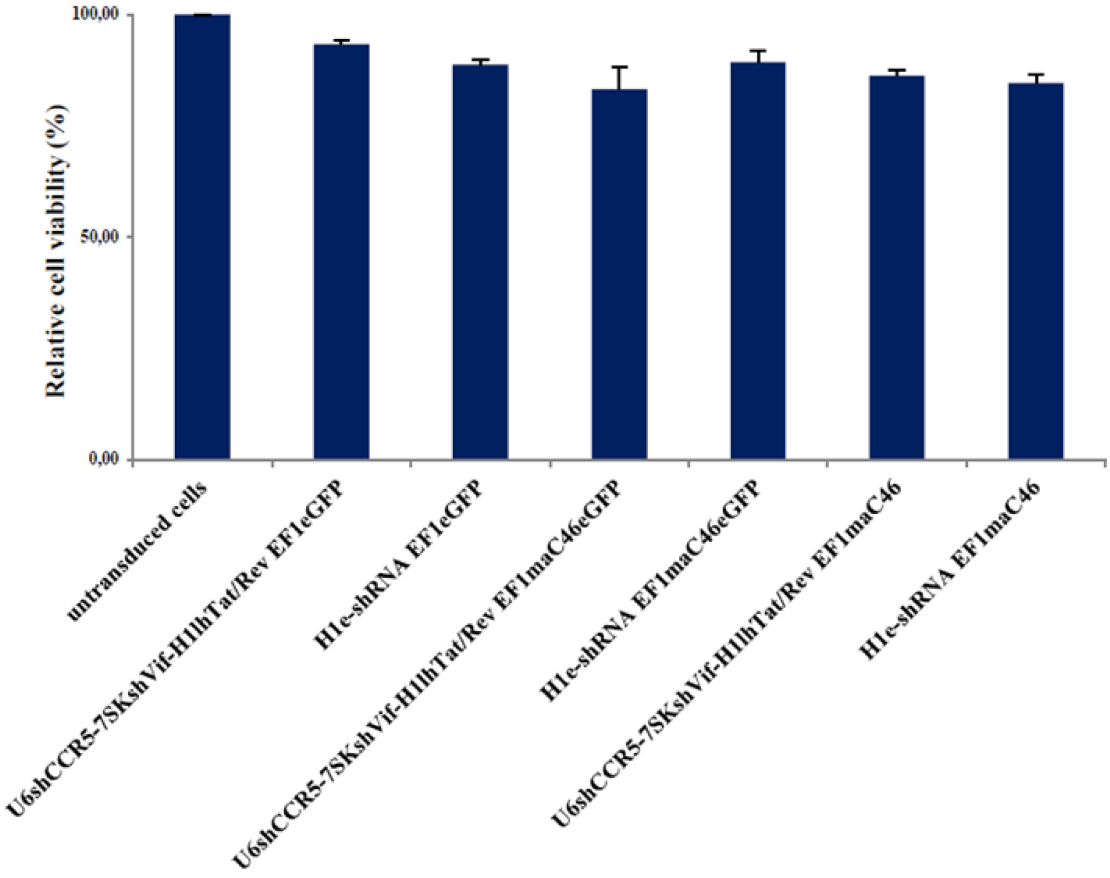

Figure 7.7. Analysis of combinatorial vector-related cytotoxicity. Three days after transduction with equivalent RT units (700000 cpm) of RLVPs, 5×10^4 transduced Jurkat cells were plated in 96-well plate and subjected to MTT assay. Cell viability was calculated by measuring the absorbance at 620 nm and the value obtained for control cells (untransduced cells) was set to 100%. Relative cell viability for other samples was calculated accordingly. All error bars indicate ± SD

	Equivalent RT units	
Lentiviral vector	**700000**	**35000**
H1e-shRNA EF1eGFP	**91.33 ± 3.52**	**81.85 ± 3.54**
H1e-shRNA EF1maC46*fused*eGFP	**99.33 ± 0.24**	**54.77 ± 2.46**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1eGFP	**91.66 ± 4.13**	**73.78 ± 4.40**
U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP	**98.67 ± 0.24**	**74.30 ± 2.69**
H1scrCCR5 EF1eGFP	**ND**	**62.36 ± 4.35**
H1scrCCR5 EF1maC46*fused*eGFP	**ND**	**55.31 ± 4.23**

Table 5. Percentage eGFP positive Jurkat T-cells transduced with RLVPs. Jurkat cells were transduced with the RLVPs shown above as already described. The titers were obtained with FACS analysis and expressed as the number of eGFP-positive cells transduced with equivalent RT units (700000; 35000 cpm) of viral supernatant. The values are averages from three independent experiments performed in duplicate ± SEM.

7.2.5 The inclusion of the maC46 peptide increases the antiviral potency of the combinatorial lentiviral platform

Finally, with the purpose of discriminating the best performing RLVPs in terms of antiviral potency, Jurkat cells were transduced and then challenged with the T-tropic laboratory-adapted HXBc2 $Vpr^{+}/Vpu^{+}/Nef^{+}$ strain of HIV-1. Cell-free culture supernatants were collected at the indicated time points up to 25 days post infection and assayed for RT activity.

First, Jurkat cells transduced with a high amount of equivalents RT units (700000 cpm) of the developed RLVPs (expressing the triple cassette or e-shRNA sequence along with the maC46 sequence) and the corresponding control vectors (lacking the peptide) and were infected with the HIV-1 HXBc2 strain using a M.O.I. of 0.1 $TCID_{50}$ per cell (**Figure 7.8**).

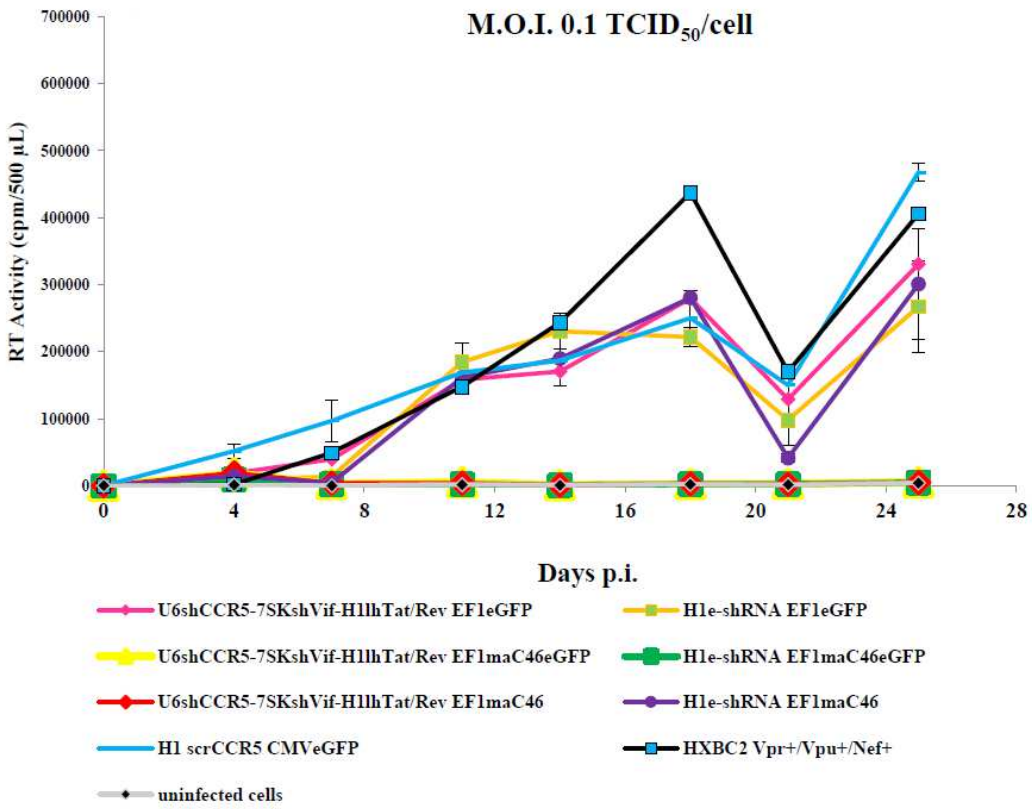

Figure 7.8. Inhibition of HIV-1 replication by the maC46 fusion inhibitor and multiple siRNA. Untransduced Jurkat cells and Jurkat cells transduced with equivalent RT units (700000 cpm) of RLVPs were infected with the HIV-1 HXBc2 Vpr+/Vpu+/Nef+ R4-tropic molecular clone at M.O.I. of 0.1 $TCID_{50}$/cell. Viral replication was assessed by measuring the RT activity in cell free culture supernatants harvested on 4, 7, 11, 14, 18, 21, 25 days post infection (p.i.). Each experiment was performed in duplicate for each sample and the average value was calculated. All error bars indicate ± SD

The results obtained clearly show that the developed RLVPs, carrying the combination of multiple siRNAs coding sequence along with the fusion inhibitor, potently blocked viral replication, compared to the ones lacking the peptide. In particular, three vectors (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP; U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; H1e-shRNA EF1maC46*fused*eGFP) conferred major protection, up to 25 days post-infection. By contrast, one of the new developed vectors (i.e. H1eshRNA EF1maC46) did not seem to be so potent in inhibiting viral replication in these challenge experiments: the protection was conferred only up to 7 days post infection. Several factors may explain the variability of these results, including the potential different levels of transduction efficiency and the level of the peptide expression, not being able to accurately detect the efficiency of transduction of constructs lacking the reporter gene.

Next, in order to identify the best combination of lentiviral vector, Jurkat cells were transduced with a lower amount of equivalent RT units (35000 cpm), to obtain a single integrated LV per cell, to avoid the shRNA overexpression and saturation of the RNAi machinery. Furthermore, specific negative controls for the expression of the peptide (i.e. H1scrCCR5 EF1maC46, H1scrCCR5 EF1maC46*fused*eGFP, H1scrCCR5 EF1eGFP) were developed and included in place of the *scrambled* vector carrying the CMV promoter, employed in the previously experiments, in order to take account of a valid control and evaluate unspecific effects. Then, Jurkat cells were challenged with the HIV-1 HXBc2 $Vpr^{+}/Vpu^{+}/Nef^{+}$ strain using a M.O.I. of either 0.1 $TCID_{50}$ per cell (**Figure 7.9A**) and 0.01 $TCID_{50}$ per cell (**Figure 7.9B**). Results indicate that, when Jurkat cells were infected with 0.01 $TCID_{50}$/cell of HIV-1 strain, one vector (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP) was able to protect up to 25 days post infection; on the other hand, in less favorable conditions, at an higher M.O.I., a more pronounced antiviral effect was observed for this lentiviral vector, compared to the others (protection up to 14 days after infection). This more robust antiviral effect could probably be due to a greater peptide stability when expressed as a fusion protein along with the reporter gene.

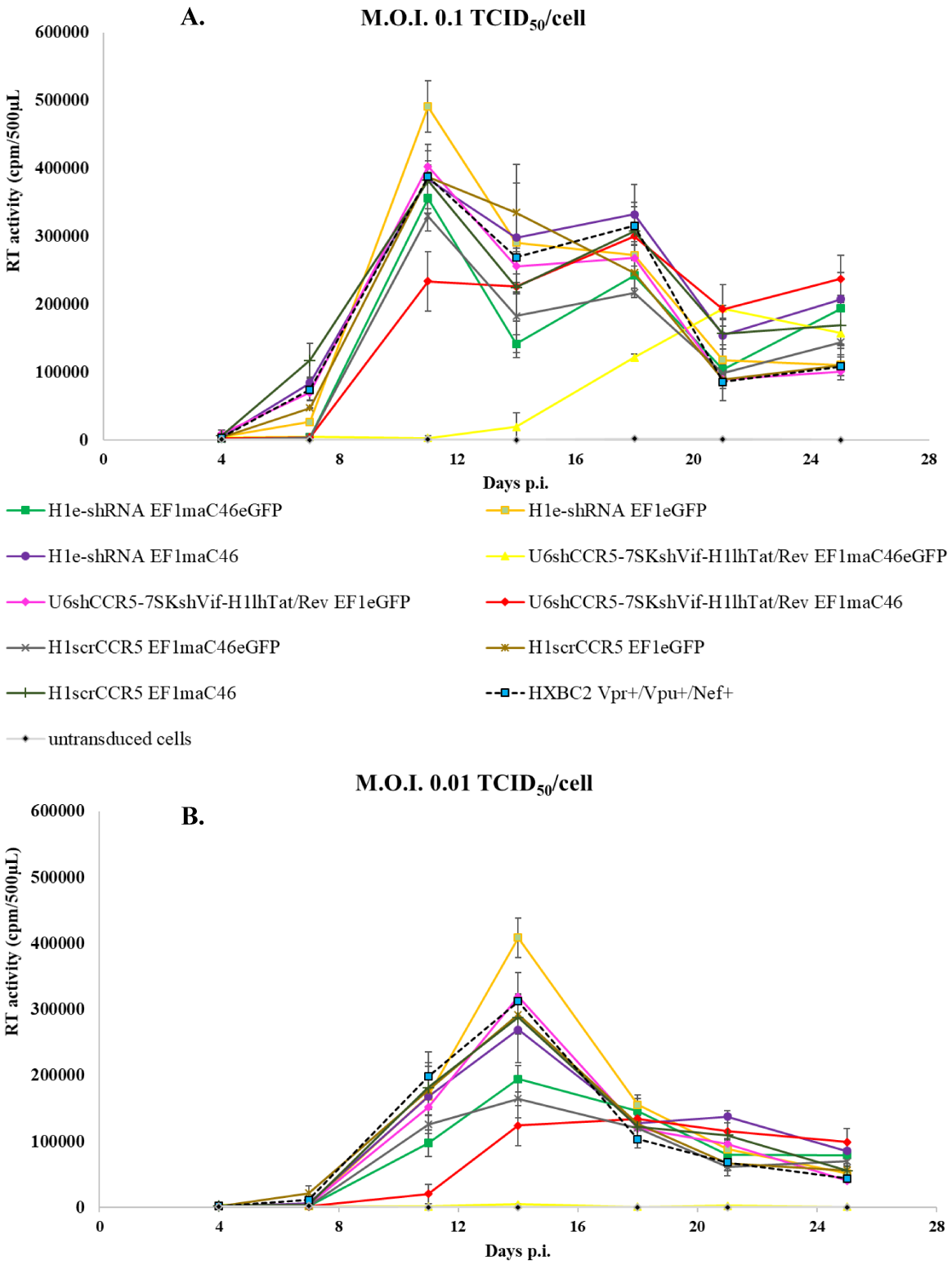

Figure 7.9. Inhibition of HIV-1 replication by the maC46 fusion inhibitor and multiple siRNA. Untransduced Jurkat cells and Jurkat cells transduced with equivalent RT units (35000 cpm) of the developed RLVPs were infected with the HIV-1 HXBc2 Vpr+/Vpu+/Nef+ R4-tropic molecular clone at M.O.I. of 0.1 (A) and 0.01 (B) $TCID_{50}$/cell. Viral replication was assessed by measuring the RT activity in cell free culture supernatants harvested on 4, 7, 11, 14, 18, 21 ,25 days post infection (p.i.). Each experiment was performed in duplicate for each sample and the average value was calculated. All error bars indicate ± SD.

7.2.6 Expression of maC46 transcript remains stable until 21 days post transduction

To understand whether differences in viral protection could be related to differences in the peptide expression, maC46 mRNA levels were quantified using qRT-PCR, as already described. 1×10^6 Jurkat cells were transduced with 35000 cpm of RLVPs containing the maC46 encoding sequence and total RNA was collected and purified at different time points (3, 15 and 21 days post transduction). Relative quantification was performed using GAPDH housekeeping gene as reference (**Figure 7.10**). Overall, we observed no significant changes in the expression of maC46 transcripts among different lentiviral vectors, or significant decrease of the peptide mRNA over time.

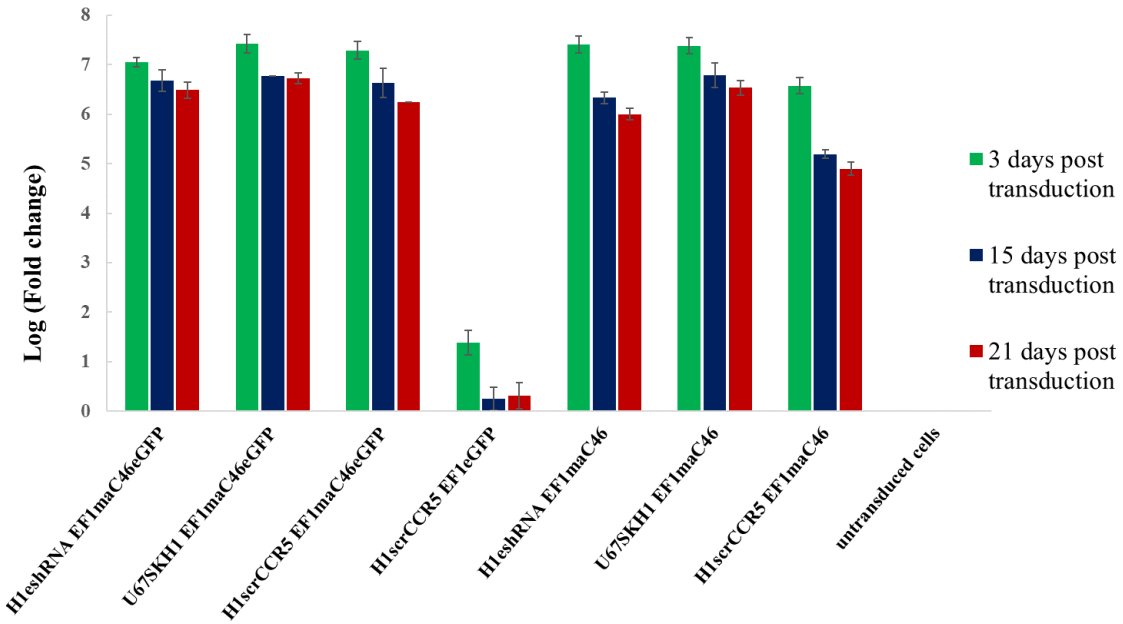

Figure 7.10. Expression of the maC46 gene in transduced Jurkat cells. maC46 RNA transcript levels were measured in untransduced Jurkat cells and Jurkat cells transduced with equivalent RT units (35000 cpm) of the developed RLVPs, by RT-qPCR, after 3, 15 and 21 days post transduction, and were normalized to GAPDH mRNA as a measure of relative C46 expression. Each experiment was performed in duplicate and the average value was calculated. All error bars indicate ± SEM.

7.2.7 The developed combinatorial lentiviral platform shows no genotoxicity potential and a low risk of insertional mutagenesis

In HSPC-GT applications for the treatment of genetic diseases, retroviral vectors are often used to efficiently transduce and integrate therapeutic genes in the genome of the target cells, thus delivering the therapeutic factors to different tissues. However, in initial HSPC-GT clinical trials using early-generation gammaretroviral vectors (GVs), vector insertions near proto-oncogenes triggered their overexpression and induced leukemia in some of the transplanted patients (Hacein-Bey-Abina et al., 2008). These unexpected adverse events have prompted the development of more advanced LVs with SIN LTRs, which preferentially integrate into intronic regions of active genes, reducing the concerns related to insertional mutagenesis (Cesana et al., 2017).

Nevertheless, assessing LVs genotoxicity remains a fundamental step before moving to the *in vivo* system. Thus, in collaboration with C. Baum's group at the Department of Experimental Hematology of the Hannover Medical School, the mutagenic potential of the combinatorial RLVPs was tested. In particular, the vectors lacking the eGFP were selected (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 and H1e-shRNA EF1maC46 referred to as U6-EFS-C46 and H1-EFS-C46, respectively), in view of the future *in vivo* applications, along with the H1scrCCR5 CMVeGFP (referred to as H1-scrambled vector) to be tested in *in vitro* immortalization (IVIM) assay (Modlich et al., 2009). Test vectors were compared with the pRSF91.GFPgPRE positive control vector referred to as RSF91, with a documented potential to induce *in vitro* immortalization by insertional mutagenesis. To this end, a large amount of selected RLVPs were produced and titrated by anti-p24 immunoassay (H1-EFS-C46 and U6-EFS-C46 105.7 µg/mL and H1-scrambled 115.16 µg/mL), and also by flow cytometry in the vector carrying eGFP reporter gene (1.8×10^8 TU/mL). Thus, to evaluate the mutagenic potential by retroviral gene transfer in a relevant target cell type, murine Lin- bone marrow cells were harvested with a purity of greater than 90% from steady state hematopoiesis of C57Bl6 mice and treated with RLVPs. Two rounds of transduction were performed on days -1 and 0 at a defined M.O.I.; the mean vector copy number (VCN) was measured on day 4; this is the first time point when proliferation reduces the number of episomal elements enough to yield reproducible mean copy numbers and early enough to exclude potential plasmid contaminations (resulting from the use of supernatants produced by transient transfection of packaging cells), influencing the overall VCN and transduction efficiency. The transgene expression (where

applicable, for the vector containing a reporter gene) was determined on day 4 and 15 as reported in the **Table 6**.

IVIM ID	Sample ID	Vector	c.MOI*	VCN **	% eGFP	MFI***	% eGFP
160803	160803-1	**Mock**	-	0.00	-	-	-
160803	160803-2	**Mock**	-	0.00	-	-	-
160928	160928-1	**Mock**	-	0.00	-	-	-
160928	160928-2	**Mock**	-	0.00	-	-	-
160928	160928-3	**Mock**	-	0.00	-	-	-
160803	160803-3	**RSF91**	60	7.74	98.00%	6096	99.90%
160803	160803-4	**RSF91**	60	9.01	97.40%	6285	93.00%
160928	160928-4	**RSF91**	60	8.37	98.70%	6423	99.50%
160928	160928-5	**RSF91**	60	7.69	97.80%	6152	97.50%
160928	160928-6	**RSF91**	60	8.09	98.60%	6392	99.10%
160803	160803-14	**H1 scrambled**	140	4.18	6.69%	154	32.80%
160803	160803-15	**H1 scrambled**	140	4.63	6.87%	137	24.00%
160928	160928-10	**H1 scrambled**	140	5.48	19.80%	145	41.10%
160928	160928-11	**H1 scrambled**	140	7.85	23.00%	170	35.80%
160928	160928-12	**H1 scrambled**	140	5.59	21.60%	172	49.30%
160803	160803-18	**H1-EFS-C46**	140	15.48	-	-	-
160803	160803-19	**H1-EFS-C46**	140	23.89	-	-	-
160928	160928-16	**H1-EFS-C46**	140	25.57	-	-	-
160928	160928-17	**H1-EFS-C46**	140	24.24	-	-	-
160928	160928-18	**H1-EFS-C46**	140	20.25	-	-	-
160803	160803-16	**U6-EFS-C46**	140	19.27	-	-	-
160803	160803-17	**U6-EFS-C46**	140	13.81	-	-	-
160928	160928-13	**U6-EFS-C46**	140	13.15	-	-	-
160928	160928-14	**U6-EFS-C46**	140	10.45	-	-	-
160928	160928-15	**U6-EFS-C46**	140	11.56	-	-	-

Table 6. Vector copy number and transgene expression in transduced Lin- cells.
Mock: control sample for which no retroviral transduction was performed
* **Cumulative MOI** (2 rounds of transduction were performed on day -1 and day 0)
** **Vector Copy Number** (for the assay to be valid, the mean VCN on day 4 must be above 1)
*** **GFP Mean Fluorescent Intensity** (The transgene expression was determined on day 4 and 15, where applicable)
The IVIM assay and the VCN determination were performed under the standard operating procedure (SOP) version 151123 and 120724 - Taqman respectively.

The tested RLVPs showed a good transduction efficiency, measured as VCN, up to 25 VCN/cell. According to this assay, when 1×10^5 lineage negative cells are transduced, and the efficiency is above 80%, with samples having a VCN of at least 3, as determined on day 4, 90% of all assays are expected to trigger immortalized insertional mutants together with a significant correlation between an increase of the VCN and immortalization phenotype (IP). However, although the extremely high VCN levels obtained for test vectors, having used a M.O.I. higher than that used for the positive control vector RSF91, no impact on cell proliferation and on cell viability was observed, as well as no vector- or supernatant-associated toxicity was evident. Indeed, the potential unspecific toxic effects of vector preparations were determined by documenting the viability and growth rate of the cells during an early phase after transduction (day 1-6) and a later phase (day 8-15) (**Figure 7.11**). A reduced proliferation rate and viability could reveal cytotoxic effects of the supernatant. A meta-analysis (MA) of Mock samples for day 1-6 (n=45) was used to determine the expected proliferation rate during the early phase (mean = 1.87 ± 0.09 division per day).

One day after transduction, a slightly lower viability was observed for all transduced samples (statistically indistinguishable from Mock), which was recovered already on day 4. Overall, both RSF91 and the test vectors showed a normal proliferation rate within the expectation range, both in the early and the late phase of the assay. Cells were then grown in cytokine-supplemented medium for another two weeks before being replated in 96-well plates. Under these conditions, mock-treated cells barely survived. With a constant number of target cells exposed to the gene transfer vectors, the incidence of cells with a transformed (replating) phenotype was determined (**Figure 7.12**).

In three independent IVIM assays, the transformation frequency per vector copy number in cells treated with our test vectors was strongly lower than that of the positive control, in particular for the test vector U6-EFS-C46, suggesting a significantly reduced risk of insertional mutagenesis and genotoxicity.

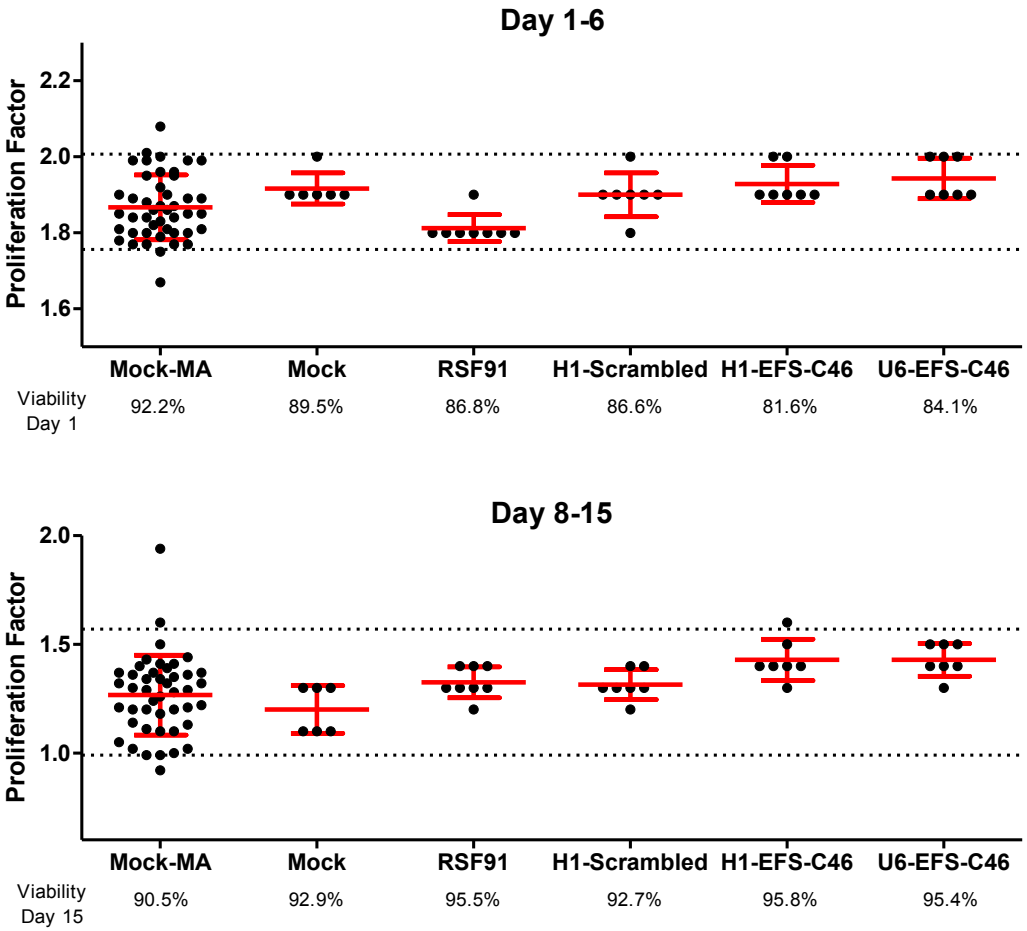

Figure 7.11. Proliferation rate during first six days and during days 8-15 of the IVIM assay. The Lin- cell number and viability were measured during the transduction procedure and the subsequent expansion phase (days 1, 4, 6, 8, 11, 13 and 15). The values from a meta-analysis (MA) of various Mock samples are shown on the left. The dotted lines indicate the 5% and 95% percentile of the MA-Mock samples and serve as an expectation range of normal proliferation. The read bars indicate means ± SD. Mean viability on day 1 and 15 is shown below in percent. Test vectors are referred to as U6-EFS-C46, H1-EFS-C46 and H1scrambled; the positive control vector is referred to as RSF91.

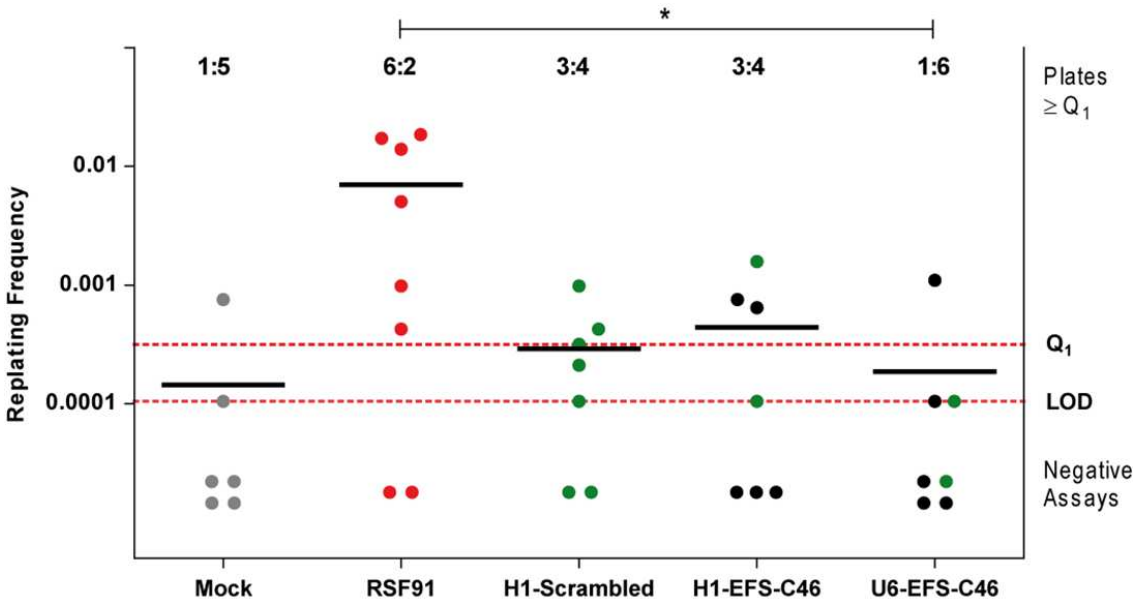

Figure 7.12. Replating Frequency (RF) of Mock (grey), RSF91 (red) and test vector transduced samples (green dots for variants encoding eGFP, black without eGFP). The ratio of replating frequency (determined by limiting dilution cloning) per vector copy number (detected by RT-PCR) is a measure of the degree of transformation. Ratios above the graph indicate the number of assays with cell growth above (left number) and below (right number) the Q1-MTT-threshold. Black bar for RSF91 indicates the mean replating frequency. The limit of detection (LOD) according to Poisson statistics (using 100 cells per well in a 96-well plate) is 1.05×10^{-4}, which corresponds to 1 positive well in the MTT-readout. All replating frequencies between the LOD and Q1 level cannot be distinguished from spontaneous cell growth. The filled circles for negative assays below the LOD were manually inserted into the graph. Every absorbance reading from the positive control or the test vectors above the MTT threshold was counted as a positive well. The difference in incidence of positive to negative assays for RSF91 to U6-EFS-C46 was significant (Fisher's Exact; p = 0.0406).

8. DISCUSSION

HIV-1 still remains one of the most widespread infection, which affects millions of people worldwide. In the latest years, the advances of Highly Active Antiretroviral Therapy (HAART) have transformed HIV-1 infection from a lethal to a manageable chronic disease. However, in spite of favorable outcomes provided by the newer therapies, HAART is not curative, due to an early establishment of chronically infected cells, called latent viral *reservoirs*. Moreover, the virus rapidly mutates its genome, often originating variants that are no longer sensitive to the drug regimen in use. On the other side, patients often suffer from the several side effects associated with HAART (such as nausea, vomiting, mood changing, appetite loss, etc.) and are still at risk of developing HIV-associated disorders (mainly opportunistic infections, due to the progressive failure of the immune system). To overcome these limitations, researches are currently focused on developing new molecules or finding alternative therapeutic strategies, to reduce the drug-related toxicity or to discover new options for a life-long remission of HIV-1/AIDS.

In this context, my PhD project explores two different approaches, in order to interfere with HIV-1 infection: on one side, a strong effort is made in the search of new antiviral compounds able to inhibit the early stages of the viral replication. Indeed, in the latest years, natural compounds and bioactive molecules have become known for their various therapeutic properties and proven effective against several health disorders and infections. On the other side, clinical and experimental evidences have already demonstrated the feasibility of hematopoietic stem and progenitor cells (HSPCs)-gene therapy, as promising approach for the treatment of AIDS, as it may facilitate a sustained inhibition of HIV-1 replication after a single therapeutic intervention, greatly limiting the occurrence of resistant strains (Allers and Schneider, 2015; Wang and Cannon, 2016). In this context, we are developing and testing recombinant lentiviral vector particles (RLVPs) to be used in the genetic manipulation of HSPCs.

8.1 Natural extracts and purified bioactive molecules in the chemotherapy of AIDS

A rich source for the discovery of new HIV infection inhibitors has been, and continues to be, the large diversity of compounds already available in nature, particularly those in

botanical extracts. Although no natural compound has yet been developed up to clinical approval for HIV treatment, several studies have proven the low toxicity correlated to a broad-spectrum of action of different molecules of natural origin (De Clercq, 2000; See et al., 1997; Singh and Bodiwala, 2010; Turville et al., 2008; Xu et al., 2015). Recently, in collaboration with E. Tramontano, our group demonstrated the properties of Lupeol, a triterpenes derived from *Hemidesmus indicus* decoction, to inhibit Reverse Transcriptase (RT)-associated RNAse H functions and α-glucosidase activity in biochemical assays (Esposito et al., 2017). Moreover, with the same collaborators, we tested some purified molecules from *Onopordum illyricum L.*, a Mediterranean plant, identifying a few compounds effective on RT-associated RNase H function and Integrase function in biochemical assays and, among them, one (i.e. 1,5-Dicaffeoylquinic acid) was able to inhibit the early stages of HIV-1 replication in cell-based assays (Sanna et al., 2018).

Moreover, we tested the ability of Oximacro®, a purified fraction of the cranberry extract, produced by Biosfered (Turin, Italy), to inhibit HIV-1 infection and replication. Oximacro® was already shown to be effective either against urinary tract infection (Occhipinti et al., 2016) and HSV-1 and HSV-2 infection *in vitro*, by preventing viral attachment to the target cells (Terlizzi et al., 2016). Ultimately, Oximacro® was tested against human Influenza virus type A and type B infection *in vitro*, and it exerted a virucidal activity, as a result of an interaction between the compound and the viral envelope glycoprotein (Luganini et al., 2018). Based on this, Oximacro® has the potential to become a valid candidate microbicide, leading the way for investigating it also against HIV-1 infection. Therefore, the identification of natural antivirals with a broad spectrum of activity could represent a promising starting point for a reliable co-therapy for HIV-1-positive individuals as well as a potent tool in the prevention of sexually transmitted diseases.

Indeed, we first tested the cytotoxicity of Oximacro® in T lymphoblastoid Jurkat cells, assessing a CC_{50} value of 19.34 ± 0.97 µg/mL. Once tested against an env-defective HIV-1 HXBc2 $Vpr^{+}Vpu^{+}Nef^{+}$, in a trans-complementation assay, the compound was able to inhibit the early phases of HIV-1 replication *in vitro* in a dose-dependent manner, with a calculated EC_{50} of 11.20 ± 0.35 µg/mL. Moreover, when the virus was pre-incubated with the compound, viral infectivity was greatly reduced, supporting the hypothesis of an interfering mechanism of action towards viral entry. Next, we investigated whether this inhibitory activity was related to the viral tropism. However, using the same trans-complementation assay, Oximacro® was able to inhibit viral replication, independently

from the co-receptor usage (i.e. CCR5 or CXCR4). The reason for this is perhaps that Oximacro® interacts with viral envelope glycoproteins in a non-specific way, resulting in an impairment in viral recognition of the cellular receptors. Finally, we tested the efficacy of Oximacro® against acute HIV-1 infection, by monitoring viral growth over time and, when required, continuously adding the compound to the infected cell culture. Once again, we saw that viral pre-incubation with the compound contributes to a higher inhibition of viral replication. In addition, continuous administration of the compound was able to protect cells from the infection over time.

We are currently investigating the mechanism of action of Oximacro® at a molecular level. In particular, we are interested in understanding how cell entry is prevented in presence of Oximacro® and whether the compound can affect other steps of the viral cycle.

Overall, these results show that Oximacro® could provide a valid candidate for the development of a broad-spectrum microbicide, representing a promising starting point for a reliable co-therapy for HIV-1-positive individuals and a potent tool in the prevention of sexually transmitted diseases.

8.2 Gene Therapy of AIDS: a novel approach combining anti-HIV-1 siRNAs and a fusion inhibitor

Gene therapy can be a valid tool in counteracting AIDS, since it can be exploited to render the entire blood cell repertoire susceptible to HIV-1 infection and/or replication resistant to the virus. This result might be achieved by genetically modifying HSPCs: once accomplished it, these cells could be transplanted back safely and efficiently into HIV-1 infected patients, who should be now protected against *de novo* infection (Pernet et al., 2016). In this context, our research group has recently developed combinatorial platforms, resembling the HAART approach, based on SIN LVs, expressing multiple siRNAs, targeting both cellular and viral transcripts involved in HIV-1 replication and infection, with the final aim of genetically modifying HSPCs. In particular, CCR5, Tat, Rev, and Vif were selected as targets of RNA interference and inserted in SIN LVs, in different combination. Two of the developed vectors, one containing multiple Pol III promoter (H1, U6 and 7SK)/shRNA cassettes within the same LVs (i.e. pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev) and the other expressing different siRNAs in a single sequence (eshRNA),

under a unique promoter (i.e. pLL3.7 H1e-shRNA), were proved to efficiently block HIV-1 replication in human primary CD4+ cells (Spanevello et al., 2016).

Even though the achieved results were promising, this strategy is mainly effective against CCR5-using viruses and not CXCR4-using viruses. Thus, the two selected vectors were further improved by conferring them the capacity to inhibit cell infection by both CCR5- and CXCR4-using HIV-1 isolates. In the last years, different studies have investigated the possibilities to confer protection against both R5-tropic and X4-tropic HIV-1 strains by potent fusion inhibitors, among which small synthetic peptides derived from the C-terminal heptad repeat of HIV-1 glycoprotein gp41 (Egerer et al., 2015; Kimpel et al., 2010). In particular, a membrane anchored form of 46 amino acids (maC46), when expressed on the surface of genetic modified cells, has proven to be safe and to confer a selective survival advantage over infected cells. maC46 peptide interacts with the N-terminal hydrophobic α-helix of HIV-1 gp41, preventing the six-helix bundle formation of HIV- 1 and subsequently the fusion to the host cellular membrane (Brauer et al., 2013; Wolstein et al., 2014). Thus, the maC46 coding sequence, either alone or fused in frame with the eGFP reporter gene, was introduced within pLL3.7 U6shCCR5-7SKshVif H1lhTat/Rev and pLL3.7 H1e-shRNA backbones, in place of the CMV-eGFP cassette present in these vectors. Of note, in the new developed LVs, the transgene expression was driven by the human Polymerase II EF1 promoter which is able to drive the expression of transgenes in lymphoblastoid cell lines as well as in HSPCs more efficiently than other promoters, including the CMV one (Sumiyoshi et al., 2009). In parallel, we also developed control LVs, expressing either the eGFP reporter gene under the EF1 promoter or a *scrambled* shCCR5 sequence under the control of H1 promoter, in order to discriminate the contribution of either the RNAi platform or the maC46 peptide, respectively. In addition, the new developed vectors were improved in their safety profile thanks to the insertion of an optimized version of the Woodchuck hepatitis virus post transcriptional regulatory element (WPRE) (Schambach et al., 2006).

A first analysis was conducted to ascertain the correct surface localization of the maC46 in transfected 293T: the obtained results demonstrated that the peptide was correctly processed into the cells and was localized at the plasma membrane. Then, T lymphoblastoid Jurkat cells were transduced with a high equivalent RT units of RLVPs (700000 cpm). First of all, we demonstrated that cells could be efficiently transduced, achieving a good percentage of eGFP positive cells (ranging from 91 to 99%), without any major cytotoxic effects. Then, transduced Jurkat cells were infected with HIV-1 HXBc2

$Vpr^+/Vpu^+/Nef^+$ R4-tropic molecular clone (M.O.I. of 0.1 $TCID_{50}$/cell). The results obtained clearly demonstrated the strong contribution of the maC46 peptide in interfering with HIV-1 replication. Indeed, three of the optimized vectors (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46; U6shCCR5-7SKshVif H1lhTat/Rev EF1maC46*fused*eGFP; H1e-shRNA EF1maC46*fused*eGFP) potently blocked viral replication up to 25 days post infection. Several factors may explain why one RLVPs (i.e. H1e-shRNA EF1maC46) was able to inhibit viral replication only up to 7 days post infection, including the potential different levels of transduction efficiency and the level of the peptide expression. Next, in order to further select the best performing vector/s to be employed in the following steps of the project, Jurkat cells were transduced with a lower amount of equivalent RT units (35000 cpm). Indeed, the identification of a minimal amounts of RLVPs, which ensures an acceptable transduction efficiency, while preventing potential side effects, is one of the goal when developing a gene therapy strategy (Herrera-Carrillo and Berkhout, 2015). Importantly, in our experimental conditions, we achieved a suitable transduction efficiency, ranging from nearly 50% to 80% eGFP-positive cells, also when 35000 cpm were employed. Results from the challenge experiment, showed that one RLVPs (i.e. U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP) was able to confer the best antiviral activity, in the challenge performed with the M.O.I of 0.01 $TCID_{50}$/cell. By contrast, at the higher M.O.I. (0.1 $TCID_{50}$/cell), the antiviral effect of this LVs was observed only up to 11 days post infection. Overall, this finding showed that the highest antiviral activity is achieved when maC46 is fused with eGFP, suggesting that the addition of the reporter gene to the peptide could confer a higher stability to the latter. This important aspect is currently under investigation. On the other hand, the triple cassette resulted more efficient than the e-shRNA in conferring anti-HIV-1 protection to transduced Jurkat cells. It is important to note that, while our results clearly indicate that the maC46 has an important role in protecting the cells against HIV-1 replication, the contribution of the peptide alone (assessed by means of *scrambled* vectors) is not sufficient to achieve a protection against HIV-1 replication in transduced Jurkat cells. In parallel, to understand whether differences in viral protection could be related to differences in the peptide expression, maC46 mRNA levels were quantified in transduced Jurkat cells at different time points (3-, 15- and 21-days post transduction), by qRT-PCR, using GAPDH housekeeping gene as reference. Overall, we observed no significant changes in the expression of maC46 transcripts among different RLVPs, or significant decrease of the peptide mRNA over time. We are currently investigating if the stability of the peptide at

the cell membrane is also maintained during the all infection period, in order to exclude a correlation between the loss in antiviral protection with an increased turn-over of the peptide at the cell level.

Moreover, we also tested the genotoxic potential of the RLVPs, in order to rule out the possibility of insertional transformation, in collaboration with C. Baum's group at the Department of Experimental Hematology (Hannover Medical School, Germany), by adopting a cell culture assay (Modlich et al., 2009). Despite the high vector copy numbers obtained for our tested vectors, no impact on cell proliferation and on cell viability was observed. Furthermore, the U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 vector showed a significantly lower incidence of insertional mutants compared to the positive control of the experiment.

In conclusion, our data indicate that the developed combinatorial platforms U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP and U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46 (even though the latter to a lesser extend), represent the best performing RLVPs, at least under the experimental conditions we adopted. Currently, in collaboration with M. Cavazzana (Institute Imagine, Paris, France), studies are ongoing, in order to assess the transduction efficiency and the antiviral potency in peripheral blood mononuclear cells (PBMCs) transduced with the U6shCCR5-7SKshVif-H1lhTat/Rev EF1maC46*fused*eGFP RLVP, as well as to evaluate the levels of transgene expression in genetically-modified HSPCs, before moving to the animal model.

Overall, the developed combinatorial platforms represent a promising strategy to render HSPCs, and, consequently, all the HIV-1 susceptible cell types, resistant to viral infection.

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