CHARACTERIZATION OF NEW SYNTHETIC OR NATURAL COMPOUNDS WITH BROAD SPECTRUM OR DUAL ANTIVIRAL ACTIVITY IN HIV-1 TREATMENT

PhD Degree Program in Experimental and Translational Medicine

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Alla Luce che guida i miei passi,
ai sorrisi innocenti che danno gioia
alla mia vita

SUMMARY

1 INTRODUCTION

1.1 CURRENTLY AVAILABLE DRUGS FOR HIV-1 TREATMENT

Human immunodeficiency virus (HIV) is a retrovirus responsible for acquired immunodeficiency syndrome (AIDS). The syndrome consists of a progressive failure of the immune system leading to life-threatening opportunistic infections and cancers. According to UNAIDS, at the end of 2017 an estimated 36.9 million people were living with HIV worldwide, being 17% higher from 2001. New infections in 2017 were 1.8 million and it is estimated that 1.0 million people died from AIDS-related causes in 2017 (www.unaids.org/sites/default/files/media_asset/unaids-data-2018en.pdf).

Since the clinical and biomedical characterization of AIDS in early 1980s, tremendous efforts have been made to identify effective therapeutics for this deadly disease. The first anti-HIV/AIDS chemotherapeutic agent azidothymidine (AZT) was introduced in 1987. It reduced the progress of AIDS by inhibiting the enzyme reverse transcriptase (RT), an enzyme that HIV uses to synthesize viral complementary DNA. Since the identification of azidothymidine, several newer classes of anti-HIV/AIDS chemotherapeutic agents have been developed [1]. With the marketing of these anti-HIV drugs, particularly the development of combinatorial antiretroviral therapy (HAART), the life span and quality of patients living with HIV improved significantly. The prognosis of AIDS patients with full access to current therapies has dramatically changed since the first cases of AIDS were reported. The life expectancy for AIDS patients was less than 1 year before AZT was introduced in 1987; today, HIV infection is often treated as a chronic infection rather than a lethal disease [2, 3].

The research in this field has led to several agents capable of arresting the HIV viral replication cycle at different stages including viral entry, fusion, reverse transcription, integration and protein maturation (Fig. **1**). At present, 31 different antiretroviral drugs have been licensed by the US Food and Drug Administration (FDA), distinguished in five different classes of molecules [4]:

- inhibitors of viral **entry** into cells by targeting the interaction between viral envelope glycoprotein gp120 and cellular CCR5 co-receptor (e.g. maraviroc);

- inhibitors of virus–cell **fusion** by targeting viral glycoprotein gp41 (e.g. enfuvirtide);
- inhibitors of HIV retrotranscriptase (**RT)** by targeting its substrate-binding site (nucleoside/nucleotide RT inhibitors or NRTIs, e.g., emtricitabine) and non-substrate-binding (allosteric) site (non-nucleoside RT inhibitors or NNRTIs, e.g., etravirine).
- inhibitors of the integration of the proviral DNA into the host cell genome by targeting HIV **integrase** (e.g. raltegravir);
- inhibitors of virus maturation and infectivity by targeting HIV **protease** (e.g., darunavir).

The present therapeutic regimen, HAART, is composed of a combination of two nucleoside reverse transcriptase inhibitors (NRTIs) to which a third agent is added. Effective inhibition of viral replication can be achieved in >90% of patients starting with such regimens. However, despite such a large armamentarium, both acute and chronic toxicities limit the prolonged use of several antiretroviral agents, and this is even more a concern when patients become older and suffer from age-related comorbidities [5]. Although most of the traditional complications associated with AIDS can now be easily prevented with HAART, some residual morbidity risk persists, including cardiovascular disease, osteopenia and osteoporosis, some cancers, liver dysfunction, renal dysfunction and neurocognitive disease [6]. Much of this excess risk is attributable not only directly to HIV infection and consequence persistent immune dysfunction and inflammation but also to antiretroviral drug toxicity. This regimen also incurs significant expense and requires a high degree of compliance as incomplete adherence to therapy results in ongoing HIV replication in the presence of drug, which invariably leads to the selection of viral variants resistant to the antiretroviral regimen and that can be, eventually, transmitted to new hosts [7]. Moreover, since 2010, no significant reduction of novel HIV-1 infection was recorded in most countries, suggesting that current efforts, including the increase access to antiretroviral treatment are not sufficient to end the AIDS epidemic (www.unaids.org/sites/default/files/media_asset/unaids-data-2018en.pdf). Implementation of preventive strategies should be an effective tool in this direction, as reducing HIV transmission, it would implement the global response to HIV-1. Unfortunately, an efficient anti-HIV-1 vaccine does not exist and, so far, pre-exposure prophylaxis (PrEP) with

antiretroviral drugs represents the sole approach available. Microbicides delivering antiviral compounds in the vagina or rectum were shown to reduce HIV-1 transmission [8]. Moreover, topical microbicides can reach high local drug concentrations for preventing HIV-1 transmission without toxicity due to the many potential benefits associated with the drug delivery route. However, efficacy of currently planned PrEP approaches may be hampered by the presence of circulating strains already resistant to available antiretroviral agents (present in the PrEP preparations) or the co-presence of other genital infections that increase patient's susceptibility to HIV-1 infection [9].

Therefore, although the drug arsenal of more than thirty approved drugs, a definitive cure still does not exist for HIV infections and molecules with new mechanisms of action are dramatically needed, especially considering the high rate of emergence of new HIV-1 resistant strains. The following chapters describe the state of art in the development of novel HIV integrase inhibitors (chapter 2) and microbicides (chapter 3), in the attempt to go behind the actual HIV treatment limits.

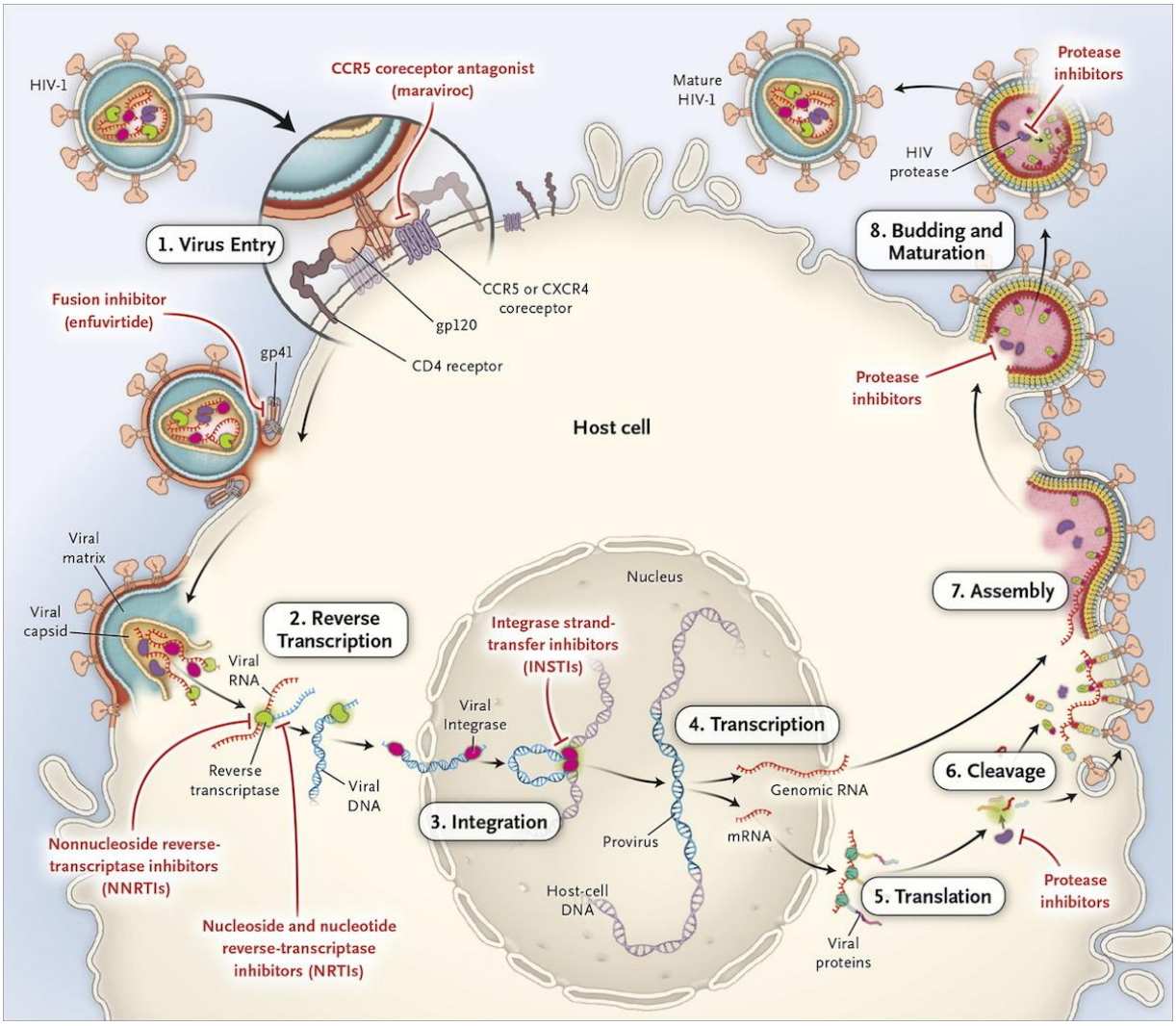


Figure 1. HIV-1 replication cycle and clinically targeted steps (highlighted in red). Adapted from Metifiot and Pommier (2010).

1.2 HIV INTEGRASE

1.2.1 MECHANISM OF VIRAL INTEGRATION

Integration of HIV genetic materials within the host cell genome is essential for viral replication. Upon entry into the host cells, the viral RNA is reverse transcribed into double-stranded cDNA by the viral RT enzyme. After this retrotranscription process, the viral enzyme HIV-1 integrase (IN), in the context of a macromolecular assemblage consisting of viral DNA and viral and host cell proteins termed the retroviral pre-integration complex (PIC), allows the viral genome integration into the host chromosome by performing two different catalytic reactions. In the cytoplasm, IN processes the viral DNA 3'-ends (3'-processing), whereas in the nucleus this processed viral DNA is a substrate for the strand-transfer (ST) reaction and is inserted into the chromosome [10].

During the 3'-P reaction, both of the viral cDNA termini are endonucleolytically cleaved by IN to remove the GT dinucleotides from the CAGT tetranucleotide sequence of the long terminal repeat (LTRs). The resulting vDNA is recessed at each 3'end and expose CA-3′-OH at both 3′-ends of viral cDNA. The trimmed viral cDNA remains bound to the IN and forms a pre-integration complex (PIC) which is then translocated to the nucleus to integrate with the host cell DNA. During ST step, the exposed CA-3′-OH of viral DNA acts as a nucleophile to attack the phosphodiester bonds on complementary strands of the host DNA through a transesterification reaction. This reaction is facilitated by two divalent metal ions, which help to stabilize the IN-DNA complex [11]. In this process, ST takes place concomitantly for both extremities of viral DNA. The 3'ends of the processed vDNA are joined to the 5'ends of the target DNA, which results in the integration intermediate. In this intermediate, the 5'ends of the vDNA and the 3'ends of the target DNA are not joined, and the two protruding 5'CA dinucleotides are left out [12]. Ligation completes the integration process by filling in the single-strand gaps between the viral and target DNA by removing the two extra nucleotides at the 5'ends of the vDNA. The viral IN is responsible for catalyzing 3'end processing and joining, but once the integration intermediate is formed, the cellular machinery carries out the remaining step and completes the integration process [13] (Fig **2**). The site selection for HIV integration shows minimal sequence selectivity toward the target DNA. However, HIV integrates preferentially inside

transcribed genes. It is plausible that cellular factors such as transcription complexes, including those bound to the IN within the PICs, as well as chromatin remodelling are implicated in the selection of the HIV integration sites within the transcribing genes.

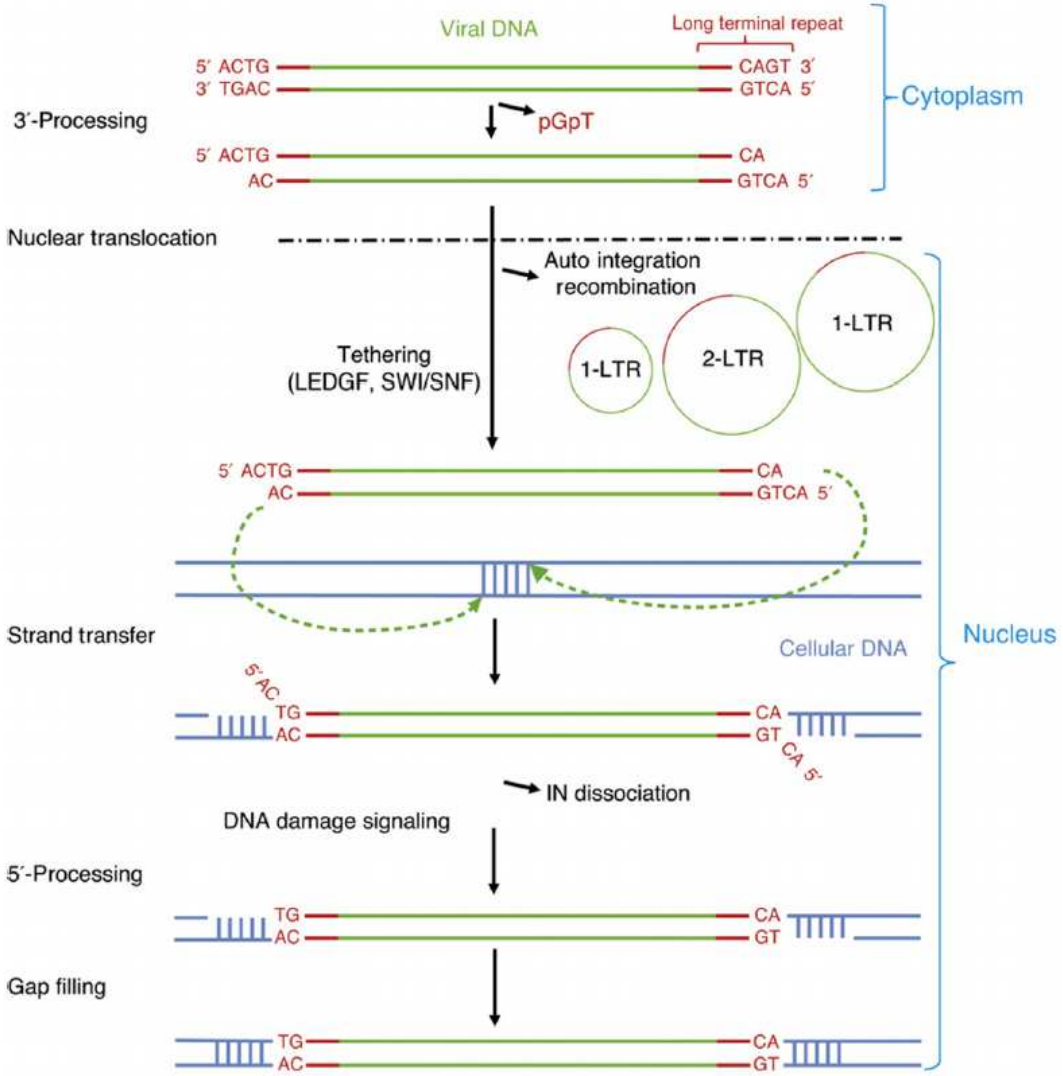


Figure 2. Mechanism of HIV integration. 3′-processing: integrase locates CAGT tetranucleotide at both 3′-ends of viral cDNA and trims off to expose sticky CA dinucleotide. Strand transfer: the trimmed viral DNA-in complex combines with cofactors to form PIC, which translocates into the nucleus and integrates with host DNA [15].

1.2.2 STRUCTURE OF HIV INTEGRASE

HIV-1 IN is a 288 amino acid long (32 kDa) protein encoded at the 3′-end of the HIV Pol gene. This multifunctional enzyme contains three structurally and functionally distinct domains: a zinc-binding N-terminal domain (NTD; residues 1–50), a catalytic core domain (CCD; residues 51–212) and a nonspecific DNA-binding domain (CTD; residues 213–288) [13] (Fig. **3A**).

- The **NTD** contains highly conserved His and Cys residues (the HHCC motif), which chelate one zinc ion and are important for oligomerization (Fig. **3B**).

- The **CCD** corresponds to the catalytic core domain with its three highly conserved acidic residues: Asp64, Asp116 and Glu152. These three residues can bind one or two divalent metal ions, such as Mn^{2+} or Mg^{2+}. All integrase activities strictly require the presence of these metallic cationic cofactors, which are coordinated by two residues of the catalytic triad (D64, D116 and E152) [1]. The DDE (Asp-Asp-Glu) motif is highly conserved among the IN superfamily and is instrumental for the catalytic activity of IN as mutation of any of these residues abolishes or reduces the catalytic activity (Fig **3C**).

- The **CTD** domain binds DNA substrates non-specifically and is responsible for stabilizing the complex once bonded with DNA (Fig. **3D**).

All three domains are essential for catalysis of 3′-P and DNA ST, although the roles of the NTD and the CTD are less well understood in comparison with CCD [16]. Due to the insolubility of the full-length HIV IN protein, its crystal structure remains unknown. However, a crystal structure of full-length IN from the prototype foamy virus in complex with its substrate DNA has been recently resolved [17]. The structural information derived from this model as well as from studies involving the three isolated domains indicated that HIV IN most likely exists as a dimer or in higher oligomeric states, such as a tetramer in solution. In particular, dimers formed at either end of the viral DNA molecule are responsible for 3'-processing activity. Pairs of dimers bring together the two ends of the viral DNA, leading to the formation of a tetramer (a dimer-of-dimers), the active form required for concerted integration [18].

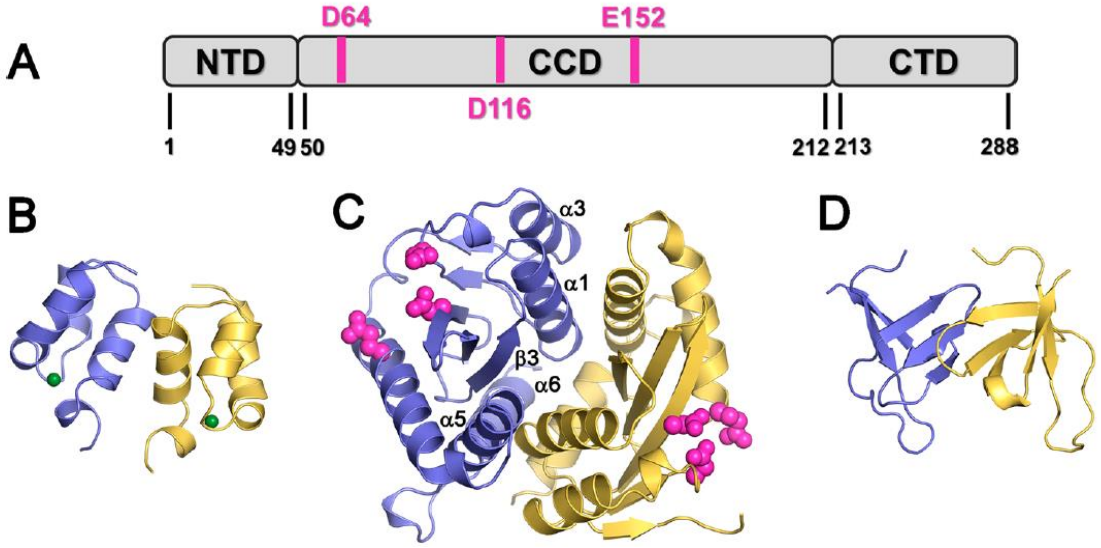


Figure 3. Structure of HIV-1 IN. (A) IN domains, where the catalytic triad is shown in pink. (B–D) Structures of single IN domains: (B) NTD; (C) CCD; (D) CTD [19].

1.2.3 HIV INTEGRASE INIBITHORS (INI)

By far, most of the anti-HIV drugs on the market are inhibitors of HIV RT and PR enzymes. HIV IN is a relatively newer therapeutic target. Its therapeutic value was proven with the discovery of raltegravir (RAL), the first IN inhibitor (INI) for human use in 2007 [20]. In comparison to other viral targets, the HIV IN stands out as a unique antiviral target because it has the least resemblance to any human protein. This unique feature makes IN an attractive anti-HIV target for reducing off-target side effects. Moreover, HIV INIs added new armament to antiretroviral therapy since they target a distinct process (i.e., integration) in the retroviral life cycle. Because of their unique mechanism of action, they have potential to reduce the chance of adaptation by the virus either when used alone or used in combination with other types of anti-HIV drugs. The structural complexity of IN and its mechanism of action suggested several strategies for therapeutic interventions: interfering the binding of IN to viral cDNA ends; interfering IN oligomerization; interfering the 3′-P activity; interfering the ST activity; and interfering with the protein–protein interactions between IN and cellular cofactors [21]. Searching IN-specific inhibitors had been focused on identification of compounds that target the IN active site. Such IN-specific inhibitors generally can be classified into two groups: inhibitors that can suppress the 3′-P and ST (referred to as 3′-P inhibitors), and inhibitors selective for the IN

ST activity (INSTIs). The 3′-P inhibitors have the preference to prevent viral DNA binding to IN. In contrast, INSTIs bind to the IN-viral DNA complex and probably interfere with the binding of host cellular DNA [22]. ST inhibition is the most productive approach by far that leads to clinical candidates. Indeed the three INs that has been approved by the FDA as new anti-HIV drugs are INSTI: Raltegravir, Elvitegravir and Dolutegravir. All these clinically approved INSTIs function by targeting the IN active site, in particular they all share the same interfacial inhibition mechanism. Interfacial inhibitors are drugs that target macromolecular complexes by binding at the interface of at least two macromolecular components, thereby stabilizing a conformation intermediate that interferes with the dynamic activity of the macromolecular complex (Fig. **4**) [13]. In the case of INSTIs, the macromolecular complex is the intasome consisting of the catalytic site of one IN polypeptide, the viral DNA, and the two catalytic magnesium cations (Fig. **4D**). All the INSTIs establish molecular contacts with each single component of the intasome complex [23]. For all INSTIs, three oxygen or nitrogen atoms coordinate the two magnesium ions in the IN catalytic triad residues (DDE motif; Fig**. 4**). At the same time, the INSTIs also bind their halobenzyl group with the 3'-penultimate cytosine of the vDNA, while the terminal adenosine of the vDNA is displaced from the active site (Fig. **4**).

1.2.3.1 INTEGRASE STRAND TRANSFER INHIBITORS (INSTIs)

Raltegravir (RAL) has been the first IN inhibitor approved by FDA in 2007. It is a potent and well-tolerated anti-HIV drug. RAL has been co administered with etravirine (NNRTI) and darunavir or ritonavir (PIs) as a salvage therapy for treatment experienced patients who were facing virological failure due to extensive multidrug resistances. This treatment achieved virological suppression similar to that observed in HIV treatment naive patients. After approval, the clinical efficacy and tolerability of RAL gave a second chance to patients who, after the failure of HAART, were left with almost no treatment alternatives. However, it has the twice-daily dosing limitation which does not favour treatment adherence and a relatively low barrier to the development of resistance (single point mutations confer high-level resistance). Indeed in the clinic use, resistance to RAL arises rapidly from three major mutation sites at positions 155 (N155H), 143 (Y143R), and 148 (G140S/Q148H double mutant), displacing the compound or the divalent metal ions

needed for compound binding from the active site. Therefore, new INSTIs have been developed to overcome RAL resistance with a once-daily administration.

More recently, another HIV INIs, **elvitegravir** (EVG) [24] was approved by the US FDA for the treatment of AIDS. EVG combined with other antiviral agents achieved the benefit of once-daily dosing, but it suffers from extensive cross-resistance with RAL [25].

Dolutegravir (DTG) offers good tolerability, once-daily dosing without a pharmacological enhancer and relatively low cross-resistance with RAL [26]. Indeed, DTG could readjust its position and conformation in response to structural changes in the mutant IN active site that became resistant to RAL [27]. This ability to maintain high potencies against mutant strains of HIV that are resistant to RAL and EVG makes it superior to earlier generation INIs when used in antiretroviral-experienced patients but mutations G140S/Q148H still confer cross-resistance to RAL and EVG [28].

In the last years, a third generation strand transfer inhibitors analog to DTG has been developed so as to find a treatment against viruses resistant to first and second generation inhibitors. Among this class, **bictegravir** and **cabotegravir** have shown strong antiviral activity as compared to dolutegravir in clinical trials [29]. Cabotegravir shares a similar drug resistance profile with DTG, retaining activity against RAL and EVG resistant mutants and is currently undergoing Phase III clinical trials. Bicitegravir displayed improved sensitivity compared with RAL, EVG and DTG, particularly for high level INSTI resistance containing combinations of mutations such as E92Q+N155H or G140C/S+Q148R/H/K. It was approved by the FDA for use in the USA and globally in February 2018 for patients who did not previously receive antiretroviral treatment or to replace the current antiretroviral regimen in those who have achieved virological suppression for more than 3 months.

Since the virus mutates fast and emergence of drug resistance is a major problem, there always will be a need to develop IN inhibitors. Thus, tremendous research efforts have been made in the field to identify novel chemical entities for the class of HIV INIs.

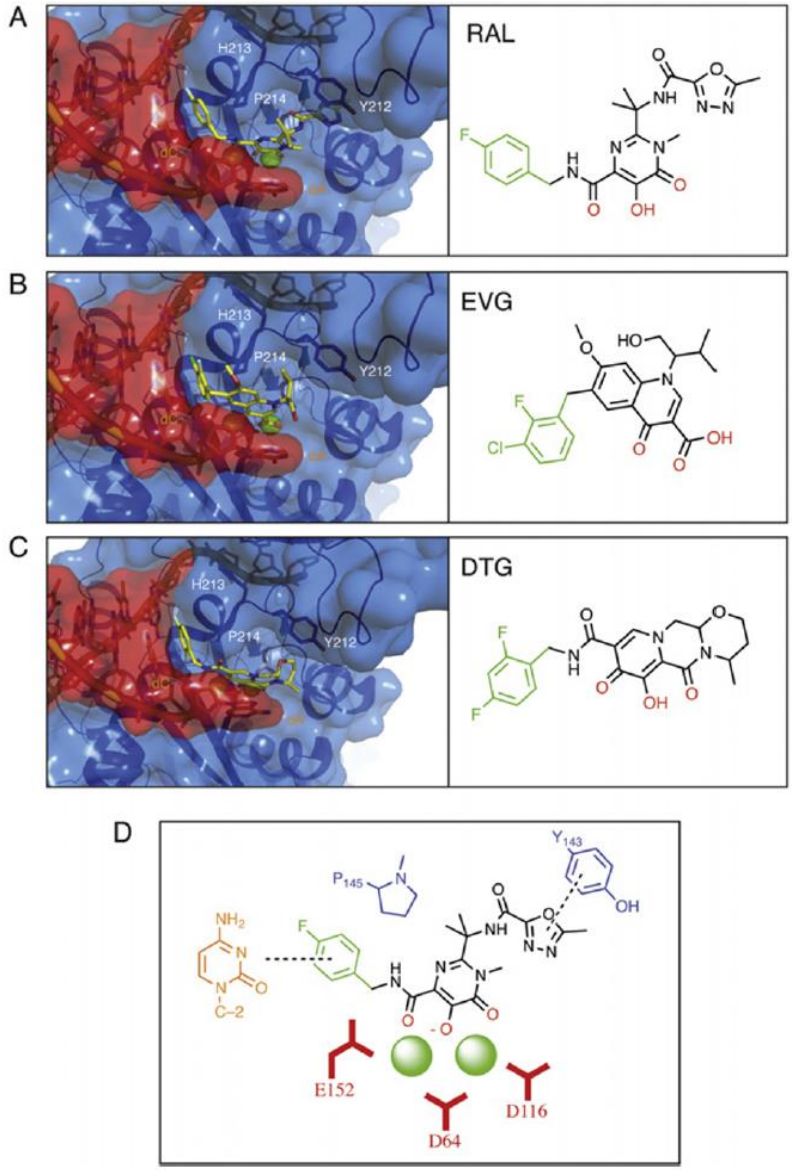


Figure 4. INSTIs interfacial binding to the intasome. (A–C, left panels) zoom-in view of the PFV (prototype foamy virus) IN active site containing metal, DNA, and drugs: RAL (panel A), EVG (panel B), and DTG (panel C). (A–C, right panels) chemical structures of RAL, EVG, and DTG. INSTIs' main features are coloured in red/dark gray and green/medium gray to highlight the chelating triad and the halobenzyl ring, respectively. (D) Scheme showing the interfacial inhibition mechanism of RAL. Interactions between the halobenzyl ring with the penultimate cytosine of the viral DNA and between the oxadiazole ring with the tyrosine residue of IN are indicated by dashed lines. Adapted from Metifiot et al., 2013 [15].

1.2.3.2 ALLOSTERIC INTEGRASE INHIBITORS (ALLINIs)

The clinically approved INSTIs function by targeting the IN active site. Because of the limited chemical space available for inhibitor design specific to IN, an overlap in resistance for the future generation INSTIs is inevitable. Therefore, although the successful design of future-generation INSTIs is attainable, this development will most likely result in only a temporary abatement in drug resistance. Therefore, future efforts should be directed to obtain compounds that block the integration process using different mechanisms of action.

Recently, research interests have moved toward the design of inhibitors with an allosteric mechanism of action or inhibitors of the interactions between cellular cofactors that are essential for integration. The first group of inhibitors would bind IN at a different region from the substrate-binding active site while still inhibiting its enzymatic activity [30]. The second group of inhibitors would inhibit the protein-protein interactions between IN and its cofactor [31]. Inhibiting cofactor binding leads to allosteric modification. Therefore, both groups can be described as IN allosteric inhibitors (ALLINIs). Since ALLINIs target a site distinct from the active site, they have a resistance profile generally different and non-overlapping with INSTIs [32].

1.2.3.2.a LEDGF-IN interaction inhibitors

The majority of ALLINIs target the LEDGF/p75, which was discovered as the first cellular cofactor crucial for the function of IN to merge viral cDNA with host genome. LEDGF is a transcriptional regulatory protein that is strongly associated with chromatin throughout the cell cycle. It is expressed as two spliced variants: the LEDGF/p52 and LEDGF/p75 proteins. During HIV infection, LEDGF/p75 works to enhance the interaction between PIC and the host chromosome [33]. More strictly, it is a nuclear protein that promotes IN chromatin tethering by allowing specific interaction between the residues Ile365, Asp366, Phe406 and Val408 in the IN binding domain (IBD) present in the C-terminal region of the LEDGF/p75 protein, and the IN dimer. It also binds to the chromatin through N-terminal PWWP (Pro-Trp-Trp-Pro) domain [34] (Fig.**5A**). LEDGF/p75 is predominantly located in the nucleus where it associates with chromatin through its PWWP domain. During HIV replication it thereby tethers IN associated with the viral genome to the host chromatin facilitating the integration into HIV-preferred sites [35]. Next to its tethering function

LEDGF/p75 protects IN from proteolytic degradation and stimulates the catalytic activity of IN *in vitro* as well as *in vivo* [36]. The protein–protein interaction surface of LEDGF/p75 and IN has been intensively characterized and provides a well-defined pocket, limited in its extension and with multiple hydrophobic and hydrogen bond interactions indicating that its disruption by small molecules is a feasible endeavour (Fig **5C**). Indeed, several small molecules have been developed that target IN-LEDGF/p75 interaction, collectively called LEDGINs [37].

Albeit different classes of LEDGINs with high variability in their biological activity have been described so far, they all share certain characteristics. In contrast to INSTIs, which bind to the catalytic site after integrase has assembled on its DNA substrate (long terminal repeat sequences, LTRs) [17], LEDGINs bind to the dimer interface of integrase irrespective of the assembly with LTRs [37, 38]. In addition they exert a dual mechanism of action blocking the LEDGF/p75–IN interaction and simultaneously modulating the multimerization state of IN resulting in the allosteric inhibition of the integrase catalytic activity [31, 37, 39]. This mechanism of inhibition has been termed 'aberrant IN multimerization', which means these compounds cause the assembly of IN into a unit that is catalytically inactive as it no longer can bind viral DNA and integrate it into host DNA [40]. LEDGIN binding to the integrase dimer interface leads to a stabilization of the dimer, restricting integrase oligomeric flexibility and as a consequence affecting the productive formation of the intasome. Hence, by modulating multimerization required for enzymatic activity, LEDGF/p75, and thus LEDGINs, has the ability to allosterically affect IN activity [41]. In addition, LEDGINs also induce a decrease of deletions at the 2-LTR junctions in the 2-LTR circles produced after HIV-replication, which is consistent with an antiviral mechanism involving the inhibition of 3′-P process [42]. By inhibiting both LEDGF/p75 interactions and catalytic activity, LEDGINs can inhibit both ST and 3′-P at the same level unlike INSTIs [43]. Moreover, it has been demonstrated that LEDGF/p75 depletion results not only in a reduced integration but also in a quiescent state of residual integrants. Upon treatment with LEDGINs, the residual proviral integration shifts away from transcription units and this LEDGIN-induced retargeting results in a silent HIV reservoir in cell lines and primary $CD4^+$ cells. This silent reservoir is refractory to reactivation by latency reversing agents. This is an important observation as pushing sufficient proviruses into latency is theoretically predicted to drive the basic reproduction number of HIV below 1,

resulting in unsustainable infection [44]. Hence, addition of LEDGINs to HAART regimens during acute HIV infection may represent a new strategy to achieve a remission of HIV infection in patients [45]. Finally, the presence of LEDGINs during virus production not only blocks provirus integration but affects as well the infectivity of the residual progeny virus [31].

Because the LEDGF/p75-binding pocket at the CCD dimer interface is distant from the active site, a new way to overcome the cross-resistance problem associated with active-site targeting INSTIs has opened [46] as LEDGINs are not cross-resistant with INSTIs. Combination experiments demonstrate that LEDGINs and INSTIs do not antagonize each other but act in an additive or even slightly synergistic way, implementing a possible design of LEDGIN/INSTI combination therapy within the HAART treatment schemes [31].

The approach of structure-based drug design led to the discovery of a series of molecules that inhibited IN-LEDGF/p75 binding and HIV-1 replication in cell culture at micromolar (BI-1001) to nanomolar concentrations (CX14442) [31]. So far, the only LEDGIN in early phase of a clinical trial is BI 224436 [47]. It targets a non-catalytic site of IN disrupting the interaction between chromatin/IN and LEDGF/p75. It was found to inhibit 3′-P step, IN interaction with LTR DNA and LEDGF without inhibiting the ST step. It is predicted to have low cross-resistance with other INIs because it binds to a distinct site on IN and it was shown to retain effectiveness against high-level RAL-resistant strains. To date, it is the first INI in clinical trial binding a non-catalytic site of HIV integrase [48].

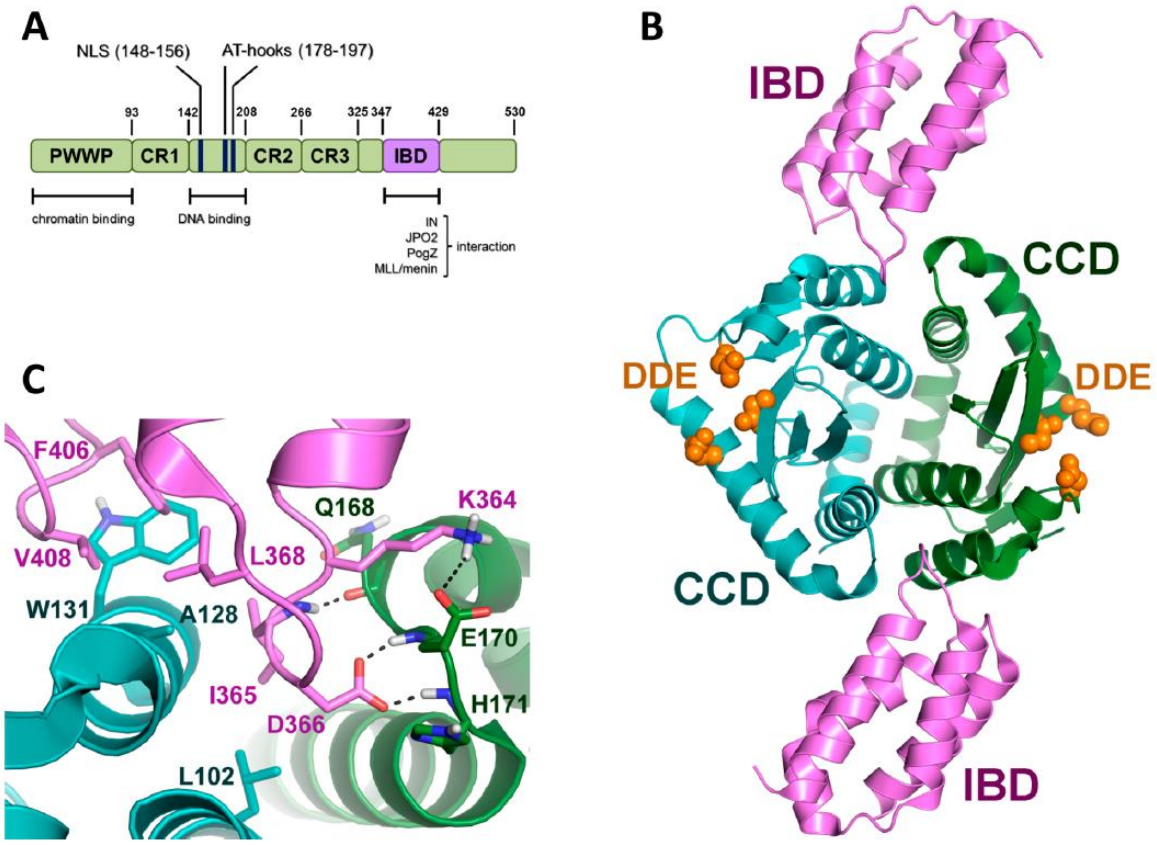


Figure 5. (A) LEDGF/p75 domains: N-terminal PWWP motif, the central DNA binding domain (blue) and the C-terminal IBD (magenta) essential for binding to IN and cellular proteins. (B) Co crystallized structure of LEDGF/p75−IBD (magenta) and the CCD dimer of integrase (green and blue). The catalytic triad is represented in orange. (C) Cartoon focused on CCD−IBD binding. IN CCDs are shown in green and blue, whereas the LEDGF/p75 IBD is in magenta. Residues of IN (dark green) and IBD (magenta) critical for the interaction are highlighted. Adapted from Di Santo et al., 2014 [19].

1.2.3.3 DUAL INHIBITORS

In order to address resistance problems associated with the current HIV drug regimen, many new strategies have been developed, including dual inhibitors drugs. The dual inhibitors contain a pharmacophore that allows them to bind to two different targets to produce additive or synergistic effects [49]. This is currently an active area of drug research across a wide range of fields and has been validated in the oncology arena. Dual inhibitors of tyrosine and phosphoinositide kinases have shown a very promising antitumor

activity [50]. More recently, this approach has also been used for antimalarial treatments [51] and it has now reached the HIV field.

Among Integrase dual inhibitors two approaches have been developed: inhibitor of both IN and RT enzymes, and IN catalytic site and LEDGF-binding site dual inhibitors.

1.2.3.3.a IN-RT dual inhibitors

The most common type of dual inhibitors are those that target IN and RNase H of RT, which share common structural geometry [52]. Both enzymes also have the same DDE motif, which is required for catalytic activity. By targeting two major enzymes essential for HIV replication with a single compound, this strategy should offer treatment regimens superior to combination therapy, reducing pill burden, improving medication compliance and decreasing the adverse effects or drug-drug interactions. Unfortunately, so far, compounds within this class either displayed high cytotoxicity in cell culture as pyrrolyl derivatives [53] or exhibited good inhibitory activity against RT RNase H but not against IN as quinolinonyl DKA derivatives [54]. Similarly, another series of potential dual RT-IN inhibitor inhibited both the IN activity and RNase H function of RT at similar concentrations (1.9 and 3.2 μM, respectively) in enzymatic assay but showed no activity against HIV infected cells [55].

1.2.3.3.b IN catalytic site and LEDGF-binding site dual inhibitors

Fan et al. used a scaffold hopping approach to design a set of small molecules capable of inhibiting the catalytic site as well as the LEDGF/p75–IN interaction [56]. As a starting point the pharmacophores of salicylic acid and catechol were fused and therefore a molecule was generated capable of chelating the catalytic Mg^{2+} ions in the active site and of binding into the hydrophobic pocket formed by the dimer interface in the IN–CCD. Four different classes of compounds were described with the most potent compound reaching moderate activity for the inhibition of IN strand transfer reaction and LEDGF-IN interaction (IC_{50} of 19μM and 8μM, respectively). The compound did not induce any cytotoxicity in cells, but no antiviral activity was reported either. Further optimization of the described structures will be required to reach higher activities *in vitro* as well as antiviral activity. Whether the strategy of designing compounds that bind both the catalytic site of IN and the LEDGF/p75 binding site, and therefore inhibit both of the functionalities of HIV-IN simultaneously, will be a valid strategy to potently inhibit integration with a

reduced risk of resistance selection or will lead to undesired side effects due to unequal affinity for both inhibitory sites still remains to be investigated.

In conclusion, besides the design of more specific and effective catalytic site inhibitors that can delay the emergence of resistant strains, a few new approaches seem to be on the horizon in the development of novel HIV integrase inhibitors. First, the design of dual inhibitors which should delay (and ideally avoid) the emergence of resistant strains and simplify the therapeutic regimen by acting on more targets. Secondly, the development of allosteric inhibitors which, targeting regions different from active site, should have a non-overlapping resistance profile with previous IN inhibitors and could be associated with them in HAART regimen as in the case of NNRTI and NRTI. In this field, inhibitors of interactions between the HIV integrase and the human cofactor LEDGF are in the pipeline.

1.3 HIV-1 MICROBICIDES

As sexual intercourse represent the main route of HIV-1 transmission, in the attempt to prevent HIV-1 spreading, specific approaches targeting directly mucosal transmission such as microbicides have been intensively investigated.

1.3.1 THE ENTRY PROCESS

Entry inhibitors represent excellent candidates for the development of topical microbicides since they block viral transmission at the mucosal surface, thus providing a method of prophylactic intervention. The following section briefly describes the mechanisms of HIV-1 transmission during sexual intercourse.

1.3.1.1 HIV-1 ENTRY PROCESS

In the context of HIV sexual transmission at genital mucosa, initial interactions between the virus and mucosal immune effector mechanisms determine the outcome of exposure. The first barrier for viral entry into the submucosa is the epithelium, whose structural conformation is variable along the genital mucosa with important implications regarding viral transmission [57]. Except for the endocervix and anal mucosa, composed by a monolayer of columnar epithelium, most of the genital mucosa consists of several layers of keratin-containing cells, known as stratified squamous epithelium that presents a major physical barrier to viral entry [58]. Accordingly, the anorectal epithelium exhibits the highest probability of HIV-transmission (0.3–5%) in comparison to female (0.05–0.5%) and male genital epithelium (0.04–0.14%), followed by the oral mucosa (0.01%) that is the least susceptible epithelium [59].

The virus, either cell-free or cell associated, can penetrate the epithelium through micro-lacerations that occur during sexual intercourse, ulcerative genital infections (caused by other infections agents), via paracellular passage after HIV-triggered epithelial disruption, or by transcytosis across epithelial cells [60]. These processes can be modulated by the presence of several factors, including variations in the expression of viral receptor and co-receptors on immune cells, local pro-inflammatory environment, mucosal antiviral factors,

HIV-cell interactions, hormonal levels, the composition of the commensal microbiota, and pathogenic co-infections [61].

The loss of tissue integrity as a result of ulcerative genital infections or abrasions that occur during sexual intercourse or possibly transcytosis can allow HIV to enter epithelial tissues and establish productive infection in target cells. The main cellular targets are $CD4^+$ T lymphocytes, $CD4^+$ cells of the macrophage lineage and dendritic cells (DCs). These cells are found in and under the epithelial cell lining of the female genital tract. After genital ulceration, intraepithelial Langerhans cell exposed to HIV-1 can internalize HIV-1 in their cytoplasmic compartments and migrate to the sub-epithelium and lymph nodes, to disseminate the virus [62]. Moreover significant populations of macrophages, known to be HIV 1 reservoirs [63], are found in the sub-epithelium and lamina propria of the vagina and cervix. The rupture of this lining can also directly expose sub-epithelial DCs to the virus, favouring the binding and internalization of HIV-1 virions via the DC-SIGN receptor [64] or the productively infection and dissemination of the virus via transfer to $CD4^+$ T cells through infectious synapses in the lymph nodes [65]. Finally, the sub-epithelium and lamina propria also host $CD4^+$ T cells, the key HIV-1 targets. The number and the activation status of such $CD4^+$ T cell influence the probability of infection after viral exposure, as activated $CD4^+$ T cells are permissive to HIV replication, while quiescent $CD4^+$ T cells can be infected but viral replication in these cells is inefficient.

Before establishing an infection per se in $CD4^+$ T-cells, the virus can interact with alternative receptors that facilitate the diffusion, attachment, and cellular entry, such as integrins, carbohydrate binding proteins (DC-SIGN) and nonspecific interactions. Nonspecific attachment results from electrostatic interactions between positively charged domains on HIV gp120 and negatively charged heparan-sulfate proteoglycans (HSPGs) or glycosphingolipids (GSLs) on target cells [66]. Next steps of HIV entry can be divided into: receptor binding, coreceptor binding and membrane fusion. Receptor and coreceptor binding is mediated by viral protein gp120 while membrane fusion is mediated by HIV gp41. Once the virus is close to the cell membrane, HIV gp120 binds to the CD4 receptor of the cell surface and various conformational changes in gp120 occur. In particular, the V1/V2 domain of gp120 modifies its position and its flexibility, protruding a high conserved region of gp120 (called V3 loop) away from the virion spike toward the cell membrane to interact with CCR5 or CXCR4 co-receptors [67]. Coreceptor binding induces

additional conformational changes that prime gp41 for membrane fusion, bringing the viral and cellular membranes together and causing the lipids from the cellular and viral membranes to mix. Lipid mixing results in the formation of a fusion pore and release of the virion contents into the cytoplasm [68]. Furthermore, HIV Env expressed on the surface of infected cells can also interact with CD4 on uninfected HIV target cells, which in turn can lead to the fusion of the cellular membranes or the formation of virological synapses and cell-cell transmission [69].

1.3.1.2 THE RELATIONSHIP BETWEEN HIV-1 AND HSV-2

As previously mentioned, the primary mode of transmission of HIV is sexual contact. This route represents the access of several other pathogens causing sexually transmitted infections (STI): Neisseria gonorrhoeae, Treponema pallidum, Chlamydia trachomatis, Trichomonas vaginalis, herpes simplex virus (HSV-1 and HSV-2), human papillomavirus (HPV) and hepatitis B virus (HBV).

Herpes simplex virus type 2 (HSV-2) is among them the most common sexually transmitted infections (20–30% of sexually active adults in North America and over 60% in sub-Saharan Africa) and is the leading cause of genital ulcers worldwide [70]. Until recently, genital herpes was more likely to be caused by HSV-2. However, the incidence of primary genital infection with HSV-1 is now increasing [71].

Both HSV-1 and HSV-2 produce similar lesions that recur with variable frequency in skin and mucosae throughout the individual's life and latently infect neurons of the trigeminal or dorsal root ganglia. HSV-2 is widespread even among people with low intercourse frequency, and, importantly, most people are not aware of being infected. Among STI, HSV-2 infection has received more attention over the years because the high prevalence (500 million persons are actually infected by HSV-2 worldwide) and because of a better understanding of the synergy between HSV-2 and HIV-1. In fact, several studies have shown that HSV-2 genital ulceration is associated with a two- to fourfold increased risk of acquiring HIV infection, thus significantly contributing to the epidemics of acquired immunodeficiency syndrome (AIDS) [9, 72]. Moreover, it has been evidenced that HSV-2 significantly increases the dissemination of HIV in the genitalia of those co-infected with these viruses [73]. More strikingly, HSV-2 reactivation at the genital level can stimulate HIV-1 replication. It was also observed that patients undergoing highly active antiretroviral

therapy had lower genital and plasma HIV-1 mRNA levels after HSV-2 treatment [74]. Interestingly, the relationship between HIV and HSV-2 is reciprocal, as infection with HIV also significantly increases the probability of acquiring HSV-2, and reactivation with HSV-2 is more frequent in HIV positive patients [75].

The molecular mechanisms that relate HSV-2 and HIV are just beginning to be elucidated. Most sexually exposures to HIV do not result in infection, likely due to protection afforded by an intact mucosal epithelium, as well as by innate and adaptive mucosal immune factors present in the genital tract. Additional important mucosal determinants of transmission are also the number and activation status of potential HIV target cells, including CCR5/$CD4^+$ T cells and DC-$SIGN^+$ dendritic cells [76, 77]. Several studies have demonstrated that the presence of HSV-2 enhances HIV susceptibility interfering with these protective mechanisms, in particular by disrupting the epithelial surface, recruiting HIV target cells to the genital tract and/or by producing a proinflammatory local milieu [78].

More strictly, one of the mechanisms by which infection with HSV-2 increases the probability of acquiring HIV is through the generation of microlesions by HSV-2 at the site of infection, which expose to HIV immune cells that reside in the epidermis and dermis to patrol these tissues (Fig.**6**) [79, 80]. On the other hand, a recent study found that the foreskin of individuals infected with HSV-2 contained significantly higher number of $CD4^+$ T cells than foreskins from non-infected individuals, thus increasing the probability that HIV encounters these target cells at this site [81]. Another clinical study found that vaginal infection with HSV-2 significantly increased the number of DCs (up to tenfold) and $CD4^+$ T cells (threefold) at the site of infection, and that these cells expressed DC-SIGN and CCR5, respectively, both receptors for HIV (Fig. **6**) [82]. Furthermore, it has been found that infection of the skin-resident macrophages by HSV increases the expression of the CCR5 receptor, a coreceptor essential for HIV entry in macrophages [83]. As most of the primary HIV infections have been attributed to CCR5 tropic viruses, this study provided one of the possible mechanisms by which HSV-2 increases the chances of acquiring HIV [84]. Langerhan cells (LCs) prevent HIV-1 transmission via the C-type lectin, langerin, which mediates the capture and degradation of HIV, thereby reducing HIV-1 transmission to T cells. De Jong et al. showed that infection of Langerhans cells with HSV-2 reduced the expression of langerin in these cells [85] promoting LC infection with HIV and consequent transfer of this virus to T cells in the tissue and lymph nodes

(Fig. **6**). Finally, it has been demonstrated that HSV-2-infected dendritic cells migrate to the proximal lymph nodes and secrete retinoic acid enabling naive T cells to infiltrate the genital mucosa, increasing the number of $CD4^+$ T cell infiltrations in the mucosal tissue. T cell migration is further potentiated due to the secretion of CXCL-9 chemokine from the infected HSV epithelial cells [86]. Taken together, infection with HSV-2 promotes the recruitment of immune cells at the site of infection that are targets for HIV, thus increasing the chances for these cells to be successfully infected by the latter virus (Fig. **6**). On the other hand, it has been recently demonstrated that human dendritic cells infected with HSV-2 secrete cytokines that promote the reactivation of HIV from cells latently infected with this retrovirus [80].

The direct influence of HSV-2 on expression of genes essential for HIV-1 to replicate in T cells has also been investigated. As HSV-2 and HIV-1 can infect the same cells, it has been shown that HSV-2 ribonucleotide reductase proteins ICP-10, ICP-27 and ICP-4 upregulate HIV-1 replication by their interactions with the HIV-1 LTR region. Further, HSV-2 protein 16 interacts with the HIV-1 Tat protein and increases HIV-1 transcription [87]. As a result, HSV-2 may not only enhance HIV-1 transmission, but it may also have a significant impact on HIV-1 viral control and disease progression among coinfected patients. In this population, HSV-2 reactivation is associated with more frequent and higher level of HIV genital shedding and increased levels of plasma HIV-1 RNA either through ulceration or HSV-2-induced increases in HIV transcription [88]. Similarly, HSV-2 suppressive treatment with acyclovir reduces not only the rates of genital HSV-2 reactivation but also the level and frequency of genital HIV shedding, and even the HIV viral load [89].

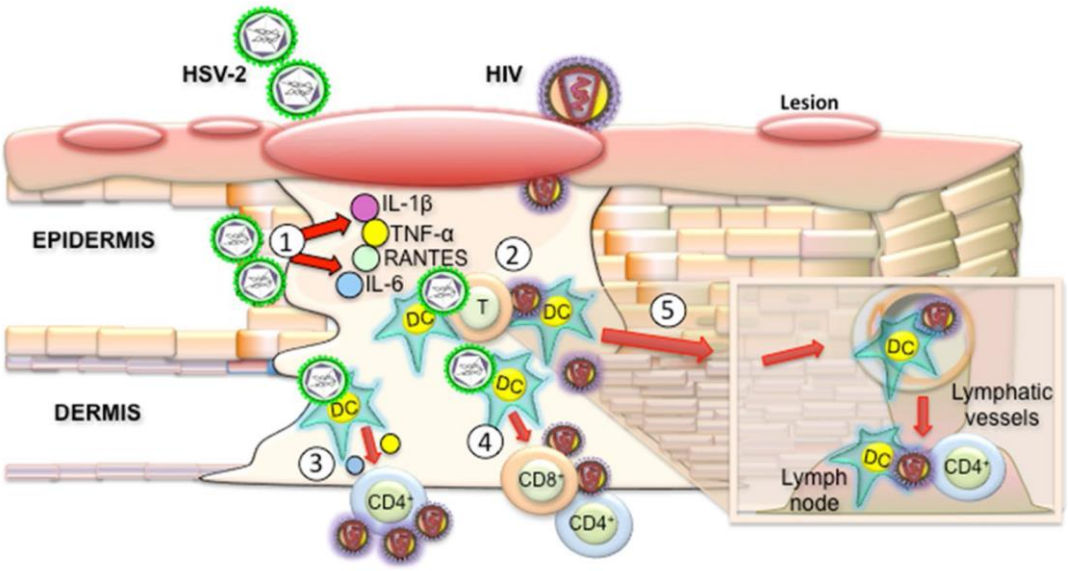


Figure 6. Relationship between HSV-2 and HIV at the site of infection. Infection with HSV-2 produces microlesions that expose the epidermis, dermis and subjacent immune cells at the site of infection to incoming viruses, such as HIV. **1**. Infection of genital epithelia by HSV-2 produces the secretion of pro-inflammatory cytokines (e.g., IL-6, TNF-α, IL-1β, RANTES) in response to infection, which attracts immune cells, such as dendritic cells and T cells to the site of infection. **2**. Residing and infiltrating immune cells (DCs and T cells) are infection targets for HSV-2 and HIV. **3**. DC infection with HSV-2 induces the secretion of soluble molecules at the site of infection that promote the reactivation of HIV from $CD4^+$ T cells in HIV^+ individuals with subsequent replication and dissemination. **4.** DCs infected with HSV-2 recruit T cells that express integrine α4β7, a molecule that facilitates infection of these cells by HIV. **5**. DCs accumulating at the site of infection can be used by HIV as Trojan horses to reach and infect T cells in lymph nodes. Adapted from Suazo et al., 2015 [90].

1.3.1.3 HSV-2 ENTRY PROCESS

As HIV, epithelial cells are the first type of cells that HSV-2 encounters. Unlike HIV, epithelial cells constitute the primary target and site of replication of this viral infection [91]. After replicating initially in epithelial cells, HSV-2 is transported retrogradely along the axon of sensory neurons to establish latency in the sensory ganglion. The latency is kept under surveillance of the immune system that also controls the virus present in the mucosa. Under certain conditions, including stress, immunodeficiency or immunosuppression, the virus is reactivated and taken through anterograde transport back

to the genital mucosa where once again HSV-2 replicates. During this replication, HSV-2 may or may not cause lesions in epithelial tissues and be transmitted to another susceptible host [92].

Entry of herpes simplex virus 2 into cells depends upon multiple cell surface receptors and multiple proteins on the surface of the virion. HSV-2 entry into host cells is mediated and modulated by the action of five fundamental glycoproteins through their interactions with their cognate receptors: gB, gC, gD and the heterodimer gH/gL [93]. The first step in HSV-2 entry is the binding of the virus through gB and gC to heparin sulphate proteoglycans (HSPGs) on the cell surface to tether the virus at the cell surface. The next step is the specific interaction between gD and multiple receptors, such as the herpes virus entry mediator (HVEM-a member of the tumor necrosis factor receptor family), nectin-1 and nectin-2 (two members of the immunoglobulin superfamily), and, to a lesser extent, specific sites in heparansulfate generated by certain isoforms of 3-O-sulfotransferases (3-*O*-sulfated HSPG) [94]. The interaction of gD with one of its receptors causes conformational changes in the C-terminal region of this protein and the formation of a fusion complex with gB and gH/gL [95]. Finally, the fusion between the viral envelope and the membrane of host cells releases the viral nucleocapsid into the cell cytoplasm.

1.3.2 TOPICAL MICROBICIDES

Efforts to prevent the heterosexual transmission of HIV/HSV-2 have been based on three approaches: behavioral changes (safer sex), the development of vaccines, and the development of antivirals or dual microbicides. Abstinence and the use of condoms to reduce the risk of cross-sex transmission have not been successful for reasons of financial insecurity, fear of retaliation, desire for pregnancy, or avoidance of sex. Abstinence and the use of condoms reduce the rate of transmission by only approximately 50%, and they do not avoid the increased risk of HIV acquisition or transmission in HSV-2-seropositive people. Despite considerable efforts to develop a vaccine against HIV and HSV-2, effective vaccines to prevent the spread of HIV/HSV-2 are not yet available [96].

Antivirals such as acyclovir have been used since long to treat infections with HSV and other herpesviruses. Acyclovir is a guanosine nucleoside analog that once internalized into

infected cells inhibits viral replication by interfering with the activity of the viral polymerase. The efficacy of this drug is significant when applied early after infection. However, it does not clear viral latency or alter the frequency of recurrences after acute treatment [92].

Because HIV-1 and HSV-2 propagate mainly via sexual transmission, specific approaches to target directly mucosal transmission such as microbicides have been investigated. Microbicides are compounds formulated as gels, creams, films, or suppositories that can be applied on mucosal surfaces (vagina, rectum) and protect the host against invasion by sexually transmitted infections (STIs), including HIV-1 and HSV.

Given the relentless spread of HIV infection worldwide, as well as the biological and social factors which predispose many women to a heightened risk of acquiring sexually transmitted pathogens, there has been considerable interest in developing a female-controlled microbicide against HIV in the last years. In particular, difficulties in negotiating condom use by male partners represent a particularly important challenge for many women and microbicides represent excellent female-controlled, preventive option that does not require negotiation, consent, or even partner knowing and could be used without requiring the explicit knowledge or consent of sexual partners.

Several microbicidal candidates targeting HIV-1 at the mucosal door of entry into the host are at various stages of development, some of them are active also on HSV-2. Eleven microbicides against HIV-1 are currently undergoing clinical evaluation, and more than 50 are in preclinical development [97]. Several steps of viral propagation after an HIV-1 encounter with mucosal surfaces could be target by microbicides. These include viral crossing of mucosal epithelia, attachment to mucosal and submucosal residing cells (LCs, DCs, macrophages, and $CD4^+$ T cells), and migration of the virus to peripheral lymph nodes. In HSV-2 infection, microbicides should prevent HSV-2 from entering the epithelial cells and sensory neurons and prevent latency (Fig.**7**).

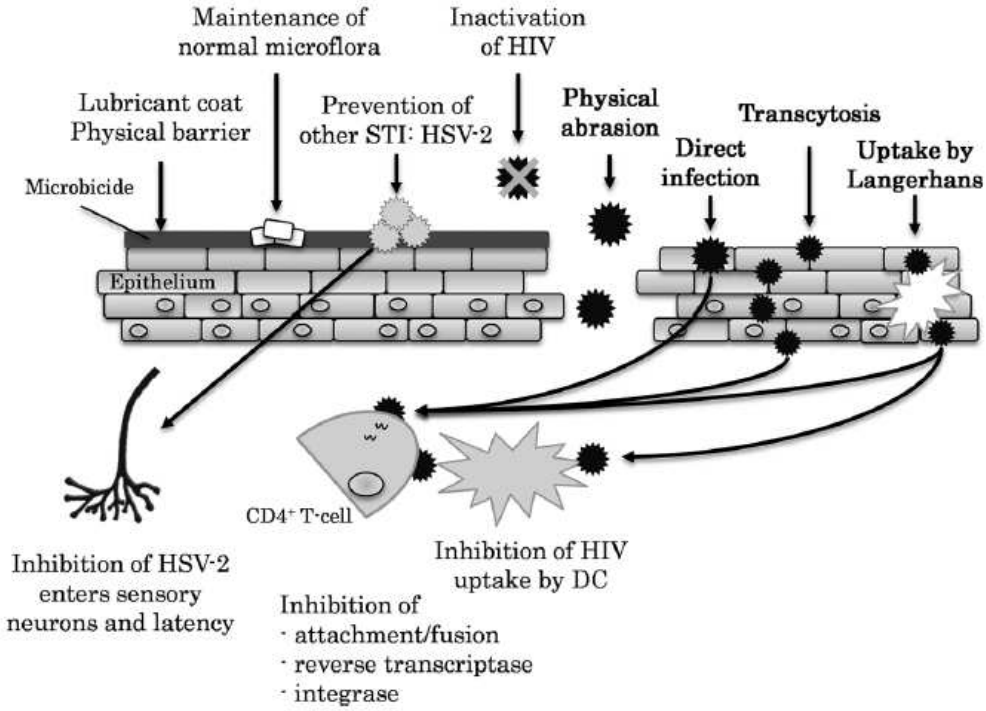


Figure 7. Potential mechanisms of action of an ideal microbicide. The microbicide should act as a lubricant coat, providing a physical barrier against HIV-1 and other sexually transmitted infections, such as HSV-2. It must prevent HIV from entering by physical abrasion, direct infection, transcytosis, or uptake by Langerhans cells. Once HIV/HSV-2 has crossed the epithelial barrier, the microbicide must prevent HSV-2 from entering the sensory neurons and establishing latency, and must prevent HIV uptake by dendritic cells (DCs), HIV attachment, entry and/or block HIV replication before integration [98].

1.3.2.1 PAST, PRESENT AND FUTURE OF ANTI-HIV-1 AND HSV-2 MICROBICIDES

Developing a safe and efficient microbicide is not without obstacles, and the first generations of microbicides have been unsuccessful. This class of microbicides is represented by detergents, such as nonoxynol-9 (N-9) that solubilise viral membranes, therefore, rendering them inactive. However, whereas *in vitro* results were excellent [99], *in vivo* data showed that N-9 increased HIV-1 and HSV-2 transmission instead of preventing them [100]. This was believed to be due to the potential pro-inflammatory impact of N-9 on vaginal mucosae [101].

Numerous other molecules have been tested as vaginal microbicides. Some of these agents are specific inhibitors of HIV-1 infection while others are non-specific. The non-specific inhibitors include compounds that modify the cervico-vaginal environment (vaginal milieu protectors) such as **buffering agents**, those that interact with the positively charged viral envelope known as **polyanionic polymers**, and envelope inhibitors such as **lectins**. The specific inhibitors include **CCR5 co-receptor antagonists**, and **reverse transcriptase inhibitors** such as Tenofovir (TFV), Dapivirine (DPV), and UC781.

1.3.2.1.a Buffering agents

During sexual intercourse the release of semen increases the pH in the female genital tract, which neutralizes vaginal acidity and renders the environment favourable for HIV-1 infection [102]. Thus, **buffering agents** were designed to counteract this process. BufferGel™ or Carbopol 974 (BufferGel, ReProtect, Baltimore, MD, USA) was a carbopol polymer formulated with a buffering agent that has the ability to buffer twice its volume in semen to pH≤5. This was a microbicide candidate designed to maintain the acidic pH of the vaginal tract. Phase I safety clinical trials showed that BufferGel™ destroyed bacteria that cause vaginosis [103], without effect on resident bacterial populations such as lactobacilli. However, BufferGel was unable to prevent HIV-1 transmission during a clinical trial conducted in Southern Africa and USA [104].

1.3.2.1.b Polyanionic polymers

Polyanionic polymers against HIV inhibit infection by interacting with the positively charged V3 loop of HIV-1 gp120 [103]. The binding of these compounds to the virus is enhanced by the conformational changes that unmask charged regions of gp120 during entry into target cells [105]. Dendrimers are the newest members of the polyanionic polymer family. These compounds are able to bind multiple locations on different cells at the same time [106]. Dendrimers are made of a core, interior branches and terminal surface groups for selective interaction with specific targets. SPL7013 is a dendrimer gel (Vivagel, Starpharma Holdings Ltd., Melbourne, Australia) that demonstrated to significantly inhibit the entry of HIV and HSV into human cells obtained from biopsies [107]. It also showed protection against the simian/human immunodeficiency virus (SHIV) chimera in a macaque model [108]. A clinical study evaluated the security, adherence and effect of

VivaGel® on the microbiome of the vagina of sexually active women and found that, although this compound modified the microbiome, this did not translate into serious complications for the individuals [107]. Because of these favorable results, it is expected that this microbicide will soon be evaluated in more advanced clinical trials in humans.

Cellulose sulphate and **PRO 2000** are two molecules that emulate sulphate heparan and display an important capacity to block the entry of HIV-1and HSV-2 into target cells [109, 110]. Although *in vitro* studies suggest that these molecules bind to the viral surface glycoprotein gB and gp120, which are essential for virus infection, clinical assays with these drugs demonstrated that PRO 2000 and cellulose sulphate would not confer significant protection against HSV-2 or HIV, and even associated their use with an increase in contracting HIV [111].

1.3.2.1.c Lectins

Different proteins are being investigated for use as microbicides [112]. These are essentially entry inhibitors with varying modes of action against the virus. Some of these proteins bind glycosylated and non-glycosylated regions of the viral envelope while others interact with receptors on target cells that are critical for infection. **Lectins** are among them the most promising envelope inhibitors studied as potential HIV-1 microbicides. Many of these compounds have a broad spectrum of activity against HIV, show no toxicity to human cells [113], have a high genetic barrier to resistance [114] and are cheap to produce [115]. **Griffithsin** (GRFT) is the most important lectin tested in a microbicide form. It is a carbohydrate binding protein extracted from the red algae Griffithsia sp. It binds mannose N-glycosilations in gp120 blocking the binding to its receptor on host cell [116]. Additionally, griffithsin has been shown in the murine model of HSV-2 infection to reduce infection with this virus, as well as pathology by blocking cell-to-cell spread in the infected tissue [117]. This lectin should now progress into macaques and then humans. Cyanovirin-N (CV-N) is also a lectin, and like GRFT has shown potent and broad inhibitory activities against HIV-1. However some studies reported it to be toxic [116].

1.3.2.1.d CCR5 antagonist

Beyond polyanionic and virucidal agents, also CCR5 co-receptor antagonists have been investigated as potential microbicides. **Maraviroc** is a potent inhibitor of CCR5 interaction

with HIV-1 [118]. Its formulation as an aqueous gel or vaginal ring protected macaques against SHIV acquisition [119]. A potential drawback with maraviroc use as a microbicide is that it is already used clinically to treat HIV-1 infection. Its application as a microbicide in infected individuals may give rise to resistant strains.

1.3.2.1.e RT inhibitors

Even if the ideal of a preventive microbicide is to act on the earlier phase of virus life cycle (entry) to prevent virus spreading, also **RT inhibitors** have been tested as potential microbicides. Studies using macaques showed that pre- and post-exposure prophylaxis with **tenofovir**, a **NRTI**, protected against intravenous challenge with SIV [120]. This report was among the first to provide evidence that tenofovir could be used for HIV-1 prevention. Since then tenofovir has become one of the leading antiretrovirals in HIV-1 microbicide research. Formulation of tenofovir as a 1% gel represents the first development of an existing antiretroviral drug as a microbicide against HIV [8]. A clinical trial conducted in South Africa, using 1% tenofovir gel within 12 h before and after sex (CAPRISA 004 trial), showed protection against heterosexual transmission of the virus. More precisely, after 30 months of trial the gel prevented transmission by 39% [8]. HIV-1 RNA genotyping of women who got infected during the trial did not reveal the presence of tenofovir resistance mutations suggesting that vaginal application of the gel did not give rise to resistant viruses [8]. However, it is not yet known what will result after a longer term use of the drug. Interestingly, in CARPISA 004 trial TDF demonstrated also anti-HSV-2 effects, reducing the transmission of this virus up to 51 % [121]. This result was surprising because tenofovir had not been previously conceived of as an anti-herpetic agent, differently from other microbicides such as PRO-2000, Carraguard, Buffergel, and others that had in part been conceived of as HSV-2 prevention tools based on data from *in vitro* systems and animal model. Consistent with this observation, subsequent *in vitro* and *in vivo* studies in the murine model and macaques have demonstrated that TDF possesses antiviral capacity against HSV-2 [122]. However, other clinical studies failed to demonstrate an anti-HSV activity of TDF [123]. It is estimated that negative results recently obtained in clinical studies with TFV would mainly be due to poor adherence to gel treatments.

Among **NNRTI**, **UC781** and **Dapivirine** represent the most interesting microbicide candidates. The thiocarboxanilide derivative UC-781 is a NNRTI characterized by high affinity for HIV-1 reverse transcriptase. UC781 is very hydrophobic, thus, able to easily penetrate the viral envelope and enter the capsid to bind reverse transcriptase even before entry into target cells [124]. A phase I trial evaluating a once-daily dose of this compound for six days proved that it was safe for use in humans [125]. Dapivirine is a molecule with a dual mode of action against HIV-1, as it inhibits both viral entry and reverse transcription [103]. A phase III clinical trial with dapivirine vaginal ring, conducted in Uganda, Malawi, Zimbabwe and South Africa (MTN-020-ASPIRE trial), reported a 27% reduction in the sexual transmission of the virus [126]. Dapivirine is the only microbicide candidate so far to be tested in humans as a vaginal ring [127].

1.3.2.2 THE DELIVERY SYSTEM

Behind the choice of the antiviral drugs, the delivery system needs also to be taken into consideration when designing a microbicide as it can influence patients' compliance and drug bioavailability. Several systems have been proposed to date: gels, vaginal rings, films or tablets.

1.3.2.2.a Semisolid gels

Semisolid gels are the most commonly used HIV-1 microbicide delivery systems [8]. Often they are based on water soluble polymers such as the cellulose derivative hydroxyl ethyl cellulose or the polyacrylic acid derivative carbopol 974. Gels have the advantage of being simple to use and show high consistency of drug delivery since they can spread easily [128]. Also their viscosity and elasticity enhance drug stability and retention. However, one of their most important disadvantages is the tendency to be messy and leaky resulting in low adherence [129]. Gels are mainly designed for application before sexual intercourse, although, they can also be used after sex.

1.3.2.2.b Vaginal rings

Vaginal rings are used for controlled release of drugs into the genital tract over long periods of time [130]. These rings can consist of polymers such as poly (dimethylsiloxane), silicon, ethylene vinyl acetate and styrene butadiene. One of the key advantages of vaginal

rings is that they provide an alternative to users sustained adherence since once inserted into the genital tract they continuously release the drug for long durations without requiring intervention [126]. Furthermore, vaginal rings are already used to deliver hormones and contraceptives into the female genital tract. However, the most significant limitations of this system are its cost, since vaginal rings are complex and expensive to manufacture, and the fact that some compounds have limited diffusion through the polymeric matrix [131].

1.3.2.2.c Vaginal films

Perhaps a solution to the messy and leaky characteristics of gels is the use of **vaginal films** which are applied in much smaller quantities. Because of this, vaginal films have a higher likelihood of acceptability among women than gels [132]. Films dissolve very rapidly once in contact with vaginal fluids allowing for a faster release of drugs. In addition, their application does not require the use of an applicator, thus, making them inherently less expensive than gels. The drawback with this system, however, is the fact that vaginal films have low overall mass that is limiting for antiviral agents requiring high dosage [133]. Moreover, there is a potential for physical abrasion from films sharp corners and edges.

1.3.2.2.d Tablet and suppositories

Tablets and **suppositories** are designed to release the microbicidal agent by melting in the genital tract. With these systems the microbicide can be delivered over several hours. Tablets are often formulated with mucoadhesive polymers to increase their retention time [128]. The main advantage of using tablets and suppositories is that they are already employed to deliver intravaginal drugs. However, the disadvantage of using this system is the possibility of leaving granny residues in the vaginal tract following dissolution [134].

In conclusion, there are many compounds targeting different stages of HIV-1 life cycle that are being studied for use as microbicides. However, the critical challenge with these studies is the progress from *in vitro* to human trials. Several compounds with promising antiviral activity *in vitro*, failed during clinical evaluations. Moreover all currently available PrEP approaches, including vaginal gels containing antiretroviral or intravaginal rings with long-acting antiretroviral drugs or implantable and injectable formulations,

inhibit HIV-1 replication but have no or very limited activity against HSV-1 or 2, thus their efficacy may be limited or not sufficient to prevent new infections especially in high prevalence settings for both viral infections.

1.4 AIM OF THE WORK

Despite the significant success achieved with antiretroviral combination therapies on HIV-1 positive people, the rate of novel HIV-1 infection worldwide still represent a major concern. Moreover, the emergence of resistant viruses, the lack of patient compliance stemming from adverse side effects and complex regimens have resulted in many therapeutic failures [135]. In addition, close to 20% of newly diagnosed HIV patients are infected with viruses resistant to existing drug classes. In this contest, the availability of better tolerated antiretroviral agents that function through novel mechanisms and lack cross-resistance to the existing drugs will be essential to the future treatment of HIV-1 positive patients and to the prevention of novel HIV-1 infections.

Essentially two main strategies were used in the field of HIV research and drugs resistance: new molecules or targets and new molecule with multiple targets. In this thesis both these possibilities were investigated. The first part of the work was dedicated to the characterization of compounds with new mechanism of action, such as dual inhibitors. Thanks to the ability to exert their action by binding simultaneously to multiple viral targets, these molecules should have the advantage of developing viral resistance with more difficulty. Starting from the computational identification of kuwanon-L as a potential allosteric inhibitor of HIV integrase, in this thesis this compound was further characterised in enzymatic assays to evaluate its activity on HIV-1 integrase. Moreover, kuwanon-L was tested in cellular-based assay to confirm its antiviral activity also on viral strains. Finally, its mechanism of action was further investigated through a time-of-addition (TOA) experiment and its innovative dual mode of action was confirmed in HIV RT enzymatic assays.

In the second part of the thesis new potential microbicides molecules with activity on both HIV and other STI (sexually transmitted infectious) were investigated. Such molecules would be very attractive as the co-presence of other genital infections, particularly those due to HSV-1 or 2, increases patient's susceptibility to HIV-1 infection and constitute a serious drawback that strongly limits the efficacy of PrEP approaches. In this work, a novel series of rhodanine derivatives was characterized *in vitro* on both HIV-1 and HSV-1/-2 viral strains. Their biological properties as novel anti-HIV agents were investigated

through *in vitro* cell toxicity and antiviral activity assays on laboratory strains bearing resistance to HIV-1 and HSV-2 currently used drugs. A preliminary ADME evaluation was performed by determining water solubility, passive membrane permeability and metabolic stability of these compounds. Finally, their efficacy as antivirals agents in prototypical genital PrEP gel preparation was tested. The obtained results suggest that these novel rhodanine derivatives could represent effective agents to be used as dual anti-HIV/HSV microbicides in PrEP approaches.

2 MATERIAL AND METHODS

2.1 MATERIALS AND METHODS (PART I)

The preparation of the extract and isolation of kuwanon-L was performed by the Department of Biotechnologies, Chemical and Pharmacy of University of Siena as previously described [136].

2.1.1 BIOCHEMICAL STUDIES

2.1.1.1 PREPARATION OF RECOMBINANT PROTEINS

Full-length IN and LEDGF proteins were expressed in Escherichia coli BL21 (DE3) as previously described [137]. Briefly, His-IN was purified by loading the precipitate of cell lysate onto a Ni-Sepharose column and eluting with a decreasing imidazole gradient (0–500 mM) in a HEPES buffer (50 mM, pH 7.5) containing NaCl (1M), CHAPS (7.5 mM), and b-mercaptoethanol (2 mM). FLAG-IN was purified by loading the precipitate of cell lysate onto a phenylsepharose and ammonium sulfate gradient (0–800 mM) in a HEPES (pH 7.5, 50 mM) buffer containing NaCl (200 mM), CHAPS (7.5 mM), and b-mercaptoethanol (2 mM). Peak fractions were pooled, loaded onto a heparin column, and eluted with an increasing NaCl gradient (200 mM to 1M) in a HEPES (pH 7.5, 50 mM) buffer containing CHAPS (7.5 mM) and b-mercaptoethanol (2 mM). Fractions containing integrase were pooled and stored in glycerol (10%) at -80°C.

His-LEDGF and FLAG-LEDGF were purified by loading the precipitate of cell lysate onto a heparin column and eluting with an increasing NaCl gradient (200 mM to 1M) in a HEPES (pH 7.5, 50 mM) buffer containing CHAPS (7.5 mM) and b-mercaptoethanol (2 mM). Peak fractions were pooled, loaded onto a Superdex 200 GL column, and eluted with a buffer containing HEPES (pH 7.5, 50 mM), NaCl (200 mM), and b-mercaptoethanol (2 mM). Fractions containing LEDGF were pooled and stored in glycerol (10%) at 80°C.

2.1.1.2 HTRF LEDGF-DEPENDENT AND -INDEPENDENT ASSAY

The IN LEDGF/p75-dependent assay allows measurement of the inhibition of 3'-processing and strand-transfer IN reactions in the presence of recombinant LEDGF/p75

protein, as previously described [136]. Briefly, IN (50 nM) was pre-incubated with increasing concentrations of compounds for 1 h at room temperature in reaction buffer containing HEPES (20 mM, pH 7.5), DTT (1 mm), glycerol (1 %), MgCl2 (20 mM), Brij-35 (0.05 %), and BSA (0.1 mg/mL). To this mixture, 100 nM DNA donor substrate, 50 nM DNA acceptor substrate, and LEDGF/p75 protein (50 nM or with omission of LEDGF/p75 protein) were added, and incubation was performed at 37 °C for 90 min. After the incubation, europium/streptavidin (4 nM) was added to the reaction mixture, and the HTRF signal was recorded with a PerkinElmer Victor3 plate reader and use of λ=314 nm and λ=668 and 620 nm for the acceptor and donor substrates, respectively.

2.1.1.3 HTRF-BASED INTEGRASE-LEDGF INTERACTION ASSAY

This assay was carried out as described [137]. Briefly, His-IN was pre-incubated with different concentrations of compound in a buffer containing NaCl (150 mM), $MgCl_2$ (2 mM), Nonidet P-40 (0.1 %), BSA (1 mg/ml), and Tris (pH 7.4, 25 mM) for 30 min at room temperature. Then, FLAG-LEDGF was added to the reaction mixture, and a mixture of anti-His6-XL665 and anti-FLAG-EuCryptate antibodies was then added. After 4 h at 4°C, the HTRF signal was recorded with a Perkin Elmer Victor 3 plate reader with use of 314 nm for the excitation wavelength and 668 and 620 nm for the wavelengths of the acceptor and donor emission, respectively. The HTRF signal is defined as the emission ratio 668/620 nm multiplied by 10 000.

2.1.1.4 HTRF-BASED IN SUBUNIT EXCHANGE ASSAY

His and FLAG-tagged INs were mixed in Tris (pH 7.4, 25 mm) buffer containing NaCl (150 mM), MgCl2 (2 mM), Nonidet P-40 (0.1 %), and BSA (1 mg/ml). Test compounds were then added to the mixture, and incubation was carried out for 2.5 h at room temperature. A mixture of anti-His6-XL665 and anti-FLAG-EuCryptate antibodies was then added, and incubation was carried out at room temperature for 3 h. The HTRF signal was recorded as above.

2.1.1.5 RT PROTEIN EXPRESSION AND PURIFICATION

The recombinant HIV-1 RT gene was subcloned into the p6HRT_prot plasmid and protein was expressed in E. coli strain M15. Briefly, bacteria cells were grown up to an OD600 of 0.8 and induced with 1.7 mM IPTG for 5 hrs. HIV-1 RT purification was carried out as described [138]. Briefly, cell pellets were resuspended in Lyses Buffer containing 20 mM Hepes pH 7.5, 0.5 M NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole, 0.4 mg/mL lysozyme, incubated on ice for 20 min, sonicated and centrifuged at 30,000 x g for 1 hr. The supernatant was applied to a His-binding resin column and washed thoroughly with wash buffer (20 mM Hepes pH 7.5, 0.3 M NaCl, 5 mM β-mercaptoethanol, 60 mM imidazole, 10% glycerol). RT was eluted by imidazole gradient and the enzyme-containing fractions were pooled, dialyzed and aliquots were stored at –80 °C.

2.1.1.6 RNASE H POLYMERASE-INDEPENDENT CLEAVAGE ASSAY

The RNase H activity associated with HIV-1 RT was measured in 100 µL reaction volume containing 50 mM Tris HCl pH 7.8, 6 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 80 mM KCl, 50 µM hybrid RNA/DNA (5'-GTTTTCTTTTCCCCCCTGAC3'-Fluorescein, 5' CAAAAGAAAAGGGGGGACUG-3'-Dabcyl) (Metabion), 3.8 nM RT and kuwanon-L or control drugs (range 50-0.001 µM). The reaction mixture was incubated for 1 hr at 37 °C, the reaction was stopped by addition of EDTA and products were measured with a multilabel counter plate reader Victor 3 (Perkin) at 490/528 nm (excitation/emission wavelength).

2.1.1.7 DNA POLYMERASE ASSAY

The RNA-Dependent DNA Polymerase (RDDP) activity associated with HIV-1 RT was measured using the Invitrogen EnzCheck Reverse Transcriptase Assay Kit, as described [139]. In 50 µL volume containing 60 mM Tris-HCl pH 8.1, 8 mM $MgCl_2$, 60 mM KCl, 13 mM DTT, 100 µM dTTP, 2 nM HIV-1 RT and poly(A)-oligo(dT). The reaction mixture was incubated for 30 min at 37 °C with or without kuwanon-L or control drugs (range 50-0.001 uM). The enzymatic reaction was stopped by addition of EDTA and products were measured with a Victor 3 (Perkin) at 502/523 nm following picogreen addition.

2.1.2 CELLULAR BASED EXPERIMENT

2.1.2.1 CELL-BASED ASSAYS

Human TZM-bl indicator cell line was obtained from NIH AIDS reagents program, cat Nr 8129 and maintained at 37°C under CO_2 (5%) in Dulbecco's modified Eagle's (DMEM) containing fetal bovine serum (10%), penicillin (50 µg/mL), and streptomycin (50 µg/mL). HIV-1 NL4.3 laboratory strain (NIH AIDS reagent program, cat. Nr.114) was titrated as follows: serial fivefold dilutions of each virus were made in quadruplicate wells in 96-well culture plates in a total volume of 100 µL of growth medium for a total of eight dilution steps. 20.000 freshly trypsinized cells in growth medium (100 µL) containing diethylaminoethyldextran (DEAE-dextran, 30 µg/ml) were added to each well, and the plates were incubated at 37°C in a humidified 5% CO_2-95% air environment. After 48 h, medium was removed, and viral infection was quantified by means of a CPRG assay. For the neutralization assay, TZM-bl cells (20 000 cells per well) were seeded in 96-well plates in complete DMEM supplemented with DEAE-dextran (30 µg/ml). 300 TCID50/mL of NL4.3 HIV strain were pretreated for 1 h at 37°C with six serial dilutions (range 20000–6.4 nM) of each compound and then added to the cells. Vehicle-treated (0.1% DMSO) cells served as negative control, an integrase inhibitor (dolutegravir) was used as positive-control drug. After two days, viral infection was quantified by means of a CPRG assay. The inhibitory curves were fitted by nonlinear regression, allowing IC_{50} calculation with the aid of Prism software. To evaluate the cell toxicity of the compounds metabolic XTT test (Sigma-Aldrich) was performed according to the manufacturer's instructions.

2.1.2.2 TIME-OF-ADDITION ASSAY (TOA)

Time-of-addition experiment was performed using a single cycle assay and the pseudotyped virus REJO4541 clone 67. For the TOA assay, 40.000 TZM-bl cells/well in a 96 multiwell plate were infected with 1500 TCID50/ml of the env-pseudotyped HIV-1 virus in complete medium supplemented with 30 µg/ml DEAE dextran (Sigma-Aldrich). Virus was incubated with cells for 1 hour at 4°C and unbound virus was subsequently removed by extensive and repeated washing with PBS to synchronize the replication. For the following seven hours, antiretroviral compounds inhibiting distinct viral replication steps (Maraviroc, Lamivudine, Dolutegravir) and kuwanon-L were added at the following

time points: at time 0, after 60, 120, 180, 240, 300, 360 and 420 minutes. To ensure a completed inhibition of viral replication we used a 40-fold IC_{50} concentration as previously evaluated for each compound on TZM- bl cells (Maraviroc 0.7 µM, Lamivudine 5 µM, Dolutegravir 1 µM and kuwanon-L 15 µM). β-galactosidase expression in cell lysates 48h post-infection was used as a marker of HIV infection and was normalized to untreated control cells.

2.2 MATERIALS AND METHODS (PART II)

2.2.1 CHEMISTRY

***General information*:** Synthesis of rhodanine derivatives was performed by Prof. Botta's collaborators in the Department of Biotechnology, Chemistry and Pharmacy of the University of Siena. All commercially available chemicals were used as purchased. Anhydrous reactions were run under a positive pressure of dry N_2. Thin-layer chromatography (TLC) was carried out using Merck TLC plates: silica gel 60 F254. Chromatographic purifications were performed on columns packed with Merck 60 silica gel, 23–400 mesh, for the flash technique. ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz on a Bruker Avance DPX400 spectrometer. Melting points were measured using a Gallenkamp melting point apparatus and are uncorrected. Microwave irradiation experiments were conducted using a CEM Discover Synthesis Unit (CEM Corp., Matthews, NC, USA). The instrument consists of a continuous focused microwave power delivery system with operator-selectable power output from 0 to 300 W. The temperature of the contents of the vessel was monitored with a calibrated IR temperature control mounted under the reaction vessel. All experiments were performed using a stirring option, whereby the contents of the vessel are stirred by a rotating magnetic plate located below the floor of the microwave cavity and a teflon-coated magnetic stir bar in the vessel.

Methyl 4-(5-formylfuran-2-yl)-2-hydroxybenzoate (5): Methyl-4-iodosalycilate **3** (1.00 mmol) and 5-formyl-2- furan boronic acid **4** were dissolved in 10 mL of DMF and 15 mL of EtOH. The reaction mixture was stirred for 10 minutes under N_2, then $Pd(PPh_3)2\ Cl_2$ (0.10 mmol) was added and finally Na_2CO_3 2M (6.00 mmol). The reaction mixture (light-orange) was stirred under N_2 at room temperature. After 1h the reaction went to completion (monitoring with TLC) and was quenched with H_2O and 2N HCl, then EtOAc was added, and the mixture was stirred until the two layers became clear. The aqueous layer was extracted three times with EtOAc, then the organic phase were washed several times with H_2O and brine, dried over Na_2SO_4, filtered and evaporated under reduced pressure. The crude product was purified by flash chromatography using PE/EtOAc= 4:1 as eluent to yield the wishes product **5** as a light orange solid (yield: 96%); mp = 150°C (decomposition); ^{1}H NMR (CDCl3 , 400 MHz): ð = 10.84 (s, 1H), 9.69 (s, 1H), 7.91-7.88

(d, 1H, *J* = 12 Hz), 7.40-7.39 (d, 1H, *J* = 4 Hz), 7.35-7.32 (m, 3H), 6.94-6.93 (d, 1H, *J* = 4Hz), 3.97 (s, 3H); ^{13}C ($CDCl_3$, 100 MHz): ð = 177.47, 161.69, 157.37, 152.53, 135.17, 130.58,122.56, 115.74, 113.73, 112.82, 109.84, 52.40, 29.59; MS (ES): m/z 245.0 $[M-H]^-$; Anal. (C13H10O5) C, H, N.

4-(5-Formylfuran-2-yl)-2-hydroxybenzoic acid (6): Compound **5** was dissolved in 25 mL of CH_3OH, then a solution of NaOH 1M (5.00 mmol) was added dropwise, after the reaction mixture was heated at reflux. The reaction mixture was stirred overnight until completion (TLC). Organic solvent was removed under reduced pressure, then some water was added, and the aqueous layer was extracted three times with Et_2O; the aqueous layer was then acidified to pH 1 with HCl 6N and a precipitated appeared. (**6**) was obtained as a brown-red solid (yield : 95%); mp = 230°C (decomposition); ^{1}H NMR (DMSO, 400 MHz): ð = 9.63 (s, 1H), 7.88-7.86 (d, 1H, *J* = 8 Hz), 7.66-7.65 (d, 1H, *J* = 4 Hz), 7.45-7.39 (m, 3H); ^{13}C NMR (DMSO, 100 MHz): ð = 178.72, 171.68, 161.69, 156.82, 152.63, 135.11, 131.72, 125.14, 116.02, 114.06, 113.29, 111.53; MS (ES): m/z 231.0 [M-H].

General Procedure for the synthesis of final compounds 9a-f: To a solution of bis (carboxymethyl)trithiocarbonate (0.22 mmol) in DME (1.0 mL) were added TEA (0.22 mmol) and the opportune amine (0.22 mmol). The reaction mixture was heated at 90 °C for 10 min under microwave irradiation. After this time, the aldehyde **6** (0.22 mmol) was added, and the mixture was heated at 110 °C for 5 min under microwave irradiation. The reaction mixture was evaporated to dryness and the residue was additioned with MeOH and a drop of HCl 2N; the final rhodanine derivatives were obtained as a pure precipitate, isolated by filtration, washed with water and hexane, and finally dried under high vacuum.

(Z)-4-(5-((3-(4-fluorophenethyl)-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan-2-yl)-2-hydroxybenzoic acid (9a). (yield: 30%); Yellow solid. Mp = 292 °C (decomposition), ^{1}H NMR: (400 MHz, DMSO-d_6) d = 7.92-7.90 (d, 1H, *J* = 8.0 Hz), 7.61 (s, 1H), 7.42-7.41 (d, 1H, *J* = 3.2 Hz), 7.38-7.33 (m, 3H),7.26-7.22 (m, 2H), 7.11-7.06 (m, 2H), 4.26.4.22 (m, 2H), 2.99-2.95 (m, 2H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6): d = 194.20, 171.72,166.65, 161.84, 156.71, 154.49, 150.43, 134.83, 134.20, 131.78, 131.00,130.91, 123.19, 119.91, 118.46, 116.16, 115.72, 115.51, 113.76, 113.04,112.41, 45.63, 31.68 ppm. MS (ES): m/z 468.0 $[M-H]^-$. HPLC: tr=4.58 min; conditions: temp = 25

°C, mobile phase composed of (A)70% acetonitrile and (B) 30% water with 0.5% formic acid at a flowrate of 1.0 mL/min; purity: 96.5%.

(Z)-2-hydroxy-4-(5-((3-(4-methylphenethyl)-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan-2-yl)benzoic acid (**9b**). (yield: 79%); Orange solid. Mp = 258 °C (decomposition);1H NMR: (400 MHz DMSO-d_6) d = 7.91-7.89 (d, 1H, *J* = 8.0 Hz), 7.60 (s, 1H), 7.42-7.33 (m, 4H), 7.10-7.08 (m, 4H), 4.21-4.18 (m, 2H), 2.92-2.88 (m, 2H), 2.25 (s, 3H) ppm. 13C NMR (100 MHz, DMSO-d_6): d = 194.03, 178.56, 171.56, 166.68, 161.84, 156.79, 150.52, 135.99, 134.93, 131.77, 129.44, 128.88, 122.95, 120.15, 118.36, 115.56, 114.37, 113.86, 112.91, 112.52, 11.32, 45.70, 32.16, 20.96 ppm. MS (ES): m/z 464.0 $[M-H]^-$. HPLC: tr= 4.65 min; conditions: temp =25 °C, mobile phase composed of (A) 70% acetonitrile and (B) 30% water with 0.5% formic acid at a flow rate of 1.0 mL/min; purity:95.9%.

(Z)-2-hydroxy-4-(5-((4-oxo-3-(3-phenylpropyl)-2-thioxothiazolidin-5-ylidene)methyl)furan-2-yl)benzoic acid (9c). (yield: 91%); Orange solid. Mp = 248 °C (decomposition); 1HNMR: (400 MHz, DMSO-d_6) d = 7.90-7.88 (d, 1H, *J* = 8.4 Hz), 7.61 (s,1H), 7.44-7.43 (d, 1H, *J* = 4.0 Hz), 7.36-7.32 (m, 2H), 7.28-7.14 (m, 5H), 4.04-4.01 (m, 2H), 2.66-2.62 (m, 2H), 1.98-1.92 (m, 2H) ppm. 13C NMR (100 MHz, DMSO-d_6): d = 194.23, 171.74, 166.91, 161.79, 156.60, 150.53, 141.20, 134.94, 131.77, 128.68, 128.55, 126.30, 122.99, 120.28, 118.28, 115.53, 113.49, 113.06, 112.39, 44.39, 32.72, 28.16 ppm. MS (ES): m/z 464.0 $[M-1]^-$. HPLC: tr= 4.36 min; conditions :temp = 25 °C, mobile phase composed of (A) 70% acetonitrile and (B) 30% water with 0,5% formic acid at a flow rate of 1.0 mL/min; purity: 96.8%.

(Z)-4-(5-((3-(4-ethylphenethyl)-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan-2-yl)-2-hydroxybenzoic acid (9d). (yield: 80%); Brown solid. Mp = 273 °C (decomposition);^{1}H NMR: (400 MHz, DMSO-d_6) d = 7.89-7.87 (d, 1H, *J* = 8.0 Hz), 7.58 (s, 1H), 7.42-7.41 (d, 1H, *J* = 3.6 Hz), 7.34-7.30 (m, 2H), 7.12-7.11 (m, 5H), 4.18-4.14 (m, 2H), 2.90-2.86 (m, 2H), 2.57-2.52 (q, 2H, *J* = 7.6 Hz), 1.15-1.11 (t, 3H, *J* = 7.6 Hz) ppm. ^{13}C NMR (DMSO-$d_6$100 MHz): d = 193.44, 171.74, 166.58, 161.80, 156.66, 150.42, 149.51, 142.40, 135.13, 134.85, 131.71, 128.92, 128.28, 123.10, 119.97, 118.35,115.49, 113.54, 113.01, 112.39, 45.69, 32.16, 28.16, 15.98 ppm. MS (ES): m/z 478.0 $[M-H]^-$.

HPLC: tr= 4.23 min; conditions: temp =25 °C, mobile phase composed of (A) 70% acetonitrile and (B) 30% water with 0.5% formic acid at a flow rate of 1.0 mL/min; purity:96.5%.

(Z)-4-(5-((3-(3-chloro-4-fluorophenyl)-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan-2-yl)-2-hydroxybenzoic acid (9e): (yield 30%) red solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ11.32 (s, 1H), 7.89 (d, J = 8.1 Hz, 1H), 7.77 (d, J = 6.4 Hz, 1H), 7.68 (s, 1H), 7.62 (t, J = 8.9 Hz, 1H), 7.54 – 7.47 (m, 1H), 7.43 (d, J = 3.3 Hz, 1H), 7.36-7.32 (m,3H)ppm; ^{13}C NMR (DMSO-$d_6$100 MHz):δ 198.0, 171.60, 167.62, 156.59, 155.80, 152.63, 152.07, 135.06, 134.53, 129.32, 126.76, 125.48, 124.06, 122.43, 120.60, 117.57, 115.92, 115.62, 111.62, 111.29, 103.95 ppm MS (ES): m/z 474.0 [M-H]$^{-}$. HPLC: tr= 4.61 min; conditions: temp =25 °C, mobile phase composed of (A) 70% acetonitrile and (B) 30% water with 0,5% formic acid at a flow rate of 1.0 mL/min; purity:97.1%.

(Z)-4-(5-((3-(4-chloro-3-fluorobenzyl)-4-oxo-2-thioxothiazolidin-5-ylidene)methyl)furan-2-yl)-2-hydroxybenzoic acid (9f): (yield 57%) brown solid, ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.28 (s, 1H), 7.85 (d, J = 8.0 Hz, 1H), 7.62 (s, 1H), 7.52 (t, J = 7.8 Hz, 1H), 7.39 (s, 1H), 7.36 – 7.24 (m, 3H), 7.15 (d, J = 8.0 Hz, 1H), 5.18 (s, 2H)ppm, ^{13}C NMR (101 MHz, DMSO-d_6) δ 204.26, 181.73, 176.87, 171.78, 168.65, 166.82, 165.98, 160.40, 146.86, 144.67, 141.68, 140.82, 135.19, 133.36, 129.92, 129.20, 128.86, 126.69, 126.19, 125.22, 123.56, 123.03, 122.07, 56.51ppm. MS (ES): m/z 488.0 [M-H]$^{-}$. HPLC: tr= 4.28 min; conditions: temp =25 °C, mobile phase composed of (A) 70% acetonitrile and (B) 30% water with 0.5% formic acid at a flow rate of 1.0 mL/min; purity:96.6%.

2.2.2 ANTIVIRAL ACTIVITY AND CELL TOXICITY

2.2.2.1 MOLECULAR CLONING

Recombinant viruses were obtained from HIV-1 infected patients failing previous raltegravir-containing regimens enrolled in the raltegravir expanded access programme (MK0518-023). Plasma samples were collected and stored at -80 °C until use. After informed consent was obtained from all patients, viral RNA was extracted using the

QIAamp viral RNA mini kit (Qiagen). The integrase region (codons 1–301) was targeted using the following nested RT–PCR protocol and the following primers: Int**1F**: 5′-CATGGGTACCAGCACACACAAAGG-3′ and Int**1R**: 5′-CCATGTTCTAATCCTCATCCTGTC-3′ for the first PCR round; and primers Int**2F**: 5′-GGAATTGGAGGAAATGAACAAGTAGAT-3′ and Int**2R**:5′-GCCACACA ATCATCACCTGCCATC-3′ for the second PCR round. The first nested RT–PCR reaction was performed in 50 µL using the SuperScript III Platinum High-Fidelity One-Step qRT–PCR system (Invitrogen) with the following thermal profile: 30 min at 50°C and 10 min at 95°C for 1 cycle, 1 min at 95°C, 1 min at 52°C, and 1 min and 10 s at 72°C for 50 cycles, followed by 10 min at 72°C. The nested reaction was performed in 100 µL using PCR SuperMix High Fidelity (Invitrogen) and the following thermal profile: 10 min at 95°C for 1 cycle, 1 min at 95°C, 1 min at 50°C, and 1 min and 10 s at 72°C for 30 cycles, followed by 10 min at 72°C. Amplified products were cloned into pNL(AD8)DeltaIntproviral vector 7 originally derived from an R5 (macrophage-tropic) virus. Individual clones were then sequenced using an ABI PRISM 3100 Genetic Analyser (Applied Biosystems) (GenBank accession numbers JN163873–JN163910).

2.2.2.2 PHENOTYPIC ANALYSES WITH FULLY REPLICATING RECOMBINANT HIV-1 STRAINS AND CLONAL VIRAL VARIANTS SELECTED IN PATIENTS FAILING INTEGRASE INHIBITORS ON TZM-BL CELLS AND HUMAN CD4+ LYMPHOCYTE

Rhodanine derivatives were tested on TZM-bl cells against HIV-1 laboratory strains NL(AD8) (NIH AIDS reagent program, cat nr 11346) and NL4.3 (NIH AIDS reagent program, cat nr 114) as reported above for kuwanon-L. Vehicle (0.1% dimethyl sulfoxide [DMSO])-treated cells served as a negative control. A CCR5 inhibitor (maraviroc) and an integrase inhibitor (raltegravir) were used as positive-control drugs. Pre-incubation virus-compounds was performed either in DMEM with 10 % FBS (complete medium) or in DMEM without FBS.

The antiviral activity and cytotoxicity of compounds **2**, **9e** and **9f** was tested also on freshly purified human $CD4^+$ T lymphocytes obtained from healthy blood donors (after informed consent) on HIV NL4.3 and AD8 laboratory strains and on recombinant viruses bearing mutations associated with resistance to Raltegravir as previously described [140]. Briefly,

$CD4^+$ lymphocytes were obtained by Ficoll-Hypaque gradient centrifugation and seeded in T25 flasks (Costar) at a density of 1.0×10^6 cells/mL in RPMI-1640 (Gibco) supplemented with 50 U/mL penicillin, 50 mg/mL streptomycin and 10% heat-inactivated mycoplasma- and endotoxin-free fetal bovine serum (Euroclone) (complete medium) supplemented with 2 mg/mL phytohaemagglutinin (PHA). After 2 days, non-adherent cells were collected and $CD4^+$ lymphocyte were purified through an immunoaffinity column and CD4 binding antibodies (MiltenyBiotec) and grown in complete medium with 50 mU/mL recombinant interleukin-2 (IL-2) in a 96-well plate. 100 TCID50/mL of each strain were pretreated for 1 h at 37°C with seven serial dilutions (range 1250 to 0.08 nM) of each compound and then added to the cells. Antiviral activity was evaluated testing p24 production (Aalto Bio Reagents, Dublin, Ireland) at day 6 as previously reported [140]. Toxicity on $CD4^+$ T lymphocytes was performed with metabolic XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] test (Sigma-Aldrich) according to the manufacturer's instructions.

2.2.2.3 HSV-1 AND HSV-2 ANTIVIRAL ACTIVITY IN VERO CELLS

African green monkey fibroblastoid kidney cells (Vero, ATCC CCL-81) were grown as monolayers in Eagle's minimal essential medium (MEM) (Gibco) supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% antibiotic-antimycotic solution.

Clinical isolates of HSV-1 and HSV-2 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. HSV-1 and HSV-2 strains were propagated and titrated by plaque assay on Vero cells. A HSV-2 strain with phenotypic resistance to acyclovir was generated by serial passage in the presence of increasing concentrations of acyclovir, as previously described [141]. The criterion used to define an HSV-2 strain as resistant was an IC_{50} greater than 10-fold above that for the parental wild-type virus tested in the same assay. Acyclovir was purchased from Sigma Aldrich (Milan, Italy).

The effect of the rhodanine derivatives on HSV infection was evaluated by plaque reduction assay. Vero cells were pre-plated 24 h in advance in 24-well plates at a density of 10^5 cells/well. Increasing concentrations of compounds (concentration range: 1 nM-50 µM) were mixed with HSV-2 (MOI 0.001 pfu/cell) or HSV-2 acyclovir resistant (MOI 0.001) or HSV-1 (MOI 0.0005) and incubated for 1 hour at 37°C. The mixtures were

subsequently added to the cells, which were then incubated at 37°C for 2 h. The virus inoculum was then removed and the cells washed and overlaid with a medium containing 1.2% methylcellulose (Sigma). After further incubation at 37 °C for 24 h (HSV-2 and HSV-2 R Acy) or 48 h (HSV-1), cells were fixed and stained with 0.1% crystal violet in 20% ethanol and viral plaques counted. The effective concentration producing 50% reduction (EC_{50}) and 90% reduction (EC_{90}) in plaque formation was determined using Prism software by comparing drug-treated with wells treated with medium and solvent. The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} value. The EC_{50} values for inhibition curves were calculated by regression analysis using the software GraphPad Prism (GraphPad Software, San Diego, California, U.S.A.) by fitting a variable slope-sigmoidal dose–response curve.

2.2.2.4 VERO CELL VIABILITY

Cell viability was measured using the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] test (Sigma-Aldrich) according to the manufacturer's instructions. 20.000 cell/well were seeded in 96-well plates were incubated with different concentrations of compounds (concentration range: 1nM - 800 µM) in triplicate under the same experimental conditions described for the antiviral assays. Absorbances were measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. The effect on cell viability at different concentrations of the compound was expressed as a percentage, by comparing absorbances of treated cells with those of cells incubated with culture medium and equal volumes of vehicle. The 50% cytotoxic concentrations (CC_{50}) and 95% confidence intervals (CIs) were determined using Prism software (Graph-Pad Software).

2.2.2.5 HUMAN PAPILLOMA PSEUDOVIRUS (HPV16 PSV) PRODUCTION

Plasmids and 293TT cells used for pseudovirus (PsV) production were kindly provided by John Schiller (National Cancer Institute, Bethesda, MD). 293TT cell line, derived from human embryonic kidney cells transformed with the simian virus 40 (SV40) large T antigen, was cultured in DMEM (Gibco) supplemented with heat inactivated 10% FCS (Gibco), Glutamax-I 1% (Invitrogen) and nonessential amino acids 1% (Sigma Aldrich). The 293TT cell line allows high levels of protein to be expressed from vectors containing

the SV40 origin, due to overreplication of the expression plasmid. Detailed protocols and plasmid maps for this study are available online (http://home.ccr.cancer.gov/lco/pseudovirusproduction.htm). HPV-16 PsVs were produced as previously described [142]. Briefly, 293TT cells were transfected with plasmid expressing the papillomavirus major and minor capsid proteins (L1 and L2, respectively), together with a reporter plasmid expressing GFP. Capsids were allowed to mature overnight in cell lysate. The clarified supernatant was then loaded on top of a density gradient of 27 to 33 to 39% Optiprep at room temperature for 3 h. The material was centrifuged at 28000 rpm for 16 h at 4°C in an SW41.1 rotor (Beckman) and then collected by bottom puncture of the tubes. Fractions were inspected for purity in 10% sodium dodecyl sulfate (SDS)-Tris-glycine gels, titrated on 293TT cells to test for infectivity by GFP detection, and then pooled and frozen at -80 C until needed. The L1 protein content of PsV stocks was determined by comparison with bovine serum albumin standards in Coomassie-stained SDS polyacrylamide gels.

2.2.2.6 HPV-16 INHIBITION ASSAY IN HELA CELLS

HeLa, Human Adenocarcinoma cells purchased from ATCC CCL2, were cultured with DMEM (Gibco) supplemented with heat inactivated 10% FCS (Gibco), Glutamax-I 1% (Invitrogen). Cells were seeded in 96-well plates at a density of 25,000 cells/well, in 100 µl of DMEM supplemented with 10% FCS. The next day, serial dilutions of tested compounds (concentration range: 1 nM-50 µM) were added to pre-plated cells together with dilutions of PsV stock at a multiplicity of infection (MOI) of 0.1. Three days post-infection, fluorescent cells were counted on an inverted Zeiss LSM510 fluorescence microscope.

2.2.2.7 INHIBITION OF ANTIVIRAL ACTIVITY BY FETAL BOVINE SERUM

To evaluate the binding of compounds to albumin also in a cell based assay, the infection with the HIV laboratory strain AD8 was performed in absence or in presence of different concentrations of Fetal Bovine Serum (FBS, Gibco): 2%, 5% and 10%. The representative compound **2** was pre-incubated at different concentrations ranging from 10 µM to 3.2 nM

with 300 TCID50 /mL of AD8 in DMEM supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin, 30 µg/mL DEAE-dextran and FBS at the concentration described above. Maraviroc was used as control. After an hour the mixture was added to 40.000 TZM-bl cells/well plated in a 96-well plate and after two days the infection was quantified using a CPRG assay (Roche) as previously described.

2.2.2.8 INHIBITION OF ANTIVIRAL ACTIVITY BY PURIFIED BOVINE SERUM ALBUMIN

To confirm the binding of compound **2** to serum albumin, infection was performed in presence of different concentrations of purified bovine serum albumin (BSA, Sigma Aldrich) starting from the concentration that is usually present in FBS serum (35 mg/mL, 1X) and increasing to a five-fold concentration (175 mg/mL) up to a ten-fold concentration (350 mg/mL). In this experiment the pre-incubation between HIV and two selected concentrations of compound **2** and maraviroc (2000 nM and 400 nM) was performed in DMEM without FBS and supplemented with BSA at the concentrations described above. After one hour, the mixture was added to 40.000 TZM-bl cells/well plated in DMEM supplemented with 50 µg/mL penicillin, 50 µg/mL streptomycin, 30 µg/mL DEAE-dextran and 10% FBS in a 96-well plate. After two days the infection was evaluated through a CPRG assay.

2.2.2.9 ANTIVIRAL ACTIVITY OF COMPOUND 2 IN A GEL FORMULATION

Antiviral activity of compound **2** on NL4.3 HIV strain was evaluated also in a gel formulation. To reproduce more physiological conditions, TZM-bl cells were seeded into transwells and the gel was applied onto the cell monolayer at 50% fixed concentration with serial drug dilutions as previously described. Briefly, 40.000 TZM-bl cells were plated into each transwell apical chamber of a 24-well plate (pore size 3µm and diameter 6,5 mm, Corning) containing medium in the bottom plate and cultured overnight. For efficacy testing, 60 TCID50 of virus was added to each apical well in the presence of 100 µL of compound **2** gel, tenofovir gel or blank gel at the final drug concentrations of 25µM, 10

µM and 5 µM. Inhibition of infection was determined as previously based on deviations from blank gel.

2.2.2.10 TIME OF ADDITION ASSAY ON COMPOUND 2

A time-of-addition experiment was performed as previously described for kuwanon-L, with minor modifications. To analyze the very early steps of infection, we performed a pre-incubation step with the virus and a representative compound (**2**) for 1 h at 37°C, prior to cell infection. For the following 4 h, antiretroviral compounds inhibiting distinct viral replication steps (IgGb12, maraviroc, T20, AZT, and dolutegravir) and compound **2** were added at time zero and after 60, 75, 90, 120, 150, 180, 210, and 240 min. To ensure the complete inhibition of viral replication occurred, we used a 40-fold IC_{50}, as previously evaluated for each compound on TZM-bl cells (7.5 µg/ml IgGb12; 0.7 µM maraviroc; 1.6 µM T20; 3.2 µM AZT; 1 µM dolutegravir and 400 µM compound **2**).

2.2.3 ADME ASSAY

ADME assays were performed in collaboration with Prof. Maurizio Botta in the Department of Biotechnology, Chemistry and Pharmacy of the University of Siena.

2.2.3.1 CHEMICALS AND EXCIPIENTS

All solvents, L-α-phosphatidylcholine, hydroxyethylcellulose (HEC), and propionic acid were purchased from Sigma-Aldrich Srl (Milan, Italy). Dodecane was purchased from Fluka (Milan, Italy). Pooled Male Donors 20 mg/mL HLM were from BD Gentest-Biosciences (San Jose, California). Milli-Q quality water (Millipore) was used. Hydrophobic filter plates (MultiScreen-IP, Clear Plates, 0.45 µm diameter pore size), 96-well microplates, and 96-well UV-transparent microplates were obtained from Millipore.

2.2.3.2 UV/HPLC-MS METHOD

LC analyses were performed by Agilent 1100 LC/MSD VL system (G1946C) (Agilent Technologies) constituted by a vacuum solvent degassing unit, a binary high-pressure gradient pump, an 1100 series UV detector and a 1100 MSD model VL benchtop mass

spectrometer was used. The Agilent 1100 series mass spectra detection (MSD) single-quadrupole instrument was equipped with the orthogonal spray API-ES (Agilent Technologies). Nitrogen was used as nebulizing and drying gas. The pressure of the nebulizing gas, the flow of the drying gas, the capillary voltage, the fragmentor voltage and the vaporization temperature were set at 40 psi, 9 L/min, 3000 V, 70 V and 350 °C, respectively. UV detection was monitored at 254 nm. The LC-ESI-MS determination was performed by operating the MSD in the negative ion mode. Spectra were acquired over the scan range m/z 50-1500 using a step size of 0.1 u. Chromatographic analysis was performed using a Kinetex EVO C18 100A column (150 x 4.6 mm, 5 µm particle size) at room temperature. Analysis was carried out using a gradient elution of acetonitrile (ACN) and an aqueous solution (HCOOH 0.1% v/v): t = 0 min ACN 5%, t = 3 min ACN 5%, t = 12 min ACN 95%, t =25 min ACN 95%. The analysis was performed at flow rate of 0.6 mL/min and injection volume was 20 µL.

2.2.3.3 AQUEOUS SOLUBILITY

Each solid compound (1 mg) was added to 1 mL of water. Each sample was mixed at 20 °C, in a shaker water bath for 24 h. The resulting suspension was filtered through a 0.45 µm nylon filter (Acrodisc). The concentration of compound in solution was determined by UV/LC-MS (performed in triplicate) by comparison with the appropriate calibration curve that was obtained from samples of the compound dissolved in methanol at different concentrations.

2.2.3.4 PARALLEL ARTIFICIAL MEMBRANE PERMEABILITY ASSAY (PAMPA)

Donor solution (0.5 mM) was prepared by diluting 1 mM dimethylsulfoxide (DMSO) compound stock solution using phosphate buffer (pH 7.4, 0.025 M). Filters were coated with 5µL of a 1% (w/v) dodecane solution of L-α-phosphatidylcholine. Donor solution (150 µL) was added to each well of the filter plate. To each well of the acceptor plate were added 300 µL of solution (50% DMSO in phosphate buffer). All compounds were tested in three different plates on different days. The sandwich was incubated for 5 h at room temperature under gentle shaking. After the incubation time, the plates were separated, and

samples were taken from both receiver and donor sides and analyzed using UV/HPLC-MS gradient method above reported. Permeability (Papp) for PAMPA, were calculated according to the following equation, obtained from Wohnsland and Faller [143] and Sugano [144] equation with some modification in order to obtain permeability values in cm s-1,

$$P_{app} = \frac{V_D V_A}{(V_D + V_A) A t} - \ln(1 - r)$$

where V_A is the volume in the acceptor well, V_D is the volume in the donor well (cm^3), A is the "effective area" of the membrane (cm^2), t is the incubation time (s) and r the ratio between drug concentration in the acceptor and equilibrium concentration of the drug in the total volume (V_D+V_A). Drug concentration is estimated by using the peak area integration.

2.2.3.5 METABOLIC STABILITY IN HLM (HUMAN LIVER MICROSOMES)

The incubation mixture (total volume of 500 µL) was constituted by the following components: HLM (0.2 mg/mL), a NADPH regenerating system (NADPH 0.2 mM, $NADPH^+$ 1 mM, D-glucose-6-phosphate 4 mM, 4 unit/mL glucose-6-phosphate dehydrogenase and $MgCl_2$ 48 mM), 50 µM of each compound in DMSO and phosphate buffer (pH 7.4, 25 mM, up to a final volume of 500 µL). The mixture was incubated at 37 °C for 1 h. The reaction was cooled down and quenched with acetonitrile (1.0 mL). After centrifugation (4000 rpm, 10 min), the supernatant was taken, dried under nitrogen flow, suspended in 100 µL of methanol and analyzed by UV/LC-MS to determine the percentage of compound that was not metabolized.

2.2.3.6 SOLUBILITY ASSESSMENT

The solubilizing capacity of formulation media was measured in the presence of 2% v/v of DMSO. Stock solution of compound **2** and tenofovir, were added to obtain a final concentration of 50, 25 and 10 µM in the final volume of 1 mL of buffer solution (pH 7.4 25 mM). After sonication, the samples were shacked in a shaker bath at room temperature

for 24 h to reach equilibrium conditions. Each pre-gel solution was analyzed before and after filtration by a 0.45-µm nylon filter (Acrodisc). The solubilized compound was determined using LC-UV-MS method above reported.

2.2.3.7 GEL FORMULATION

All the semi-solid formulations were prepared adding at the pre-gel solution reported above hydroxyethyl cellulose (HEC) (1.8% w/v). In all samples, 0.2% v/v propionic acid was added to aqueous solution in order to preserve form bacteria and mold contamination. HEC was dispersed in the pre-gel solution at 25 °C under magnetic stirring, overnight.

2.2.3.8 STORAGE STABILITY AND RHEOLOGICAL CHARACTERIZATION

Gel containing compound **2** was stored at 25 °C in the dark. At predetermined data points, the formulations were visually inspected to evidence aggregate formations and the pH was measured. Rheological evaluations were performed using a rotational viscometer (Bohlin Model Visco 88, Bohlin Instruments, UK). Appropriate measuring spindles (C14 DIN) were used during viscosity measurements. Samples were loaded into the cup and allowed to equilibrate for 10 min at desired temperature (25 ± 0.5 °C) and apparent viscosity (mPa s) was determined using a shear rate of 552 s^{-1} over a 1 month period.

2.2.3.9 BINDING FLUORIMETRIC ASSAY

The binding of each compounds to HSA (human serum albumin) and BSA (bovine serum albumin) were monitored by fluorescence spectroscopy in order to determine the two dissociation constant (Kd). A quantitative analysis of the potential interaction was performed by fluorimetric analysis using 96 multiwell plates: in each well a fixed concentration of HSA or BSA (10µM in phosphate buffer 1 mM), was added with different amounts of tested compound (0.1µM to 500µM by stock solutions in DMSO). Plates were gently shacked and after allowing 30 minutes at room temperature for equilibration, after excitation at 295 nm, spectra were recorded from 300 to 400 nm with a Perkin Elmer EnVisionMultilabel Reader 2014 spectrofluorimeter, and acquired with EnVision Manager

ver.1.13 software. The obtained fluorescence quenching percentages were plotted against drug concentrations and the relative K_D values were obtained using GraphPad software.

3 RESULTS

3.1 RESULTS (PART I)

3.1.1 HOMOGENEOUS TIME-RESOLVED FLUORESCENCE (HTRF) ASSAYS

In a previous work, docking studies on the sucrose binding pocket of HIV integrase aiming the selection of novel allosteric inhibitors, identified kuwanon-L as the most promising ligand in a library of 473 natural compounds [136]. In this thesis kuwanon-L has been evaluated in biochemical assays to confirm these computational results. Firstly, it was investigated if this compound was able to inhibit the HIV-1 IN catalytic activity, both in presence and in the absence of the LEDGF/p75 protein, as observed for other allosteric inhibitors such as LEDGINs [137]. Results showed that kuwanon-L, similarly to the LEDGIN derivative CX0516 used as a control, inhibited the HIV-1 IN strand-transfer catalytic activity both in the presence and in the absence of the LEDGF/p75 protein with comparable IC_{50} values of 42 µM and 34 µM, respectively (Table **1**). Secondly, we tested kuwanon-L to evaluate its ability to target the binding between integrase and LEDGF/p75. As shown in Table **1**, kuwanon-L also inhibits the integrase/LEDGF/p75 binding with an IC_{50} value of 22 µM, which is similar to that of the HIV-1 IN activity. Finally, we tested kuwanon-L in a HTRF IN subunit exchange assay in which, at increasing concentration of inhibitor, when a compound inhibits IN dimerization, the HTRF signal decreases, whereas when a compound promotes IN multimerization the HTRF signal increases. As shown in Figure **8**, when the maximum HTRF signal, obtained at 100 µM concentrations of both CX0516 (used as positive control) and kuwanon-L, was set as 100% of HTRF signal, CX0516 and kuwanon-L were shown to promote IN multimerization with concentrations of compound that were able to inhibit the multimerization increase by 50% (MI50 values) of 20 µM and 38 µM, respectively. The obtained results are in agreement with the hypothesis that kuwanon-L binds to the sucrose pocket, near to the LEDGIN binding pocket, and inhibits both the IN-LEDGF binding and the IN catalytic activity through the compound-mediated premature protein multimerization.

Compound	HIV-1 IN LEDGF-dependent IC_{50} (µM)	HIV-1 IN LEDGF-independent IC_{50} (µM)	HIV-1 IN-LEDGF binding IC_{50} (µM)
Kuwanon-L	42±3	34±0.5	22±0.5
CX0516	9±2	10±0.5	13±4
RAL	0.058±0.01	0.061±0.01	n.d.

Table 1. Effect of kuwanon-L, CX0516 and raltegravir on HIV-1 IN LEDGF-dependent and –independent strand-transfer activity and on HIV-1 IN-LEDGF binding.

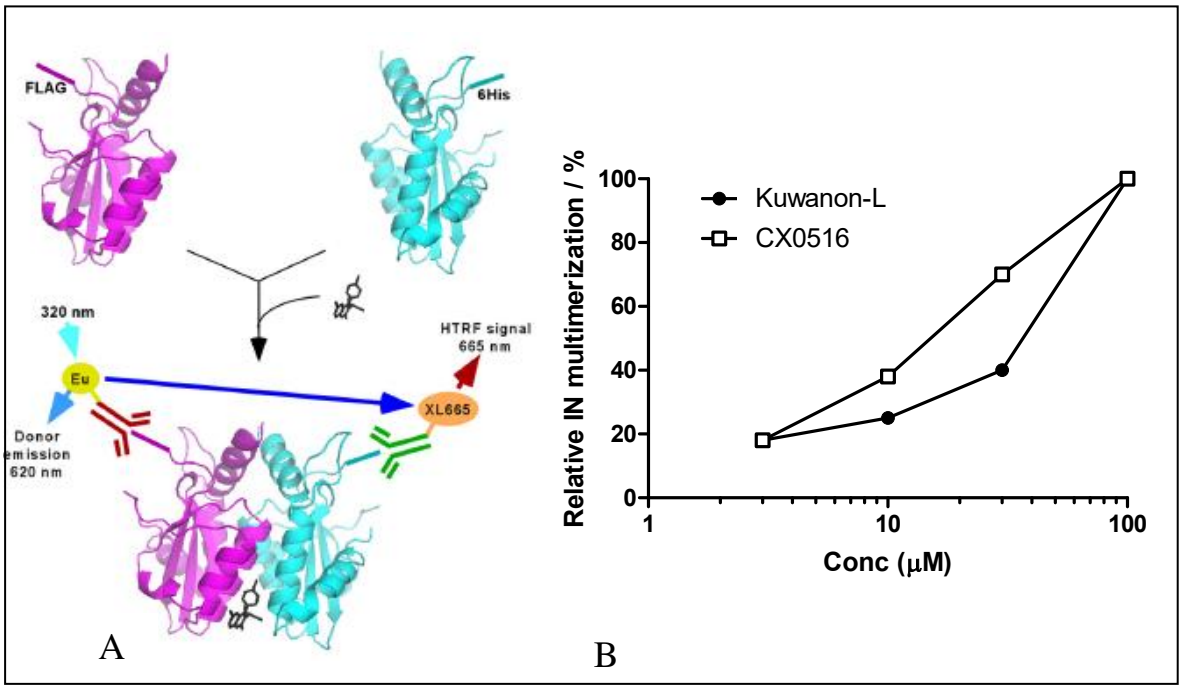


Figure 8. Effects of kuwanon-L and CX0516 on integrase multimerization.

A) HTRF assay design. The assay monitors the interaction between two integrase molecules: one containing His6 and the other containing the FLAG tag. Anti-His_6-XL665 and anti-FLAG-EuCryptate antibodies allow fluorescence energy transfer upon interaction of two full-length, wild type HIV-1 integrase proteins, one containing an N-terminal His_6 tag and the other, an N-terminal FLAG tag. B) Effects of kuwanon-L (●) and CX0516 (□) on HIV-1 IN multimerization. Each data point represents the mean of three independent reactions.

3.1.2 CELL-BASED HIV REPLICATION ASSAY

3.1.2.1 CELL BASED ASSAY ON FULLY REPLICATING HIV-1 LABORATORY STRAIN

We wanted to verify whether kuwanon-L was able to inhibit HIV-1 replication in cell-based assays. For this reason, we tested it on a fully replicating HIV-1 laboratory strain (NL-4-3) and observed that kuwanon-L was able to inhibit viral replication with an IC_{50} value of 1.9 µM (Figure **9**), showing no toxic effect at the highest tested concentration (CC_{50} >40 µM). In this case, the HIV-1 IN inhibitor dolutegravir was used as control.

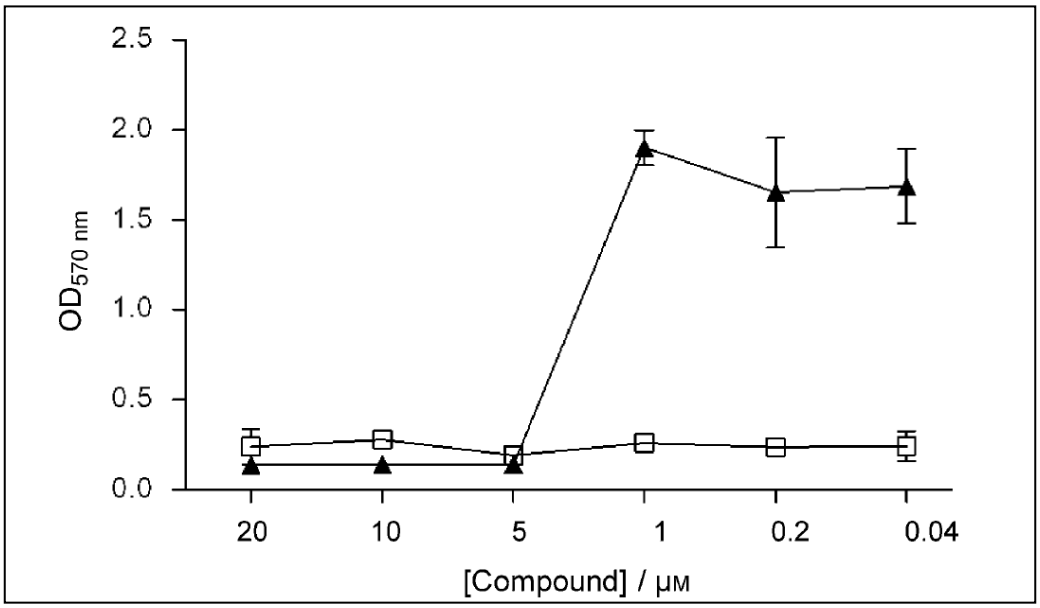


Figure 9. Effects of kuwanon-L (▲) and Dolutegravir (□) on HIV- 1 viral replication in cell culture. Optical density is reported in the ordinate. Mean values and standard deviations are shown.

3.1.2.2 CELL BASED ASSAY ON INSTI-RESISTANT MUTANTS

An important characteristic of novel antiretrovirals for HIV treatment is the lack of cross-resistance with mutations for established drugs. Since kuwanon-L was shown to bind HIV integrase, cross-resistance with INSTIs has to be excluded. Retain activity of kuwanon-L on HIV molecular clones bearing the most clinically relevant resistance mutations for INSTIs (Q148H, G140S and Y143R), was, then, evaluated. The activity of kuwanon-L and a reference compound (raltegravir) was quantified by the fold change (FC) ratio between the IC_{50} value on resistant virus and the IC_{50} value with the wild type one (wt), a measure of the compound efficacy on resistant mutant viruses (Table **2**). Interestingly, kuwanon-L

displayed an FC ratio of 1 or lower against both resistant viruses (IC_{50}: WT= 1,9 µM; Q148H,G140S = 1,7 µM; Y143R = 2 µM) contrasting the results with reference compound raltegravir (IC_{50}: WT= 10 nM; Q148H,G140S= 800 nM; Y143R = 460 nM).

Compound	**NL 4.3 WT EC_{50}(nM)**	**Q148H, G140S EC_{50}(nM)**	**Y143R EC_{50}(nM)**
Kuwanon-L	1900±25	1700±30	2000±43
RAL	10±2	800±12	460±10

Table **2.** Activity of kuwanon-L and raltegravir on a reference laboratory wild type virus (NL4.3) and two recombinant viruses bearing mutations that confer resistance to raltegravir.

3.1.2.3 TOA EXPERIMENT

In the attempt to further characterize the mechanism of action of kuwanon-L, we perform a time of addition experiment (TOA). This type of experiment has been widely used to pinpoint the stage of the HIV-1 virus life cycle that is inhibited by antiretrovirals. In this study, a series of HIV replication inhibitors of various mechanistic classes were profiled in TOA experiments in comparison with kuwanon-L (Fig. **10**). The compounds were added at different time points after infection of TZM-bl cells with an env-pseudotyped HIV-1, and infection was measured at 48 h post-infection. In our TOA assay the target of the antiviral compound kuwanon-L was identified by comparing its activity in the time scale to that of reference drugs maraviroc (CCR5 coreceptor inhibitor), lamivudine (RT inhibitor) and dolutegravir (DTG, integrase inhibitor). As shown in Figure **10**, this experiment revealed a very particular profile characterized by a double loss of activity highlighted by the two red arrows. In a careful analysis of the inhibition profile, we noted that, whereas the second time point of drug addition is congruent with the inhibition of viral IN, as was already known, the first is unexpected, but consistent with the inhibition of the RT. In fact the first loss of activity due to kuwanon-L was similar to that produced by the RT inhibitor lamivudine (Figure **10**). We, therefore, speculated that it could be due to the ability of kuwanon-L to bind different targets during viral infection.

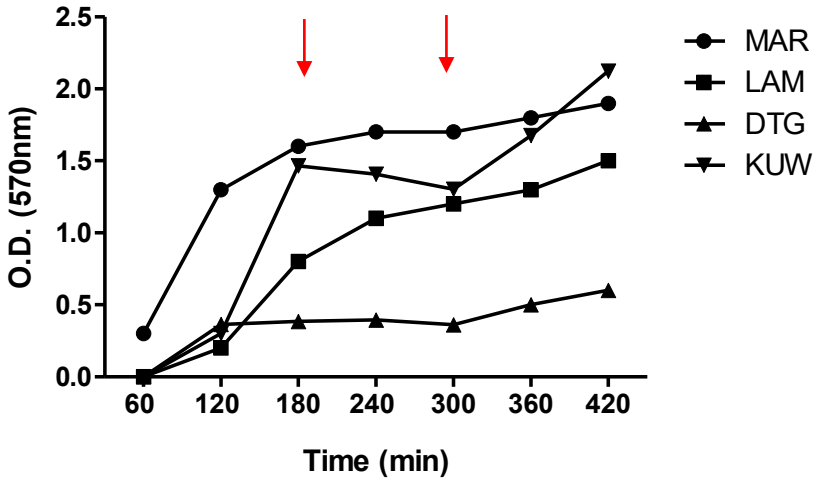


Figure 10. Time of Addition assay. MAR= maraviroc; LAM= lamivudine; DTG=dolutegravir; KUW= kuwanon-L; O.D.= optical density.optical density measured on TZM-bl indicator cell lines 48 h post-infection by use of the CPRG assay.

3.1.3 BIOCHEMICAL ASSAYS ON RT FUNCTIONS

Following the idea that kuwanon-L could actually bind both IN and RT, and already having in our hands the enzymatic activity on IN, we tested it also on RT in order to confirm what suggested by TOA. The assay conducted has served to evaluate its ability to inhibit both HIV-1 RT-associated RNA-dependent DNA polymerase (RDDP) and Ribonuclease H (RNase H) function, using efavirenz (EFV) and the RNase H active site inhibitor RDS1759 as reference controls [145](Table **3**). Effectively, kuwanon-L displayed a good potency in the disruption of both HIV-1 RT-associated activities as it inhibits RNase H function with an IC_{50} of 0.57 µM and the RDDP function, with an IC_{50} value of 0.99 µM. These results are very interesting and suggest that the natural product kuwanon-L is able to inhibit different RT and IN functions at the same time.

Compound	HIV-1 RT RDDP IC_{50} (μM)[a]	HIV-1 RT RNase H IC_{50} (μM)[b]
Kuwanon-L	0.99 ± 0.25	0.57 ± 0.06
Efavirenz	0.025 ± 0.0005	> 50
RDS1759	> 50	7.4 ± 0.2

Table **3**. Effect of kuwanon-L and control drugs on HIV-1 RT-associated activities.

[a]Concentration of compound required to inhibit HIV-1 RT- associated RDDP activity by 50%.

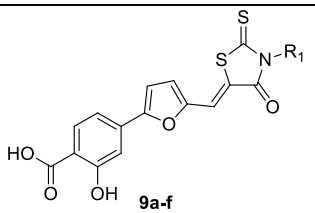
[b]Concentration of compound required to inhibit HIV-1 RT-associated RNase H activity by 50%.

3.2 RESULTS (PART II)

3.2.1 ANTIVIRAL ACTIVITIES OF RHODANINE DERIVATIVES

3.2.1.1 ANTIVIRAL ACTIVITY AND CYTOTOXICITY ON TZM-BL CELL LINE INFECTED WITH HIV-1 LABORATORY STRAINS

The novel series of synthesized rhodanine compounds (Table **4**) was tested *in vitro* to evaluate the ability of these molecules to inhibit HIV replication on human TZM-bl cells infected with HIV-1 NL4.3 (CXCR4-tropic strain) or AD8 (CCR5-tropic strain). All compounds showed antiviral activity at nanomolar concentrations with the best compound, namely **2**, having an EC_{50} of 6.9 nM and 4 nM on AD8 and NL4.3 HIV strains, respectively. Compound **9a** also showed a very interesting inhibitory effect (7.5 nM and 5.4 nM on AD8 and NL4.3 HIV strains, respectively) but a worst safety profile with respect to the other compounds. Indeed, almost all compounds of the series showed low cytotoxicity in TZM-bl cell line, with CC_{50} values higher than 20 µM. Only for compound **9a** and **9d** lower CC_{50}values were found (2.2 µM and 12.3 µM, respectively). Interestingly all compounds displayed a reduction of antiviral activity when pre-incubation with virus was performed in presence of complete medium containing fetal bovine serum (FBS). The activity of compound **2** against AD8 strain decreased 90 times in presence of serum, shifting from 6.9 nM to 650 nM. Also compound **1** showed a high loss of activity against NL4.3 strain, with an EC_{50} that moved from around 50 nM to 3 µM, corresponding to a 64-fold activity reduction. Compounds **9b-f** showed high to moderate loss of activity, in the range of 10 to 38 times. Commercially available drugs such as maraviroc and raltegravir did not show any alteration of antiviral activity when pre-incubated with serum.



9a-f

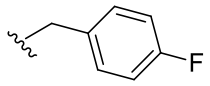
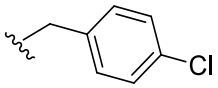
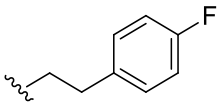
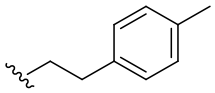
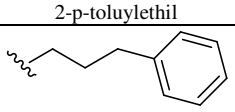
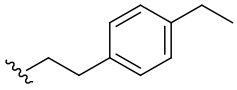
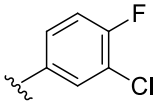
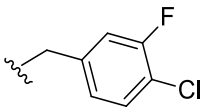
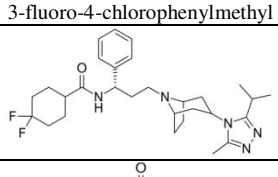
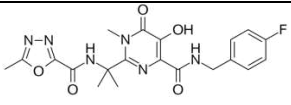
Cpd ID	R_1	AD8			NL4-3			Cytotoxicity
		EC_{50} (nM)[a]	EC_{50} (nM)[b]	ratio	EC_{50} (nM)[a]	EC_{50} (nM)[b]	ratio	CC_{50} (µM)
1	 p-fluorophenilmethyl	32.3±3	1395.0±10	43	49.5±2	2951.0±15	64	>20
2	 p-clorophenilmethyl	6.9±1	650±21	94	4±2	380±9	95	>20
9a	 2p-phluorophenileti l	7.5±3	115.5±24	15	5.4±3	204±12	38	2.2
9b	 2-p-toluylethil	46.7±9	404.0±20	9	23±4	457±5	20	>20
9c	 3-phenylpropyl	27.5±4	378±15	14	15.3±2	457±11	30	>20
9d	 2-p-ethylphenylethyl	36±3	360±9	10	23.4±3	228±10	10	12.3±3
9e	 3-cloro-4-fluorophenyl	26±6	400±14	15	30±1	420±7	15	>20
9f	 3-fluoro-4-chlorophenylmethyl	20±4	560±10	28	17±2	570±9	34	>20
MAR		36.7±6	25.5±5	1	>20000	>20000	n.c	>20
RAL		6.5±1	13.5±2	2	21±3	16±4	1	>20

Table 4. Antiviral activity of the novel series of rhodanine derivatives on TZM-bl cell line infected with two laboratory strains (NL4.3 and AD8). Maraviroc (MAR) and raltegravir (RAL) were used as reference compounds. Values represent mean±S.D of three independent experiments. Differences between pre-incubation in complete medium containing FBS are shown, together with the corresponding fold change (ratio). EC_{50} = Half maximal effective concentration. CC_{50} = Half maximal cytotoxic concentration. [a]Pre-incubation in medium without fetal bovine serum (FBS).
[b] Pre-incubation in complete medium with fetal bovine serum (FBS)

3.2.1.2 ANTIVIRAL ACTIVITY AND CYTOTOXICITY ON HUMAN $CD4^+$ LYMPHOCYTES

The antiviral activity and cytotoxicity of three promising compounds (**2**, **9e** and **9f**) were evaluated also on freshly purified human $CD4^+$ cells. $CD4^+$ T lymphocytes were infected with the two reference laboratory wild type strains NL4.3 and AD8, as well as with two recombinant viruses bearing mutations of specific integrase residues (Q148H, G140S and Y143R) that confer resistance to raltegravir (RAL). As reported in Table **5**, all compounds showed activity at nanomolar concentrations (6-25 nM) on both NL4.3 and AD8 HIV strains also in CD4+ human lymphocytes, with CC_{50} in the range of 800 nM and selectivity indexes ranging from 73 (**9f**) to 34 (**9e**). Interestingly, in contrast to raltegravir, all compounds maintained similar EC_{50} values also on integrase mutating recombinant viruses.

Compound	NL 4.3 WT EC_{50}(nM)	AD8 WT EC_{50}(nM)	Q148H, G140S EC_{50}(nM)	Y143R EC_{50}(nM)	CC_{50} (nM)	SI
2	13±3	11±1	9±2	8±3	810±20	62
9e	25±2	25±4	29±6	70±7	850±30	34
9f	6.2±5	16±3	12±3	19±2	800±15	73
RAL	3±2	2±3	800±12	460±10	n.a	n.a

Table 5.CC_{50} and EC_{50} values of three selected compounds (**2**, **9e** and **9f**) and the control drug raltegravir (RAL) on human $CD4^+$ T lymphocytes. Infections were performed with two reference laboratory wild type viruses (NL4.3 and AD8) and with two recombinant viruses bearing mutations that confer resistance to raltegravir. SI= Selectivity Index.

3.2.1.3 ANTIVIRAL ACTIVITY AND CYTOTOXICITY ON VERO CELL LINE INFECTED WITH HSV-2 LABORATORY STRAIN AND HELA INFECTED WITH HPV-16 PSVs

In the attempt to identify novel agents with a broad spectrum of activity on the most common sexually transmitted infections, the rhodanine derivatives were also tested *in vitro* toward HSV-2 on Vero cells and HPV-16 in HeLa cells. No antiviral activity was shown for all the compounds up to 50 μM for HPV-16. Instead, the compounds were subjected to a plaque reduction assay against HSV-2, in which they were pre-incubated with the virus for 1h at 37°C; then the mixtures were added on cells. As shown in Table **6**, all the compounds were found to be active at nanomolar concentrations and showed EC_{50} values lower than that obtained for acyclovir, which was used as reference compound. Particularly, the tested rhodanines proved to be up to 130-fold more active than acyclovir (compound **9b**) while presenting a very good safety profile, with selectivity indexes ranging from 600 (**9d**) to 9400 (**2**). Again, compound **2** showed the best HSV-2 antiviral profile, with an EC_{50} in the low nanomolar range (11.9 nM) and the highest selectivity index. Therefore, we further investigated the antiviral activity of this compound on HSV-1 and on an acyclovir resistant strain of HSV-2. Compound **2** was found to be about 10 times less active on HSV-1 with respect to HSV-2 (Table **7**). Anyway, this rhodanine derivative showed an EC_{50} (132 nM) slightly lower than that obtained for acyclovir (168 nM) and maintained a good safety profile (SI > 752). Moreover, compound **2** completely retained its antiviral activity toward the acyclovir resistant strain of HSV-2. However, as expected, compound **2** showed a remarkable reduction of HSV-2 inhibitory activity (among seventy times) when pre-incubated with the virus in presence of serum (table **7**).

Compound	HSV-2		Cytotoxicity (Vero cells)	SI
	EC_{50}(nM)[a]	EC_{90} (nM)[b]	CC_{50} (μM)	
1	168±14	2107±91.8	224±78.5	1134
2	11.9±5.7	481±29.9	112±11.6	9403
9a	345±16.8	426±56.3	300±32	869
9b	4.89±1.6	57.8±22.6	11±9.5	2209

9c	52±2.1	422±22.5	204.7±30.1	3936
9d	28±7.1	140±61.2	17±4.31	604.3
9e	50.3±10.5	206±85.5	100±15	1988
9f	26.4±11.1	153±15.9	7.5±2.98	298
ACV	622±21.1	-	732±46.2	1177

Table 6. Antiviral activity of the rhodanine derivatives on Vero cell line infected with HSV-2. Acyclovir (ACV) was used as reference compound. EC_{50} =Half maximal effective concentration. CC_{50} = Half maximal cytotoxic concentration. The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} value. Values represent mean ±S.D of two independent experiments.

Compound	**Virus**	**EC_{50} (nM)**	**Vero cell CC_{50} (µM)**	**SI**
2	HSV-1	131±59	98±23	752
ACV	HSV-1	168±41	698±72	4155
2	HSV-2 with serum	719±157	n.d.	n.d.
2	HSV-2 acyclovir resistant	10.7±6.3	111.9±11	10400
ACV	HSV-2 acyclovir resistant	31915±1159	732±46	23

Table 7. Antiviral activity of compound **2** on Vero cell line infected with HSV-1, HSV-2 with serum and HSV-2 acyclovir resistant strains. Acyclovir (ACV) was used as reference compound. EC_{50} =Half maximal effective concentration. CC_{50} = Half maximal cytotoxic concentration. The selectivity index (SI) was calculated by dividing the CC_{50} by the EC_{50} value.

3.2.1.4 BINDING TO FETAL BOVINE SERUM

In order to better elucidate the mechanism underlying the loss of activity of rhodanine derivatives when pre-incubated with complete medium, the selected compound **2** and the control drug maraviroc were pre-incubated with the AD8 HIV-1 laboratory strain in presence of concentrations of FBS ranging from 0% to 10%. As shown in Figure **11**, the increase of FBS concentration from 2% to 10% corresponded to a progressive loss of activity, corroborating the inhibitory effect of serum on compound **2**. Such alteration was not seen on maraviroc, whose antiviral efficacy did not change with the modification of FBS concentration.

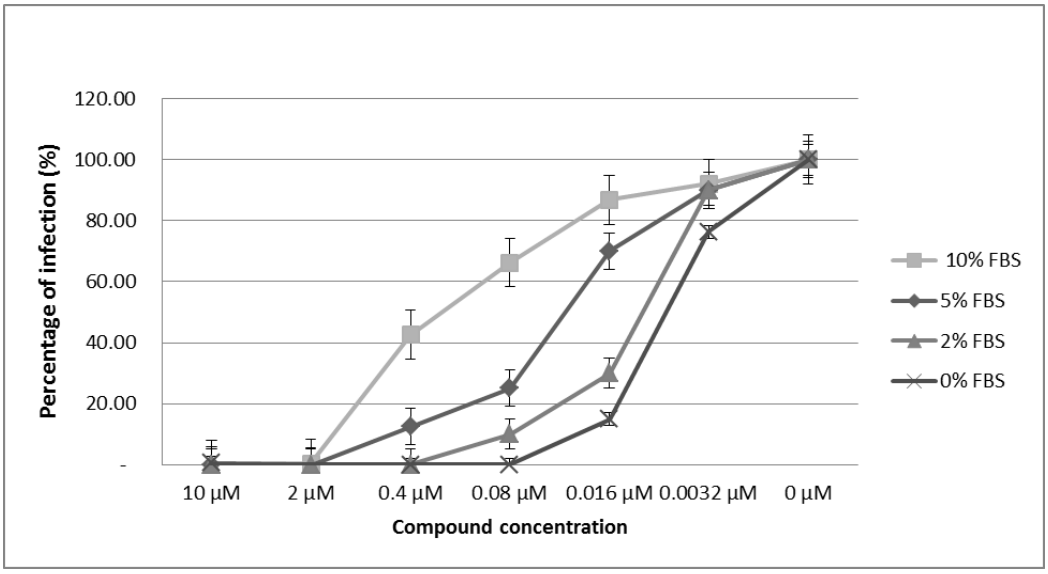


Figure 11. Effect of different concentrations of Fetal Bovine Serum (0%, 2%, 5% and 10%) on the antiviral activity of compound **2** in TZM-bl cells infected with AD8 HIV-1 laboratory strain. Experiments were performed in triplicates.

3.2.1.5 BINDING TO BOVINE PURIFIED SERUM ALBUMIN

To evaluate if the inhibitory effect of serum on compound **2** was due to the binding to serum albumin, compound **2** and the reference drug maraviroc were pre-incubated with the virus and increasing concentrations of purified bovine serum albumin (BSA), starting from the physiological concentration present in the bovine serum up to ten-fold values. A physiological concentration of albumin altered only modestly the antiviral activity of compound **2** (20% loss), when tested at a concentration of 2 µM (Figure **12**). However, at a concentration of 400 nM the loss of the compound's activity was found to be stronger (about 50%) and increasing the BSA concentration of 5 to 10 folds led to a complete loss of activity. As expected, the antiviral activity of the control drug maraviroc was not influenced by any concentration of BSA.

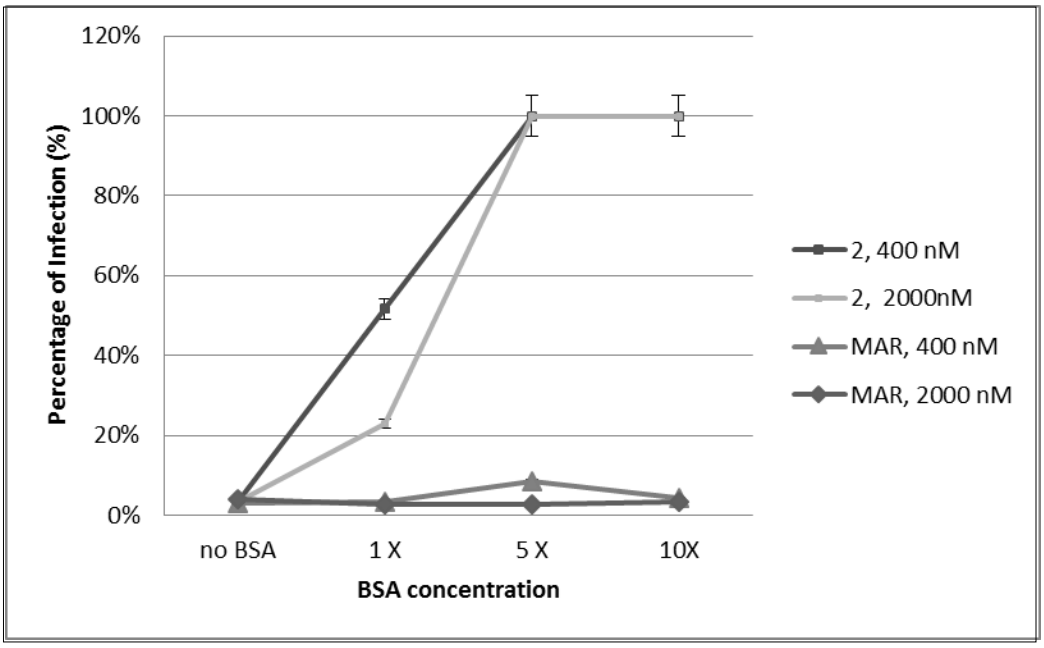


Figure 12. Effect of different concentrations of purified Bovine Serum Albumin (absence = No BSA, 35 mg/mL = 1X, 175 mg/mL = 5X and 350 mg/mL = 10X) on two selected concentrations of compound **2** and maraviroc on TZM-bl cells infected with AD8 HIV-1 strain.

3.2.1.6 ANTIVIRAL ACTIVITY OF COMPOUND 2 IN A MICROBICIDE GEL FORMULATION

We evaluated the activity of compound **2** and the reference compound tenofovir in a microbicide gel formulation using a transwell experiment. Such experiment allowed to test the compounds in a gel formulation at a high concentration (50%) for all drug dilutions without affecting cell viability and antiretroviral activity evaluation. As shown in Fig **13**, compound **2** maintained its antiviral activity when used in a gel formulation, despite the increase in EC_{50} observed in the transwell experiment (EC_{50} of 13 µM) which was however less than 3-fold higher than that observed for tenofovir (EC_{50} of 4.5 µM).

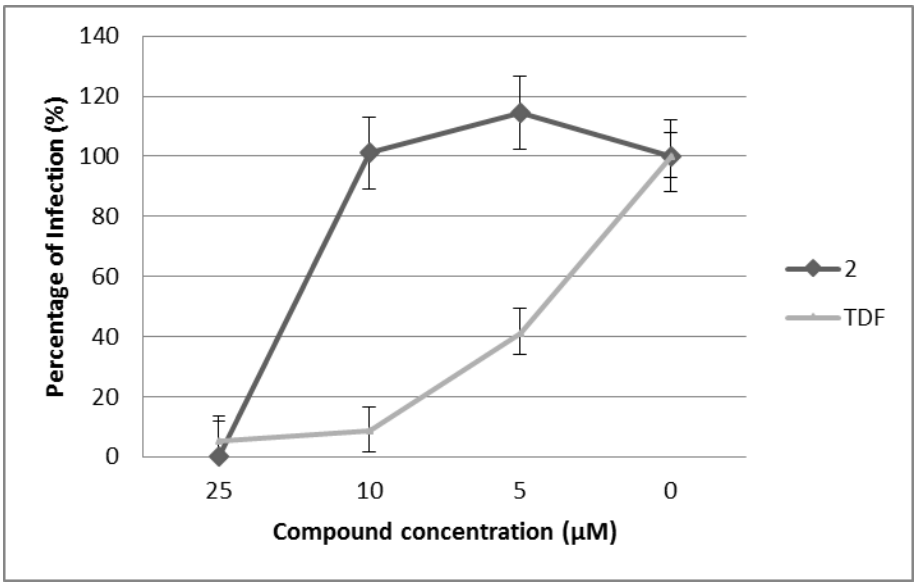


Figure 13. Anti-HIV-1 activity of compound **2** (black line) and tenofovir (TDF, grey line) in gel formulation on human TZM-bl cell line. Each concentration of both compounds was evaluated in triplicate.

3.2.1.7 TIME OF ADDITION ASSAY ON COMPOUND 2

A time-of-addition experiment was carried out to determine the exact inhibition target of this novel series of rhodanine derivatives. In this case we chose the following reference compounds with known mechanism of action: IgGb12 (an anti-gp120 antibody), maraviroc (a CCR5-receptor antagonist), T20 (a fusion inhibitor that acts by binding to the envelope glycoprotein gp41), AZT (a NRTI that inhibits the reverse transcription process) and dolutegravir (an integrase inhibitor). As the profile of IgGb12, an antibody that targets the

CD4-binding site of gp120, was the same as that seen with compound **2** (Fig. **14**), we further supposed that this compound acted in a very early phase of HIV infection such as entry process.

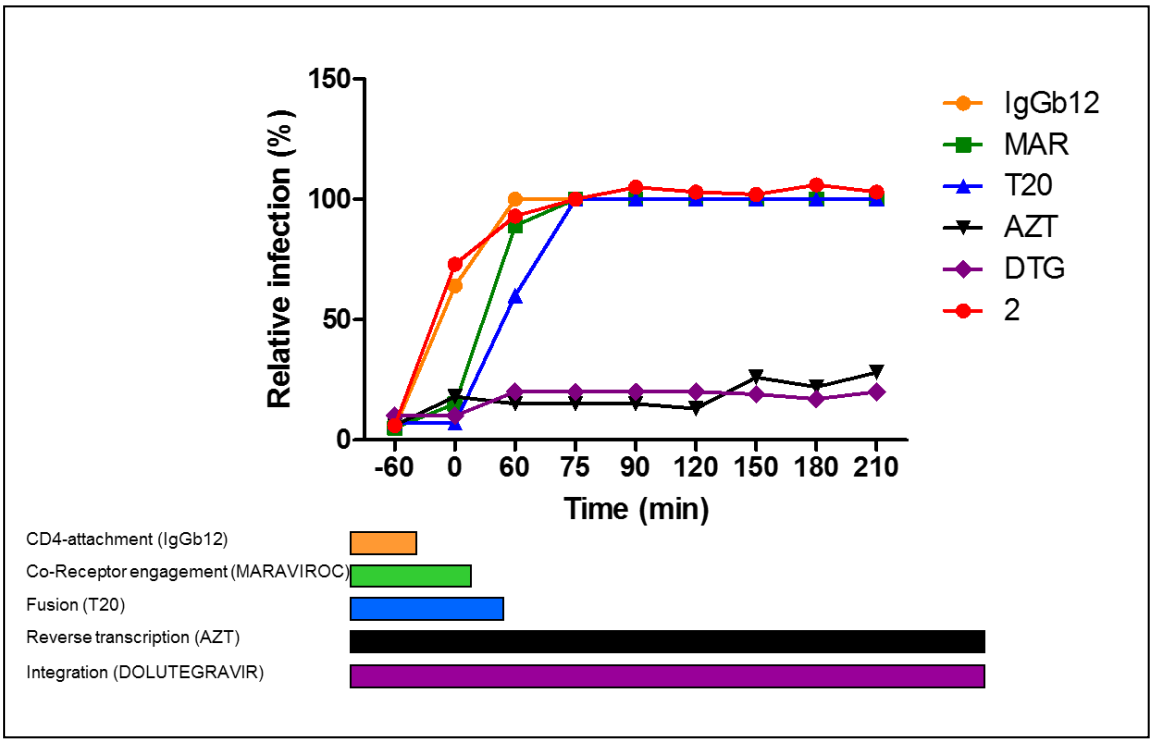


Figure 14. The time-of-addition experiment. The target of the antiviral compound **2** was identified by comparing its activity in the time scale to that of reference drugs. In the assay a panel of reference drugs sequentially targeting distinct replication steps of HIV-1 from entry to the integration into cell chromosome were used: IgGb12 (an anti-gp120 antibody that binds the CD4-binding site), maraviroc (a CCR5 coreceptor inhibitor), enfuvirtide (T20, a fusion inhibitor), azidothymidine (AZT, an RT inhibitor) and dolutegravir (DTG, an integrase inhibitor).

3.2.2 *IN VITRO* ADME STUDIES

3.2.2.1 SOLUBILITY, PERMEABILITY AND METABOLIC STABILITY

Selected compounds were profiled *in vitro* for aqueous solubility (thermodynamics solubility), liver microsomal stability and membrane permeability (Table **8**). Their aqueous solubility (ranging from 0.1 to 0.88 μg/mL) was found to be rather low. However, according to the results obtain from solubility assessment, this aspect did not prevent the efficacy of the gel formulation. In fact, the concentrations of the compounds in the pre-gel solutions obtained using our experimental conditions were identical to those of the control solution. In the same way, passive membrane permeability in a PAMPA assay indicated a low membrane permeability value for all compounds (ranging from 0.4 to 2.32 · 10^{-6}cm/s). However, this feature is considered to be drug-like, since it increases the time of exposure of the microbicide to the site of application, thus increasing its local activity. Moreover, stability tests disclosed that all compounds showed a good metabolic stability in human liver microsomes (>90%) (Table **8**).

3.2.2.2 ALBUMIN BINDING FLUORIMETRIC ASSAY

The selected method was first validated with drugs having known K_D for human serum albumin (paracetamol, diazepam and warfarin) and experimental results were in good agreement with literature data [146]. Each compound analyzed by fluorimetric titration showed decreased intrinsic fluorescence of Tryptophan and the percentage of bound albumin (HAS and BSA) at different concentrations was calculated. The percentages obtained were plotted against the concentrations used and the obtained K_D values are reported in Table **8.** Overall, all the compounds showed low K_D values, demonstrating a high binding affinity to serum albumin. In fact, it is well known that compounds showing K_D values lower than 10 μM are characterized by binding affinity to plasmatic proteins greater than 90% [146].

3.2.2.3 STORAGE STABILITY OF COMPOUND 2 GEL FORMULATION

The sample, at final concentration of 50 μM, was found to be stable at 25 °C stored in the dark. Compound **2** recovery after 1 month of storage amounted to about 100%. In addition, the apparent viscosity and pH of this gel remained stable during storage.

Compound	*In vitro* ADME				
	Water Solub. (μg/mL)	**P*app* ($1 \cdot 10^{-6}$ cm/sec)**	**Metabolic Stability (%)**	**K_D HSA (μM)**	**K_D BSA (μM)**
1	0.88±0.10	1.28	>90%	0.63±0.1	0.68±0.1
2	0.52±0.13	1.26	>90%	0.96±0.1	0.63±0.1
9a	0.54±0.08	0.4	>90%	1.51±0.2	1.08±0.2
9b	0.10±0.08	2.32	>90%	1.57±0.2	1.55±0.3
9c	0.30±0.07	1.23	>90%	1.19±0.1	1.05±0.1
9d	0.25±0.09	1.50	>90%	1.09±09	0.99±0.9
9e	0.72±0.12	0.92	>90%	2.19±1.0	1.07±0.9
9f	0.73±0.14	0.87	>90%	2.23±1.2	1.09±0.9

Table 8. Results of *in vitro* ADME analysis for selected rhodanine derivatives. Values represent mean±S.D. of three independent experiments

4 DISCUSSION

HIV infection is one of the main causes of morbidity and mortality worldwide [147]. Drug discovery and development have transformed HIV-1 infection into a chronic condition that can be controlled for many years through combination therapies with different classes of antiretroviral drugs, known as highly active antiretroviral therapy (HAART) [148]. Nevertheless, the ability of the virus to develop resistance to current antiviral treatments remain a major obstacle to long-term control of viral replication [149]. As previously mentioned, two main strategies were used in the field of HIV research and drugs resistance: new molecules/targets with novel mechanisms of action and new molecules with multiple targets. In this thesis both these possibilities have been investigated.

HIV integrase represents one of the newest targets for drug development, with the first inhibitor approved in clinical use in 2007. It is a very interesting target as it has the least resemblance to any human protein, potentially reducing off-target side effects. However, the expected emergence of IN strand transfer-specific drug-resistant mutations upon clinical use of this chemical class of antiretroviral agents emphasized the need for HIV-1 IN inhibitors that target allosteric binding pockets. Allosteric inhibition refers to a mechanism of action where the inhibitor binds at a region distinct from the substrate binding active site to inhibit protein function. The binding of an allosteric drug ligand can indirectly modulate the enzyme's active site geometry creating an unfavorable substrate binding pocket, can block the protein-protein interaction(s) that may be required for enzyme function, and/or can disrupt the formation of necessary oligomeric complexes for enzymes that function as higher ordered protein structures. In the HIV-1 drug design and discovery field, the development of the non-nucleoside reverse transcriptase inhibitors (NNRTIs) has been the greatest accomplishment for allosteric antiretroviral drug design to date. IN is a highly suitable target for allosteric drug discovery, because several allosteric inhibitory approaches are possible [150]. First, besides the catalytic domain, IN is composed of other two fundamental domains: the N-terminal and C-terminal domains that play critical roles for IN function through the promotion of DNA substrate binding and multimeric subunit formation. These domains present alternative protein regions for the design of allosteric IN inhibitors. Second, IN catalysis requires a multimeric state of the

protein associated with substrate DNA, providing the opportunity to design inhibitors effective at disrupting the formation of oligomeric nucleoprotein IN complexes. A third effective allosteric inhibitory approach is targeting the disruption of IN-cellular cofactor interactions. In this regard, small molecule inhibitors of the LEDGF/p75 binding site (LEDGINs) of IN, which block the HIV-1 replication and modulate IN oligomerization, have recently been identified [42]. In addition, a fragment-based screening recently allowed the identification of an allosteric binding site at the dimer interface. It consists of residues Ala105, Gly106, Arg107, Trp108, Pro109, Val110, Lys111, Ala133, Gly134, Ile204, Thr206, and Ile208 from one monomer and Tyr83, Trp108, Asn184, Phe185, Lys186, Arg187, Ser195, Gly197, Glu198, Ile200, and Val201 from the other monomer [151]. Furthermore, a second binding pocket at the IN dimer interface has been discovered through the crystallization of a complex containing a molecule of sucrose within the CCD of IN [152]. Further studies demonstrated that sucrose promotes the formation of the IN dimer and it cooperatively acts with raltegravir and inhibitors of the IN-LEDGF/p75 protein–protein interaction, enhancing their IN inhibition potency [136]. In the attempt to identify new allosteric ligands that, similarly to sucrose, could establish profitable interactions inside the pocket and inhibit HIV-1 integrase, a structure-based virtual screening approach on a small library of natural compounds was performed [136]. Natural products have historically represented a rich source of new drugs and even now some new leads in the field of medicinal chemistry are natural substances. In recent years, with the development of modern technologies and improvement in the means of extraction, separation and identification, structurally diverse natural products were discovered and screened for inhibitory activities against HIV-1 [153]. These computational studies on HIV integrase have led to the identification of kuwanon-L, a natural compound extracted from the roots of the plant *Morus nigra*. In this thesis the antiviral activity of kuwanon-L as well as its mechanism of action have been investigated in enzymatic and cellular assays. Since it has been reported that HIV-1 IN allosteric inhibitors display a different mode of action, being able to inhibit the IN-viral DNA assembly and also to impair integrase-LEDGF binding [37], we wonder if also kuwanon-L, selected as allosteric IN inhibitor, behave similarly. HTFR assays effectively demonstrated that this inhibitor impairs both integrase-LEDGF binding and the inherent catalytic activities of integrase, which do not rely on LEDGF, with very similar IC_{50} values in the micromolar range. It has previously been

reported that compounds that bind to the LEDGIN binding pocket can allosterically modulate the dynamic interplay between IN subunits, promoting and stabilizing the multimerization form of IN, as well as inhibiting the integrase subunit exchange required for IN·DNA complex formation [154]. To verify whether or not kuwanon-L would also behave similarly, we tested it in a HTRF IN subunit exchange assay. Actually, kuwanon-L, similar to control drug CX0516, promoted IN multimerization in a dose-dependent manner, confirming that when bound by the inhibitor, IN is trapped in a multimeric form in which the molecular movements of the individual subunits are restricted, and such reduced flexibility leads to inhibition of the catalytic process.

Overall, these results suggest that kuwanon-L binds to a site close to LEDGIN binding pocket and inhibits the IN catalytic activity with a mode of action comparable to that shown by LEDGIN CX0516.

After enzymatic characterization, we wonder if kuwanon-L was able to inhibit HIV-1 replication in a cell base assay, as numerous allosteric inhibitors with promising enzymatic activities failed to demonstrate antiviral activity *in vitro*. This is not true for kuwanon-L that confirmed its antiretroviral proprieties inhibiting the replication of a fully replicating HIV-1 laboratory strain without toxic effect also at the highest tested concentration. This is a very interesting point, as the development of other promising dual inhibitors was interrupted because of the high toxicity exert on human cells [53].

As kuwanon-L was selected on HIV-1 integrase scaffold in the attempt to generate IN inhibitors different from commercially available drugs, we wondered if this compound retained activity on HIV molecular clones bearing the most clinically relevant resistance mutations for INSTIs (Q148H, G140S and Y143R). Interestingly, kuwanon-L displayed antiviral activities similar to wild type virus on both mutants, further confirming the binding in a site different from catalytic one and the absence of cross-resistances with INSTIs. Once again this is of fundamental importance since the retain effectiveness against high-level RAL-resistant strains opens the hypothetical possibility of a combinational therapy within the HAART treatment schemes.

Giving in hand a compound with very interesting properties, we also performed a time of addition experiment to further characterize its mechanism of action. In the TOA assay, the target of the antiviral compound kuwanon-L was identified by comparing its activity in the time scale to that of reference drugs targeting distinct replication steps of HIV-1. We were

surprised by the result of this experiment as we expected an inhibitory profile similar to that of dolutegravir, the anti-integrase reference drug used in this assay. Behind the loss of activity of kuwanon-L at 5 to 6 hours after virus challenge, comparable with that produced by dolutegravir, we noted also a pick at 2-3 hours after infection, compatible with what happened with RT inhibitor lamivudine. The activity on IN had already been investigated and was expected, but this was not the case for the other enzyme. This prompted us also to test the compound on isolated RT to confirm the binding capacity of kuwanon-L also on this fundamental HIV enzyme. Indeed, the HIV-1 reverse transcriptase (RT) is the viral enzyme responsible for the reverse transcription of single-stranded viral RNA to double-stranded DNA, which is then integrated in host genome. HIV-1 RT is a multifunctional enzyme carrying out, among others, RNA-dependent DNA polymerase (RDDP) and ribonuclease H (RNase H) functions. The first activity accounts for the formation of the RNA:DNA intermediate, while RNase H activity is required to cleave the RNA strand of the RNA/DNA hybrids in the reverse transcription step.

In the attempt to verify an activity also on HIV-1 RT, we tested kuwanon-L on both these RT functions. Effectively, biochemical assays revealed the ability of this natural compound to inhibit both enzymatic functions with a similar efficiency. This is particular important as so far, all of the RT inhibitors (RTIs) approved for the treatment of HIV-1 infection inhibit polymerase function of RT, while none of them is active towards its RNase H activity. As it cannot be ignored that RNase H also plays an essential role in the HIV-1 life cycle and RNase H deficient viruses are non-infectious [155], this function represents an attractive target for new anti-HIV drugs design that might provide new opportunity to treat patients resistant to currently available antiretroviral therapies. This could be particularly true for compound able to inhibit both RT enzymatic activities. Even if till now research in this direction have failed to identify lead drugs for human experimentation, several promising compounds showed efficacy on both RT functions in enzymatic assays. All these known compounds with dual inhibitory activity have the same binding pocket in common [156]. This allosteric pocket of RNase H is located in a strategic point close to the pocket where most of known RT polymerase inhibitors bind. As it was suggested, this pocket communicates directly with the NNRTI pocket. By binding to this allosteric pocket and near to NNRTI pocket these compounds seem to acquire dual activity. We can speculate

that this could be the mechanism of action also of kuwanon-L but further experiments are needed to demonstrate this hypothesis.

Interestingly, enzymatic results are in accordance with what was previously observed in the TOA experiment, as kuwanon-L showed an anti-RT activity that was higher than the anti-integrase one both in the enzymatic assays (IN IC_{50}: 22 μM; RT RDDP IC_{50}: 1 μM; RT RNase H IC_{50}: 0.5 μM) and in the TOA experiment. In fact, in the latter we observed the greatest loss of antiviral activity when kuwanon-L was added after the RT step, whereas if it was added after HIV integration we found a still significant but more modest loss of antiviral activity. All these findings confirm our hypothesis of multiple target binding. This is a very interesting characteristic, because a dual inhibitor, binding to more than one target, should have a new favourable drug resistance profile [157] as already demonstrated in the cases of other diseases such as tuberculosis infections [158]. Moreover, in a field in which the pharmacological treatment is lifelong, this characteristic could also be important in terms of reduction of toxicity in association with other therapies. In the context of a multidrug regimen, the use of one single compound to block different steps of the viral infection can, ideally, reduce drug–drug interactions and, in consequence, the risk of toxicity.

Behind multiple target binding, another approach to go further the limits of the actual HIV-1 treatments is the implementation of prevention strategies. An interesting approach is HIV prevention through PrEP (Pre-exposure prophylaxis) approaches. Despite the approval of the antiretroviral combination of two NRTI, emtricitabine-tenofovir disoproxil fumarate (FTC/TDF), as oral daily PrEP for high risk individuals, several issue still limit its diffusion: its use for this indication relies on patient and provider acceptance and its effectiveness requires patient adherence and retention in care during periods of high-risk behaviors. Concerns regarding the use of PrEP in healthy individuals persist and include medication adverse effects including renal dysfunction and bone mineral density loss. Moreover, sexually transmitted infections and the development of drug resistance in the event of seroconversion are serious drawbacks of actual PrEP approach [159].

Another interesting strategy to overcome these limits is topical PrEP. Indeed, several studies confirmed the efficacy of topical PrEP approaches to limit the burden of novel HIV-1 infections and to enhance global efforts to eradicate this virus [8]. Microbicides

delivering antiviral compounds in the vagina, in the rectum or in the oral cavity, were shown to reduce HIV-1 transmission and can thus be considered effective tools to implement the global response to HIV-1. In fact, the CAPRISA 004 trial showed that 1% tenofovir (TFV) microbicide vaginal gel used as pre-exposure prophylaxis approach showed a 39% reduction of infection risk, demonstrating that topically used drugs can have a huge impact in limiting HIV-1 spread. However several co-factors may limit the efficacy of these topical PrEP approaches, in particular the presence of circulating strains already resistant to available antiretroviral agents present in the PrEP preparations. In fact, as expected, mutations which confer resistance to TFV have been reported in oral TFV PrEP regimens [160]. Similar observations may arise in the future in the case of other RT-inhibitors or integrase-inhibitors (INIs) PrEP-based devices, in parallel with the increased usage of molecules within these drug classes (with cross-resistance profiles) in currently used highly active antiretroviral therapies (HAART) worldwide, including developing countries. Another factor that may limit PrEP approach is the co-presence of other genital infections such as those due to HSV-1 or 2, which increase patient's susceptibility to HIV-1 infection. In some regions, especially in those with the highest prevalence of HIV-1 infection, such as in many African countries, up to 80% of young adults are serum-positive for genital herpes infection, with a significant fraction of herpes virus recurrences [161]. All currently available PrEP approaches, including vaginal gels containing antiretroviral or intravaginal rings with long-acting antiretroviral drugs or implantable and injectable formulations, selectively inhibit HIV-1 replication but have no or very limited activity against HSV-1 or 2 [121], thus their efficacy may be limited or not sufficient to prevent new infections especially in high prevalence settings for both viral infections.

Novel molecules with anti-HIV and HSV activity and non-overlapping resistance profile may hamper these problems. In recent years, several rhodanine-containing compounds have been identified with anticancer-, antimicrobial- but also antiviral activity, mainly against HIV-1 [162]. In the second part of this thesis, a novel series of rhodanine derivatives was tested *in vitro* on HIV-1, HSV-1 and HSV-2 laboratory strains with the aim to identify novel agents to be used as dual anti-HIV/HSV microbicides.

Interestingly, all eight compounds showed potent antiretroviral activity (from 4 nM to 46 nM) and safety profile (all except two, did not resulted cytotoxic even at the highest concentration) with compound **2** showing the best efficacy and safety ratio. Indeed, it

inhibited both R5 topic (AD8) and X4-tropic (NL4.3) HIV strains at nanomolar concentrations with values similar to the reference drug raltegravir and also preferable to maraviroc. Moreover, it did not exert any cytotoxic effect also at the highest concentration tested (>20 μM) and displayed a selectivity index 20 time higher (namely, 2800) than the minimum range acceptable (typically from 10 to 100).

On the other hand, compound **2** showed also a very interesting anti-HSV activity with an IC_{50} even fifty fold better than that of the reference drug acyclovir and a selectivity index almost eight times better than acyclovir. As this compound showed the best antiviral profile on HSV2, it was further characterized on HSV-1 strain, as retain activity on HSV-1 is of fundamental importance due to the increasing incidence of primary genital infection with HSV-1 [71]. Compound **2** inhibited HSV-1 with a minor potency, however displayed an activity similar to reference drug acyclovir.

To evaluate the antiretroviral activity of such compounds not only on TZM-bl cell line but also on primary human T $CD4^+$ lymphocytes, infections was performed also on freshly purified $CD4^+$ T cell. The three compounds with the best selectivity index (**2**, **9e** and **9f**) were pre-incubated with HIV R5 or X4 viruses. This experiment demonstrated that the three molecules retained their antiretroviral activity at nanomolar concentrations on both NL4.3 and AD8 HIV strains also on $CD4^+$ lymphocyte, a more physiological target than cell line. Moreover the three compounds were tested also on two recombinant viruses bearing mutations that confer resistance to raltegravir. Interestingly, all compounds maintained similar EC_{50} values also on integrase mutating recombinant viruses, suggesting a non-overlapping resistance profile of such compounds with already existing and well used drug such as integrase inhibitors. This is an important aspect, as it is a genuine concern that the use of antiretrovirals as microbicides may in the long term give rise to resistant viruses. For this reason, microbicide formulations combining different classes of anti-HIV-1 molecules should be investigated. Such formulations may provide better protection against the virus than a single compound. This is supported by several *in vitro* studies that showed synergistic effects between some HIV-1 inhibitors [163]. For example, microbicide formulation containing maraviroc and reverse transcriptase inhibitors resulted in enhanced activities of maraviroc and inhibited viruses that are resistant to this drug in cellular and colorectal explant models [164]. The possibility to combine rhodanine

derivatives and already clinically available drug is, therefore, of fundamental importance to avoid the emergence of resistance strains.

Similarly, compound **2** retained its antiviral activity toward the acyclovir resistant strain of HSV-2 suggesting a different mechanism of action and highlighting the potential of this series of rhodanines for the treatment of HSV-2 infections resistant to acyclovir.

Unfortunately, the activity of all rhodanine derivatives decreased of several times (from 20 to 100) once they were pre-incubated with serum before the *in vitro* assay. To better elucidate this behavior, the binding affinities of the new derivatives to the Human and Bovine Serum Albumin (HSA/BSA) were determined by a fluorescence-spectroscopy measurement and the Kd values were estimated. As a result, high affinity binding of the studied molecules to albumin was found, which could explain the loss of activity observed *in vitro* in the case of serum or purified recombinant albumin pre-incubation. The high binding to albumin observed for this class of rhodanine derivatives, could be a problem for systemically delivered drugs as the binding of small drug molecules with serum albumin can affect their absorption, transport and distribution *in vivo*. In particular strong binding of drug with albumin can decrease the concentration of free drug in plasma and only unbound drug exerts pharmacological activity [146]. These pharmacokinetic properties, which should be better explored in the future to modulate a long-acting release or activity of this compound even for systemic antiretroviral treatments, could be extremely useful to reduce the toxicity of these novel compounds when used in topical preparations. Indeed, high locally active drug concentrations may be reached at the mucosal interface, with a significantly reduced risk of systemic toxicity due to the rapid albumin binding and inactivation of the compounds. Moreover, the retention of drugs in the site of administration is further confirmed by the evidence of pharmacokinetic properties suitable for topical formulation such as good metabolic stability and low membrane permeability observed in the ADME experiments.

Considered the promising antiviral activity of compound **2** on both HIV-1 and HSV-1/2, as well as the negative effect of serum on its efficacy, we envisioned that a topical administration of the compound would have overcome the problem of albumin binding and represented a suitable strategy for a pre-exposure prophylaxis (PrEP) approach for the treatment of HIV and HSV infections. For this reason compound **2** was formulated in a gel and its activity was investigated in a transwell experiment, confirming a retained activity

on HIV-1 also in this formulation. The partial reduced activity displayed in this assay is not a major concern as topical formulation allows the usage of the drugs at concentrations many times higher than that achieved with oral or systemic delivery, without increasing systemic side effect and, possibly, reducing the emergence of drug resistant viruses.

The inactivity against HPV-16, a not enveloped virus implicated in sexually transmitted infections, suggests us that the viral envelope could be implicated in the mode of action of our compounds. To further elucidate the mechanism of action we finally performed a time-of-addition assay. This experiment indirectly demonstrated that these novel molecules inhibited a very early step of HIV replicative cycle, as the addition of our representative compound could not be postponed to virus seeding before completely losing its antiviral activity in cell culture. The chemical structure of rhodanine derivatives, their affinity for lipids, their behavior in TOA assay as well as their broad antiviral spectrum suggest us that their mechanism of action may involve a damage of the viral envelope. In our opinion this would lead to altered fusion properties of this component and consequent reduced infectivity of the virions, but further experiment will be necessary in the future to demonstrate this hypothesis. The fact that these molecules block a very early step in HIV-1 replication, preventing viral entry into cells may be relevant to increase the bioavailability and the efficacy of these antiretroviral agents in topical preparations. In fact, almost all currently available PrEP approaches target intracellular steps of virus replication, suggesting that available drugs will have to penetrate both the mucosal barrier and the target cell membranes in order to be effective when used in topical preparations. In contrast, entry inhibitors dispensed on the genital mucosa would be immediately available and would potentially prevent at all the transmission of the sexual infections by preventing viral entry in host cells.

5 CONCLUSIONS

In recent years many advances have been made in the fight against HIV-1 infection. However, the lack of a vaccine, together with the increasing resistance to the highly active anti-retroviral therapy (HAART), make HIV-1 infection still a serious global emergency. Development of inhibitors that operate by a novel mechanism of action could expand the options for clinicians to address these unmet medical needs for the HIV-1-infected patient population or could represent novel options for an effective prevention.

In this contest, one possibility is the development of single drug with multiple target binding, such as kuwanon-L. Up the present, kuwanon-L represents the first compound able to inhibit both the activity associated with HIV-1 IN and the two activities associated with HIV-1 RT. This new approach can be considered a solid starting point that demonstrates that the multiple-target-binding strategy can be successfully applied against HIV-1. By targeting two major enzymes essential for HIV replication, it is promising that this novel approach could offer treatment superior to early generations of anti-HIV-1 regimens that generally required the combination of multiple antiviral drugs, reducing the number of drugs needed to be administered to HIV-1-positive patients, increasing patients' compliance and decreasing the selection of drug-resistant strains and the drug toxicity. Molecular docking studies have identified the putative binding mode of kuwanon-L to integrase. Enzymatic and cellular assay have further characterized the mechanism of action of this compound on HIV integrase. In the future, further docking experiments on RT enzyme and experiments with molecular clones bearing resistance against currently available NNRTI drugs will be done to further elucidate the mechanism of inhibition of this promising natural compound.

On the other hand, in absence of an efficient anti-HIV-1 vaccine, compounds with novel mechanism of action and targeting very early steps of virus life cycle, as the novel series of rhodanine derivatives investigated in this work, may represent potent anti-HIV-1 agent suitable for prevention approaches. Their activity not only on HIV but also on HSV-1/2 viruses and their favourable pharmacokinetic profile further emphasize their usage as topical microbicides. The research in this field has come to light important challenges for future microbicides. In particular, it will be fundamental to assure constant administration

of active principles, avoid toxic effects over the genital tissues and identify early detrimental effects, such as increasing infection with other pathogens [166]. For these reasons, *in vitro* assays to assess the impact of our microbicidal candidates on bacterial flora in the vagina will be performed incubating various lactobacilli with the microbicide and evaluating possible perturbations in the bacterial balance. It is also important to consider the effectiveness of these compounds in presence of seminal plasma. Several studies reported that human semen can reduce the inhibitory activities of some anti-HIV-1 compounds [167]. This may be one of the reasons why compounds that looked promising *in vitro*, or even in animal trials, failed to protect during human trials [168]. In the future rhodanine derivatives will be test in the presence of human semen to evaluate this aspect. Moreover, to evaluate the activity of these compounds in a more physiological system, in the future these molecules will be tested in explant models, with STI target cells present in the context of the correct environment and architecture. Finally, we will perform an *in vitro* selection of resistant viruses against these microbicidal candidates that will help us to predict the mutations that could reduce the susceptibility of the virus to the microbicides and may help to better understand their mode of action as mutations will develop in the target viral proteins. In this contest, we will further investigate the hypothesis in which rhodanine derivatives act impairing viral envelope, evaluating their peroxidant activity.

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