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ANASTASIA SELYUTINA

When biologist meets chemist: a search for HIV-1 inhibitors

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When biologist meets chemist: a search for HIV-1 inhibitors

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following publications, that are referred to by their Roman numerals in the text:

I. Paju A, Päri M, **Selyutina A**, Zusinaite E, Merits A, Pehk T, Siirde K, Müürisepp AM, Kailas T, Lopp M. (2010). Synthesis of novel acyclic nucleoside analogues with anti-retroviral activity. Nucleosides Nucleotides Nucleic Acids, 29(9):707–720.

II. Ausmees K*, **Selyutina A***, Kütt K, Lippur K, Pehk T, Lopp M, Zusinaite E, Merits A, Kanger T. (2011). Synthesis and biological activity of bimorpholine and its carbanucleoside. Nucleosides Nucleotides Nucleic Acids, 30(11): 897–907.

III. Ilisson M, Tomson K, **Selyutina A**, Türk S & Uno Mäeorg. (2015). Synthesis of Novel Saccharide Hydrazones. Synthetic Communications: An International Journal for Rapid Comunication of Synthetic Organic Chemistry, 45 (11): 1367–1373

IV. Viira B*, **Selyutina A***, García-Sosa AT*, Karonen M, Sinkkonen J, Merits A, Maran U. Design, discovery, modeling, synthesis, and analysis of low toxicity, novel s-triazine derivatives as HIV-1 non-nucleoside reverse transcriptase inhibitors. Under revision

*These authors contributed equally to this work.

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Author's contributions:

I. I performed toxicity tests on HeLa cells, as well as tests for anti-HIV and anti-HCV activity. I analyzed the data and wrote the biological portions of the manuscript.

II. I performed tests for toxicity, as well as anti-HIV and anti-HCV activity. I analyzed the data and wrote the biological portions of the manuscript.

III. I designed and performed toxicity and antiviral activity tests. I analyzed the data and took part in writing the manuscript.

IV. I designed and performed all of the biological experiments. I analyzed the data and wrote the biological portions of the manuscript.

LIST OF ABBREVIATIONS

3TC	lamivudine
aa	amino acids (residues)
ABC	abacavir
AIDS	acquired immunodefficiency syndrome
APOBEC	apolipoprotein B mRNA-editing enzyme catalytic polypeptide
ART	antiretroviral therapy
Att region	attachment region
AZT	azidothymidine
CA	HIV-1 capsid protein
CC50	50% cytotoxic concentration
CCR5	C-C chemokine receptor type 5
CD	cluster of differentiation
CRF	circulating recombinant form
CXCR4	C-X-C chemokine receptor type 4
d4T	stavudine
ddC, ddCTP	dideoxycytidine
ddI	didanosine
DDX3	DEAD box helicase 3
DIS	dimerization initiation signal
DMSO	dimethyl sulfoxide
(c) (v) DNA	(complementary) (viral) deoxyribonucleic acid
DSIF	DRB (5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole) sensitivity inducing factor
EGFP	enhanced green fluorescent protein
Env	envelope protein
ESCRT	endosomal sorting complex required for transport
FDA	Food and Drug Administration of the USA
FSS	frameshit stimulatory signal
FTC	emtricitabine
Gag	group-specific antigen
gp	glycoprotein
HCV	hepatitis C virus
HIV-1	human immunodefficiency virus type-1
HLA	human leukocyte antigen
HPLC	high-performance liquid chromatography
Hsp70	heat shock protein 70
HSV-1	herpes simplex virus type-1
IC50	50% inhibitory concentration
ICAM-1	intercellular adhesion molecule 1
IN	HIV-1 integrase
IRES	internal ribosome entry site
LTR	long terminal repeat

MA	HIV-1 matrix protein
MRP	multidrug resistance protein
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	HIV-1 nucleocapsid protein
Nef	negative factor
NELF	negative elongation factor
NMR	nuclear magnetic resonance
NNRTI	non-nucleoside reverse transcriptase inhibitor
NPC	nuclear pore complex
N site	nucleotide binding site
nt	nucleotide (residue)
N(t)RTI	nucleoside (nucleotide) reverse transcriptase inhibitor
p6	HIV-1 peptide 6
p51, p66	HIV-1 protein 51, 66 (reverse transcriptase subunits)
PDB ID	protein data bank identifier
PBS	primer-binding site
PIC	pre-integration complex
PM	plasma membrane
PMA	12-myristate 13-acetate
Pol	HIV-1 polymerase (reverese transcriptase)
Poly(A)	polyadenilation signal
PPi	pyrophosphate
(c, 3') PPT	(central, 3') polypurine tract
PR	HIV-1 protease
P site	priming site
P-TEFb	positive transcription elongation factor b
R region	redundant region
Rev	regulator of expression of virion proteins
RHA	RNA helicase A
RNase H	ribonuclease H
RNP	ribonucleoprotein
RT	reverse transcriptase, reverse transcription
RTC	reverse transcription complex
RRE	Rev-responsive element
SI	selectivity index
SIV	Simian immunodeficiency virus
SU	HIV1-antireceptor (surface unit)
TAR	trans-acting responsive element
Tat	trans-activator of transcription
TDF	tenofovir disoproxil fumarate
TFV	tenofovir
TK1	thymidine kinase 1
TM	HIV-1 fusion protein (transmembrane protein)

TP	triphosphates
U5 (U3) region	unique region for 5'-end (3'-end)
UNG	uracil DNA glycosylase
UTR	untranslated region
Vif	virion infectivity factor
VLP	virus-like particle
Vpr	viral protein R
Vpu	viral protein U
WHO	World Health Organization

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a causative agent of AIDS – a severe lifelong disease. Approximately 35 million people were living with HIV-1 and 1.5 million people died from HIV-1-related diseases in 2013 (WHO http://www.who.int/hiv/data/en/). There is no preventive (or therapeutic) vaccine for HIV-1. Repeated attempts to produce such a vaccine have either completely failed or, at best, have achieved marginal results. As there is no vaccine in use or even on the horizon, the treatment of HIV-1 relies on the use of antivirals that can suppress its infection cycle. Over time, 28 different compounds have been licensed as anti-HIV drugs, and currently there are 23 compounds that have been approved by the Food and Drug Administration (FDA) for the treatment of HIV-1 infection/AIDS. Antiretroviral therapy (ART, also known as highly active antiretroviral therapy, or HAART) has been in use since the mid-1990s and is based on the simultaneous use of a combination of at least 3 drugs with different modes of action (http://aidsinfo.nih.gov/contentfiles/lvguidelines/AdultandAdolescentGL.pdf). Such treatment is effective but is lifelong. Furthermore, frequently, severe adverse effects and resistance to these compounds can be developed. As such, many laboratories around the world are seeking new compounds that are better than existing ones by several aspects:

1. They are effective against wild type (wt) virus at lower concentrations.
2. They are equally efficient against wt and known resistant forms of the virus and have higher genetic barriers against the development of resistance.
3. They have lower toxicity (acting only on the virus and not on the organism).
4. They have favorable pharmacokinetic characteristics, including i) good shelf life, enabling storage at room temperature, ii) possibility of administration in pill-form; and iii) stability in an organism for at least 24 hours, enabling once daily administration.
5. They are cheaper to produce.

To develop such compounds, chemical and biological laboratories should work together, which is the case in our Center of Excellence in Chemical Biology. The current thesis presents the results of collaboration between our (virology) laboratory and three different chemical laboratories. Together, we have studied the antiretroviral properties of acyclic thymine nucleoside analogues (article I), bimorpholines (article II), saccharide hydrazones (article III) and diaryltriazine derivatives (which were found during experimental validation of *in silico* screening; article IV), as well some other compounds (unpublished data).

Thus far, the data presented in article IV represent the most successful attempt at developing an HIV-1 inhibitor that satisfies at least some of the requirements listed above. One of the studied compounds has an efficiency that is comparable to the known HIV-1 inhibitor nevirapine; however, similar to this commercial inhibitor, its activity was mostly restricted to wt virus (it did not work against known resistant forms). However, it was never expected that any of studied compounds would immediately become a new drug that can replace

or supplement the existing drugs on the market. Inventing a drug is a long and difficult process, and only a few out of many thousands reach the goal successfully. Finding compounds with new scaffolds and promising properties is often the first, and sometimes the most crucial, step in the direction leading to the discovery of new paths and methods of combating this remarkably flexible and difficult-to-counter virus.

LITERATURE REVIEW

If the term "over-studied" could be applied to any virus, it would certainly be HIV-1, which is by far the most studied virus. As the number of papers published on HIV-1 (around 270,000 by the summer of 2015) is roughly equal to the number of atoms in the HIV-1 genome, it is also very likely the most studied biological object as well. Unfortunately, this does not mean that we know all that there is to know about this virus. However, it does mean that the available literature is immense and full of repeats (the same thing discovered over and over again) and contradictions. Even the literature that is available on HIV-1 inhibitors (well over 10,000 papers) is extremely massive. As it is beyond anyone's capacity to work through all of the data available on HIV-1, this review cannot deal with all of the known (or even all of the important) aspects of HIV-1 biology. In many cases, preference was given to classical studies, especially when dealing with the basic properties of the virus and its replication; when discussing HIV inhibitors, the literature that seemed to be the most relevant (unfortunately, it is simply impossible to read all of the papers to determine which are truly the most relevant) has been reviewed.

1. VIRUS

1.1 History of discovery

HIV-1 infects humans, and the progression of its infection leads to AIDS. At the final stage of this disease, the immune system of an infected person becomes severely damaged; as a result, this person becomes susceptible to a number of opportunistic infections (typically rare in people with healthy immune systems) and eventually dies.

Such symptoms were first described in homosexual men from the USA in 1981 (Brennan and Durack, 1981; Friedman-Kien, 1981; Gottlieb et al., 1981; Siegal et al., 1981). The discovery of the causative agent of AIDS is the result of work carried out in two laboratories: Robert Gallo's group in the USA and Luc Montagnier's group in France. In 1983, Luc Montagnier's group reported that a new retrovirus was isolated from patients with AIDS (Barré-Sinoussi et al., 1983); a year later, it was proved that this virus is the causative agent of AIDS (Levy et al., 1984; Popovic et al., 1984). Luc Montagnier and his colleague Françoise Barré-Sinoussi were awarded the Nobel Prize in 2008 for their discovery of HIV-1.

1.2 Origin and classification of HIV-1

HIV-1 belongs to the family *Retroviridae*, genus *Lentiviruses*, group of primate lentiviruses. This group includes HIV-1 and HIV-2, as well as numerous simian immunodeficiency viruses (SIVs). SIVs infect around 40 species of nonhuman primates. Intriguingly, in their natural hosts, SIVs replicate successfully but do not cause a disease similar to AIDS, which most likely reflects long-term host-viral coevolution (Apetrei et al., 2004). It is believed that HIVs were transmitted from monkeys to humans during hunting, meat-cutting or through the bites of domesticated animals.

There are 2 different species of HIV: HIV-1 and HIV-2. Although they are very similar and the progression of the disease leads to nearly identical clinical pictures, some important differences still exist. HIV-1 is a pandemic virus and is the cause of most AIDS cases globally. HIV-2 is endemic and is mostly found in West Africa. Current research is focused on HIV-1, which will be discussed in the remainder of this work unless otherwise noted.

It should also be noted that even HIV-1 is genetically heterogeneous. The major types of HIV-1, M, N, O and P, probably originated from different monkey (or, more accurately, ape) to human transmissions, which occurred around 100 years ago. Of these, only type M has become pandemic, causing the vast majority of HIV-infections in the world. The M type of HIV-1 is also heterogeneous; the existing subtypes are referred to as clades (around 10 are known), which are divided into sub-subtypes. To add to the complexity, there are a number of recombinants between the clades that are also in circulation (termed circulating recombinant forms or CRF's). Clade (subtype) B is responsible for

most of the infections that occur in America, western Europe, Australia and Japan; not surprisingly, this clade has also been the main target of anti-HIV-1 drug development. In eastern Europe, clade A is dominant. Surprisingly, Estonia is an exception, as the HIV-1 epidemics that have occurred in this country have almost exclusively been caused by the rare CRF06_cpx virus and its recombinant with A-clade virus, which is termed CRF32_06A1 (Adojaan et al., 2005; Avi et al., 2014).

1.3 Structure of the mature HIV-1 virion

HIV-1 is an enveloped virus with roughly spherical virions of variable size (its diameter is between 106 and 183 nm) (Figure 1) (Briggs et al., 2003, 2006). Its viral membrane is derived from the plasma membrane of the host cell and contains HIV-1 envelope proteins and some cellular membrane proteins. There are 2 viral envelope proteins, both originating from the cleavage of the same precursor (env protein): SU (surface protein, also known as gp120, which lies on the outer surface of the membrane) and TM (transmembrane protein, also known as gp41, which anchors SU to the viral membrane). SU and TM are heavily glycosylated; they bind to each other non-covalently and form heterodimers. Three heterodimers cluster together to form mushroom-shaped trimers. In contrast to other retroviruses, HIV-1 particles contain very few (≈10, values between 4–35 have been reported) evenly distributed trimers (Zhu et al., 2003). Beneath the membrane, there is an incomplete layer of matrix protein (MA) (Briggs and Kräusslich, 2011).

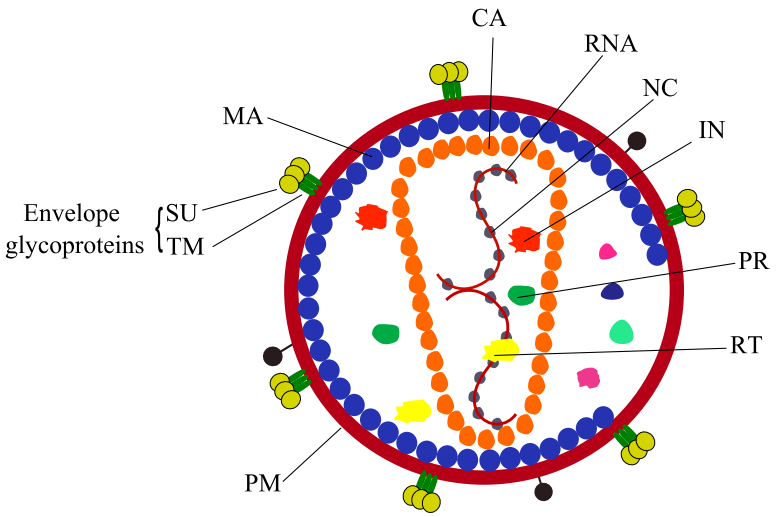


Figure 1. Structure of a mature HIV-1 virion. Clockwise from the left: PM – virion envelope derived from the plasma membrane of an infected cell, TM – transmembrane protein, SU – surface unit protein, MA – matrix protein, CA – capsid protein, NC – nucleocapsid protein, IN – integrase, PR – protease, RT – reverse transcriptase.

Inside the particle, there is a cone-like core structure. The shell of the core is formed by approximately 250 hexamers and exactly 12 pentamers of capsid protein (CA), which adopt fullerene-type geometry. Inside the core, there is a ribonucleoprotein (RNP) complex consisting of nucleocapsid protein (NC) and genomic RNA (there are 2 copies of RNA per virion). There are ≈5000 copies of each of MA, CA and NC protein and p6 peptide per particle. Importantly, the core also contains ≈250 copies of each of the essential viral enzymes: protease (PR), reverse transcriptase (RT) and integrase (IN).

The HIV particle also contains some accessory proteins: virion infectivity factor, Vif (1–150 copies per particle); viral protein R, Vpr (≈700 copies per particle); and some amount of negative factor, Nef. The other accessory proteins (*trans*-activator of transcription, Tat; regulator of expression of virion proteins, Rev; and viral protein U, Vpu) that are encoded by the HIV-1 genome have not been found in the particle (Swanson and Malim, 2008; Turner and Summers, 1999).

HIV particles also incorporate a number of host cell proteins. Some of these proteins have no known role in viral replication and are presumably incorporated into the particle non-specifically (simply because they are located near the budding site of a virus). Other cellular proteins, however, are important for viral infectivity and life cycle. These latter proteins can be found either in the viral membrane (ICAM-1 (CD54), HLA-II) or inside of the particle (lysyl-tRNA synthetase, heat shock protein 70 (Hsp70), proteins of the actin cytoskeleton and some others) (see (Ott, 2008) for details).

1.4 HIV-1 genome organization

The viral genome is a single-stranded RNA of positive polarity; the same strand is also used as an mRNA for protein (gag-pro-pol) synthesis. It is about 9200 nucleotides long and has a 5' cap and a 3' polyadenylated tail (Figure 2A). Each HIV-1 virion contains 2 copies of this RNA; they form a non-covalent dimer. HIV RNA has 9 open reading frames (ORFs) that encode 15 mature proteins (Frankel and Young, 1998) (Figure 2C).

HIV RNA contains a number of important regulatory sequences (Berkhout, 1996; Watts et al., 2009):

1. *The R (redundant) region*. There are 2 identical R regions at each end of the viral RNA genome, and they play an important role in reverse transcription (during the first-strand transfer reaction; see below). The R regions contain TAR hairpin and poly(A) hairpin elements.
2. *The TAR hairpin.* The trans-acting responsive element binds viral (tat) and cellular proteins and is involved in the regulation of transcription. As it is a part of the R-element, there are 2 copies of TAR per genome; however, only the element at the 5'-end of the genome is functional.

3. *The poly(A) hairpin*. This element contains a polyadenylation signal. Again, there are 2 copies of it per genome. In the case of this element, only the poly(A) hairpin that is located at the 3'-end of the genome is active.
4. *The U5 region.* This region is unique to the 5'-end of the genome. It contains an *att* site, which is important for the integration of viral cDNA into the host genome.
5. *The primer-binding site (PBS).* This element is complementary to the 3'-end of $tRNA^{Lys3}$, which acts as a primer for HIV-1 reverse transcriptase.
6. *The dimerization initiation signal (DIS)*. This element contains a palindromic sequence and is the site where the dimerization of 2 genomic RNAs begins.
7. *The RNA packaging signal (ψ).* This element is involved in genome packaging (some other sequences participate in this process as well).
8. *The polypurine tracts.* There are two PPTs with identical sequences in the HIV-1 genome: a central PPT (cPPT) and a PPT close to the 3'-end (3' PPT). Both of these elements serve as primers for plus-strand DNA synthesis.
9. *The U3 region.* This region is unique to the 3'-end of the genome. It contains a second *att* site, which is important for the integration of viral cDNA into the host genome, as well as the majority of sequences that regulate the gene expression of HIV-1 provirus (see below).

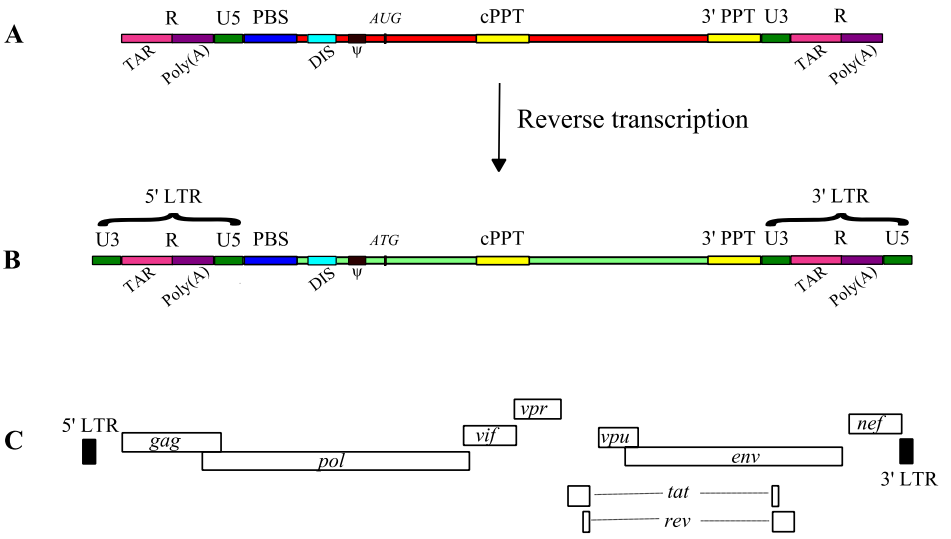


Figure 2. Cis-acting elements in HIV-1 genomic RNA (**A**) and in proviral DNA (**B**). (**C**) Organization of the coding regions inside of the HIV-1 provirus.

During the process of reverse transcription, viral RNA is copied into viral cDNA, which has long terminal repeats (LTRs) at both ends (Figure 2B). The LTRs are 2 identical sequences, each consisting of U3, R and U5 regions. They are important for the integration of viral cDNA into the host genome and for subsequent synthesis of viral RNAs.

1.5 HIV-1 infection cycle

1.5.1 Cell binding and entry

Binding and entry into the host cell is the first step of the HIV-1 replication cycle. It begins with viral attachment to the host cell and results in a fusion of virion envelope with cellular membrane, leading to the delivery of the viral core inside the cell (Wilen et al., 2012). This process is complex and can be divided into several phases:

1. *Nonspecific binding* to the cell via attachment factors. The attachment factors bind to the HIV-1 virion, but they are not essential for infection (in contrast to viral receptors) (Connell and Lortat-Jacob, 2013; Friedrich et al., 2011; Geijtenbeek et al., 2000; Ugolini et al., 1999).
2. *Specific binding* of the viral envelope complex to its cellular receptor CD4. This interaction induces conformational changes in SU, exposing the binding site for the HIV-1 co-receptor.
3. *Transfer* of bound virion along the host plasma membrane to the sites where HIV-1 co-receptors are located.
4. *Co-receptor binding*. Virions can bind one of the two HIV-1 co-receptors, the chemokine receptors CCR5 and CXCR4. HIV-1 strains are classified based on their co-receptor usage. R5 HIV-1 can use only CCR5, X4 HIV-1 can use only CXCR4, and R5X4 HIV-1 can use both CCR5 and CXCR4.
5. *Membrane fusion*. Co-receptor binding triggers further conformational changes in the envelope protein. This leads to the exposure of the hydrophobic fusion peptide of TM, which inserts itself into the cellular membrane. This, in turn, triggers rearrangements in TM trimers, and as result a six-helix hairpin structure is formed, bringing the viral and cellular membranes into close proximity and resulting in the formation of the fusion pore (Blumenthal et al., 2012).

   Subsequently the *pore dilates* to create an opening wide enough (≈50 nm) for the viral core to enter the cytoplasm. This is a very energy-demanding step, and some cellular processes (cell signaling and actin rearrangements) are potentially involved in it (Blumenthal et al., 2012).

1.5.2 Early post-entry events

After the virion core structure enters the cell, the virus must synthesize its cDNA, transport it to the nucleus and achieve its integration into the host

genome. The initial events of reverse transcription (synthesis of viral cDNA on the template of genomic RNA) sometimes can start in the core of the intact particle (Lori et al., 1992; Trono, 1992). In the cytoplasm the core is gradually transformed into the RTC (reverse transcription complex), where reverse transcription continues. The RTC travels across the cell using microtubule-directed transport, and in the perinuclear area its movement is actin-directed (Arhel et al., 2006; McDonald et al., 2002). During maturation, the RTC enlarges (100 nm wide and 400–700 nm in length, different shape) (McDonald et al., 2002), loses some viral proteins (the capsid protein is removed in a highly regulated "uncoating" process; most of the reverse transcriptase and Nef are lost) and acquires some cellular proteins (Fassati and Goff, 2001; Iordanskiy et al., 2006; Nermut and Fassati, 2003). When DNA synthesis is complete, the PIC (pre-integration complex) is formed, which is transported to the nucleus through the nuclear pore. A detailed description of the replication process and the involved enzymes is given in the subsequent sections.

1.5.3 HIV-1 cDNA integration

For successful replication to occur, a product of reverse transcription, viral cDNA, must be integrated into the genome of the host cell (for review, see (Craigie and Bushman, 2012)). Integrated viral cDNA is called provirus and serves as a template for the synthesis of viral RNAs. The integration reaction requires the presence of integrase (IN), a viral enzyme that is present in the infecting viral particle, which enters the cell in the virion core and is thus a part of the RTC and PIC. IN is a tetramer (dimer of dimers) that is complexed in the PIC with the ends of newly synthesized viral cDNA. The integration reaction is performed in basic 3 steps (Brown et al., 1989; Fujiwara and Mizuuchi, 1988):

1. *3' end processing*. IN cuts off the last 2 nucleotides from each 3' end of viral cDNA, leaving conserved CA nucleotides at the 3' ends.
2. *DNA strand transfer*. The 3' ends of the viral cDNA act as nucleophiles: they attack two phosphodiester bonds that are located on the opposite strands of the host DNA, break these bonds and join viral cDNA to the host DNA in one step (Engelman et al., 1991). The target sites are separated by 5 bp. As a result, an integration intermediate is formed, in which the 3' ends of the viral cDNA are covalently linked to the host DNA and the 5' ends are free.
3. *Reparation and completion*. The 5'-overhangs of the viral cDNA are removed, the single-strand gaps between the viral and host DNA are filled (as a result, the provirus is flanked by 5 base-pair duplications), and the viral DNA is ligated with the host DNA. This step is probably performed by cellular enzymes.

The integration of the viral genome into a chromosome leads to a successful replication. In some cases, the viral cDNA forms circles, which are dead-end products of the virus replication cycle (Farnet and Haseltine, 1991).

1.5.4 HIV-1 latency

After integration, the provirus can undergo different fates. In most cases, the replication cycle proceeds and new virions are formed, but in approximately 5% of the cases the proviruses remain in a latent form. The mechanisms that control HIV latency are complex and incompletely understood. They include transcriptional interference (the effect of neighboring cellular promoters), transcriptional activator and repressor influence (their activities and availabilities partially depend on the state of the host cell), chromatin structure and nucleosome modification, and some other factors. Latent provirus can be awakened and can serve as a source of new virions (Van Lint et al., 2013; Ruelas and Greene, 2013). HIV-1 latency represents the major obstacle in anti-HIV-1 therapy, as current antivirals cannot eliminate latent provirus, and there are no drugs that can activate (and subsequently eliminate) latent proviruses.

1.5.5 Transcription and transport of viral mRNAs to the cytoplasm

Viral mRNAs are synthesized, processed (spliced, capped, polyadenylated) and transported out of the nucleus by cellular mRNA synthesis/processing machinery (for review, see (Karn and Stoltzfus, 2012; Leblanc et al., 2013; Stoltzfus, 2009)). Viral mRNAs are produced by RNA polymerase II, which recognizes the viral promoter in the left LTR. Transcription begins at the 5'-end of the R region, and the poly(A) tail is added to the 3'-end of the R region in the right LTR. Due to alternative splicing, not only a full-length RNA transcript but also more than 40 different spliced viral mRNAs are produced (Ocwieja et al., 2012; Purcell and Martin, 1993). They can be divided into 3 groups:

- a group of *completely spliced* 1.8 kb mRNAs (encoding the regulatory proteins Tat, Rev, and Nef),
- a group of *incompletely spliced* 4 kb mRNAs (encoding Env and the accessory proteins Vif, Vpr, and Vpu),
- single *unspliced* 9 kb mRNA (encoding the Gag and Gag-pol polyproteins and serving as genomic RNA for new virions).

First, only completely spliced mRNAs are produced because only they can be transported out of the nucleus by cellular machinery. Their quantities are low due to the inefficiency of the elongation reaction. In the absence of Tat, RNA polymerase II pauses after it has synthesized the TAR element, a stem-loop structure present at the 5' end of the viral RNA. The cellular negative elongation factors DSIF and NELF bind to the paused RNA polymerase II and TAR element and prevent further RNA synthesis. However, this block is not absolute, and some amount of the mRNAs that encode the Tat and Rev proteins are still synthesized and exported to the cytoplasm. Once the Tat and Rev proteins

are produced, they enter the nucleus, where they help produce incompletely spliced and unspliced RNAs in large quantities.

Tat increases the total amount of newly synthesized viral mRNA. Tat stimulates elongation from the viral promoter by binding to TAR RNA and recruiting P-TEFb to the complex (Mancebo et al., 1997; Tahirov et al., 2010; Zhu et al., 1997). P-TEFb is a kinase and a positive transcription elongation factor. Upon binding with Tat, it is activated; it then phosphorylates the C-terminal domain of RNA polymerase II, as well as NELF and DSIF. This leads to the dissociation of NELF from the complex, the conversion of DSIF from repressor to activator of transcription and the enhancement of RNA polymerase II processivity (Fujinaga et al., 2004; Ivanov et al., 2000; Kim et al., 2002; Yamada et al., 2006).

Rev helps transport incompletely spliced and unspliced mRNAs out of the nuclei of infected cells. This is mediated by the binding of Rev to the Rev-responsive element (RRE), a stem-loop structure that is present in the *env* gene of the viral mRNAs (Heaphy et al., 1990; Malim et al., 1989). When Rev binds to HIV-1 RNA, it interacts with the proteins that make up the nuclear pore complex (NPC) and thus both Rev and HIV-1 mRNAs are transported into the cytoplasm. After that, Rev is released from the viral RNA and re-enters the nucleus (Fischer et al., 1995; Henderson and Percipalle, 1997).

1.5.6 Translation of HIV-1 mRNAs

HIV-1 mRNAs are translated mainly similar to cellular mRNAs: one mRNA encodes for one protein and is translated by a cap-dependent mechanism (for review, see (Bolinger and Boris-Lawrie, 2009; de Breyne et al., 2013; Guerrero et al., 2015). Cap-dependent translation is initiated when translation initiation factors recognize the cap structure at the 5' end of the mRNA. After that, the 40S ribosomal subunit is engaged, and a 43S ribosomal complex is formed. This complex scans the mRNA until it finds an AUG initiation codon within a proper Kozak consensus sequence; this is followed by the assembly of the 80S ribosome and the start of translation.

All transcripts of HIV-1 have the same 5' leader region, which is rather long (~300 nt) and highly structured. This region contains important regulatory sequences, including TAR, PBS and several others, which may have specific RNA secondary structures that complicate ribosome scanning. Therefore, many cellular and viral factors are engaged to increase translation efficiency, including the cellular RNA Helicase DDX3 (Soto-Rifo et al., 2012, 2013) and RNA Helicase A (RHA) (Fujii et al., 2001), the viral protein Rev (Groom et al., 2009) and others.

In addition, some HIV-1 mRNAs use special mechanisms for translation:

- The mRNA encoding Vpu-env is both structurally and functionally bicistronic; it contains two open reading frames (encoding the Vpu and Env proteins), and both of them can be translated (Schwartz et al., 1990, 1992). The mechanism used is called leaky scanning: start codon for Vpu occurs first, but as it is surrounded by a weak Kozak sequence, the 43S ribosomal complex sometimes skips it and initiates translation from the Env start codon.
- The unspliced mRNA (genomic RNA) encodes the Gag and Gag-Pol polyproteins. These two polyproteins are synthesized using a –1 ribosomal frameshift. Gag polyprotein, coding sequence of which is localized upstream of the frameshift signal, is approximately 20-fold more abundant. The –1 ribosomal frameshift occurs at a frequency of ~5% and results in the synthesis of the Gag-Pol polyprotein (Jacks et al., 1988). The two main factors that are required for frameshifting to occur are located ~200 nt upstream of the Gag termination codon. They include the heptanucleotide slippery sequence (UUUUUUA) and the downstream stem-loop pseudoknot structure, which is called the frameshift stimulatory signal (FSS). The FSS forces the ribosome to pause and to then shift one nucleotide backwards on the slippery sequence. As a result, the mRNA reading frame is changed, and the *gag* termination codon is shifted out of frame (Dulude et al., 2002; Gaudin et al., 2005; Staple and Butcher, 2005). Leaky scanning and frameshift mechanisms help achieve the correct ratio of viral Gag and Pol proteins.

Although (as was mentioned above) all viral mRNAs have the same leader sequence, only translation from the unspliced mRNA can be initiated via an additional IRES (internal ribosome entry site)-dependent mechanism. It has been shown that the genomic mRNA contains 2 IRES sites: one within the 5' UTR (called HIV-1 IRES) (Brasey et al., 2003) and the other within the gag coding region (called HIV-1 Gag IRES) (Buck et al., 2001). IRES activity depends on cellular and viral proteins, which can act as RNA chaperones and are required for its proper conformation. Cap-dependent initiation occurs under normal physiological conditions (Berkhout et al., 2011; Ricci et al., 2008), but the virus can switch to the IRES-dependent mechanism during stress (Gendron et al., 2011; Monette et al., 2009), virus-induced cell cycle arrest at the G2/M phase or under conditions of protein translation reduction (Brasey et al., 2003; Vallejos et al., 2011).

1.5.7 Assembly, budding and maturation of HIV-1 virions

To create a new infectious virion, all of the required components must meet and correctly assemble on the plasma membrane, after which the formed particle buds from the cell and matures (Kuzembayeva et al., 2014; Meng and Lever,

2013; Ono, 2010; Sundquist and Kräusslich, 2012). This is a complex process that is driven mostly by the Gag polyprotein.

Two viral integral membrane proteins, Vpu and Env, are synthesized from the same bicistronic mRNA; translation occurs in the rough endoplasmic reticulum, and the synthesized proteins are co-translationally inserted into the membrane. They are transported to the plasma membrane via vesicular transport (Checkley et al., 2011; Strebel et al., 1989).

Gag, Gag-Pol and other viral proteins are synthesized by cytosolic polysomes and are transported to the plasma membrane probably via Gag's interactions with cellular trafficking pathways (Camus et al., 2007; Dong et al., 2005; Martinez et al., 2008).

Gag consists of several domains (staring from the N-terminus of the polyprotein): the matrix, capsid and nucleocapsid proteins and finally the C-terminal p6 peptide. There are also two spacer peptides, p1 and p2, that flank the nucleocapsid domain. The matrix domain (MA) anchors Gag to the plasma membrane and probably recruits the Env protein to the site of particle formation (Checkley et al., 2011). The capsid domain (CA) is involved in different protein-protein interactions: it is partially responsible for Gag multimerization and the incorporation of some viral and host proteins into the particle. The nucleocapsid domain (NC) binds RNA and recruits a dimer of viral genomic mRNA. P6 is a protein-binding domain: it binds the accessory viral protein Vpr and attracts proteins from the cellular ESCRT (endosomal sorting complex required for transport) pathway that are required for particle budding.

Viral genomic RNA is synthesized in the nucleus and is then transported to the cytoplasm. Genomic RNA forms dimers, which are recognized by Gag and transported to the plasma membrane, where they nucleate new particle assembly (Kutluay and Bieniasz, 2010). New particles bud from the cell with the help of the cellular ESCRT complex (Meng and Lever, 2013). After release of the particle it matures, the viral protease cleaves Gag into individual proteins; this process leads to major structural changes in the formed virions.

Virion release is not the only way in which HIV-1 spreads within an infected organism. Virus can also be efficiently transmitted via direct cell-to-cell contacts through virological synapses; this process is similar to that described above (including the virion assembly, budding and maturation steps) (Bourinbaiar and Phillips, 1991; Haller and Fackler, 2008; Martin et al., 2010).

2. REVERSE TRANSCRIPTASE AND REVERSE TRANSCRIPTION OF HIV-1

Reverse transcriptase (revertase, RNA-dependent DNA polymerase, RdDp) is a DNA polymerase, and its main function is DNA synthesis (Herschhorn and Hizi, 2010). Reverse transcriptase has a lot in common with other DNA polymerases (in structure, sequence of polymerase active site and mechanism of DNA synthesis), but unlike most other DNA polymerases, which can use only DNA as a template, reverse transcriptases can use either DNA or RNA as a template. Reverse transcriptases have been found in different types of viruses, prokaryotes and eukaryotes, but in this chapter I will describe only HIV-1 reverse transcriptase.

2.1 History of discovery

Reverse transcriptase and the process of reverse transcription were discovered in 1970 (Baltimore, 1970; Temin and Mizutani, 1970); this discovery had a great impact on science and medicine (Menéndez-Arias and Berkhout, 2008). First, it complemented the central dogma of molecular biology. Previously, it was thought that information could be transmitted only from DNA to RNA to protein (or from RNA to RNA to protein for some RNA viruses). The discovery of reverse transcription proved that information can be transmitted in the reverse order (from RNA to DNA; this is also how the enzyme obtained its name (Crick, 1970)). Reverse transcription was first discovered in oncogenic retroviruses (Rauscher mouse leukemia and Rous sarcoma viruses), which facilitated the understanding of some of the mechanisms that these viruses used to drive oncotransformation. Just before the discovery of HIV-1, reverse transcription was being extensively studied, and researchers were trying to find ways to inhibit reverse transcriptase to fight cancer (Gallo, 2002). These technologies and findings became very useful for anti-HIV-1 drug development. For example, the first antiretroviral drug that was approved by FDA for clinical use in HIV-infected patients was the reverse transcriptase inhibitor azidothymidine (AZT or zidovudine) (Mitsuya et al., 1985), which was originally designed as an anti-cancer drug candidate. Reverse transcriptase inhibitors are still very important in ART: currently, they constitute roughly half of all anti-HIV-1 drugs and are divided into two main classes according to the principles of their actions (see below for details).

2.2 Biogenesis of HIV-1 reverse transcriptase

HIV-1 reverse transcriptase is synthesized as a part of the Gag-Pol polyprotein and because it contains Gag it becomes packaged into budding particles. During

the maturation of HIV-1 virions, reverse transcriptase is cleaved from the polyprotein by the viral protease. Active HIV-1 reverse transcriptase is a heterodimer and consists of two subunits: p51 and p66. The smaller subunit is similar to the larger one except that it is truncated at the C-terminus (Lowe et al., 1988; di Marzo Veronese et al., 1986). It is known that initially the viral protease cleaves two full polypeptides from the precursor. Then, two subunits form a p66/p66 homodimer, enabling the final protease cleavage step that leads to p66/p51 heterodimer formation (Sluis-Cremer et al., 2004).

2.3 Structure of HIV-1 reverse transcriptase

Of the two subunits of HIV-1 reverse transcriptase, only the larger (p66) subunit is enzymatically active (Figure 3). This subunit consists of 560 amino acid (aa) residues and has two enzymatically active domains: the N-terminal DNA polymerase domain and the C-terminal RNase H domain. The smaller subunit (p51) is shorter; it contains a 440-aa-long DNA polymerase domain but lacks an RNase H domain (Kohlstaedt et al., 1992; di Marzo Veronese et al., 1986).

The 3D-structure of the DNA polymerase domain of the p66 subunit is similar to the Klenow fragment of *Escherichia coli* DNA polymerase I and has a "right-handed" structure (Figure 4). It has 4 subdomains: fingers, palm, thumb, and connection. These subdomains and the RNase H domain are arranged side-by-side and therefore the p66 subunit has an elongated structure (Jacobo-Molina et al., 1991). The p66 subunit contains two active sites: a polymerase active site (responsible for DNA synthesis) and an RNaseH active site (responsible for the destruction of RNA contained in DNA:RNA heteroduplexes). Between the polymerase and RNaseH active sites, there is a binding cleft where a nucleic acid substrate lies (Jacobo-Molina et al., 1991). Reverse transcriptase mostly binds the sugar-phosphate backbones of DNA and RNA (Ding et al., 1998). The distance between the polymerase and RNase H active sites is approximately 60 Å, which can fit a DNA-DNA duplex that is 17-nucleotides long or an RNA-DNA duplex that is 18-nucleotides long. Near the polymerase active site, the DNA-DNA duplex structure is close to a B-form, while near the RNaseH active site its structure is close to an A form. To fit into the binding cleft, an RNA/DNA heteroduplex is bent by approximately 35º (Jacobo-Molina et al., 1991; Nowotny et al., 2007; Sarafianos et al., 2001).

The DNA polymerase domains of the p66 and p51 subunits have identical primary and secondary structures, with the same fingers, palm, thumb and connection subdomains. However, the spatial arrangement of these subdomains in p51 is drastically different from that in p66. As a consequence, the p51 subunit is more tightly packed and has a structural role; it also participates in $tRNA^{Lys3}$ binding and affects the RNase H activity of p66 (Mishima and Steitz, 1995; Sevilya et al., 2001, 2003). The fact that two polypeptides with identical sequences can adopt two very different conformations and perform different functions helps the virus get more out of its limited genome (Kohlstaedt et al., 1992).

The polymerase active site of p66 lies within the palm subdomain and consists of three aspartic acid residues (Asp110, Asp185, and Asp186), which bind two divalent cations (Mg^{2+}) that are required for catalysis (Ding et al., 1998). Asp185 and Asp186 are a part of the YXDD (Tyr(X)AspAsp) motif, which is highly conserved among many polymerases and may be a sign of their common origin (Argos, 1988; Kamer and Argos, 1984). The other part of this motif is represented by Tyr183 and Met184. These residues interact with nucleic acids and position the template-primer complex correctly in relation to the polymerase active site (Ding et al., 1998).

Next to the polymerase active site, there are several special sites where reverse transcriptase interacts with nucleic acid substrate:

- *The P site* priming site, where 3'-OH group of the primer is situated (Huang et al., 1998; Jacobo-Molina et al., 1991).
- *The N site* nucleotide binding site that binds dNTPs (Huang et al., 1998). It includes several important residues: 1. Tyr115 (binds to the deoxyribose ring and discriminates between rNTPs and dNTPs) (Boyer et al., 2000; Gao et al., 1997; Martín-Hernández et al., 1996), 2. Arg72 and Lys65 (interact with the triphosphate group) and 3. Gln151 (binds to the 3'-OH group) (Huang et al., 1998).
- *The template grip* consists of residues from the palm and fingers and interacts with the template strand.
- *The primer grip* consists of residues from the palm and thumb and interacts with the primer strand. Template and primer grips help to correctly position primer and template ends relative to the active site (Jacobo-Molina et al., 1991).

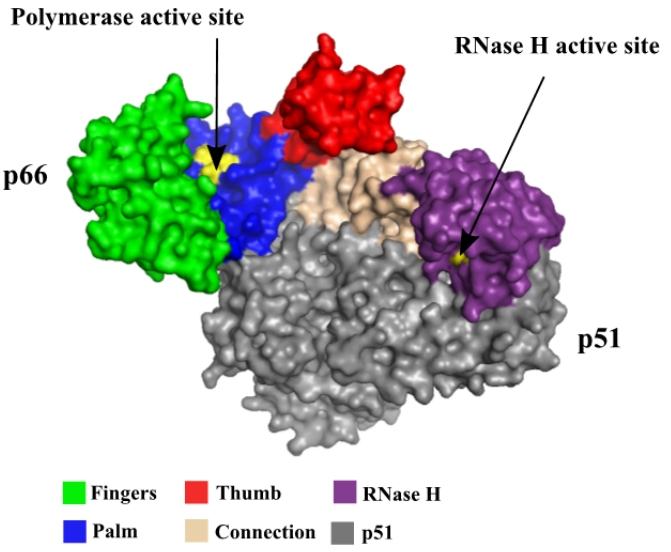


Figure 3. Structure of HIV-1 reverse transcriptase (taken from PDB ID: 1IKW).

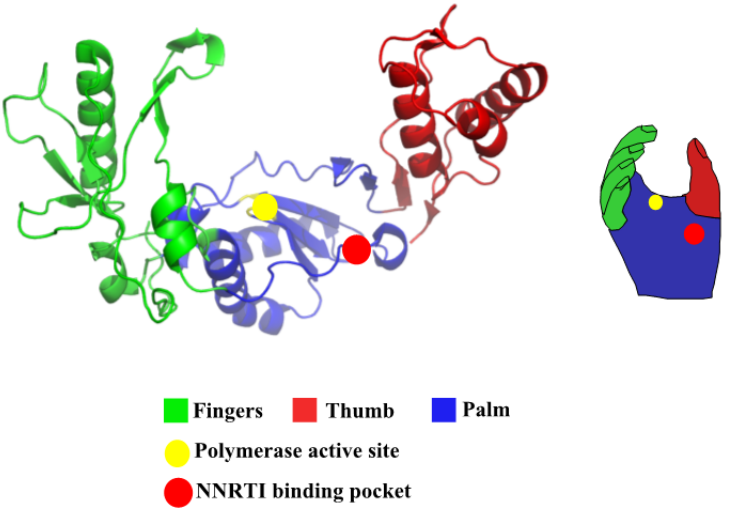


Figure 4. Ribbon representation of fingers, thumb and palm subdomains of HIV-1 reverse transcriptase p66 subunit (taken from PDB ID: 1IKW).

2.4 The process of reverse transcription

Reverse transcriptase of HIV-1 (and other retroviruses) has two different activities: 1) it can synthesize DNA using RNA or DNA as a template (DNA polymerase activity), and 2) it can destroy RNA in RNA:DNA heteroduplexes (RNaseH activity).

The main steps of HIV-1 reverse transcription are as follows (Götte et al., 1999; Hu and Hughes, 2012):

1. *Initiation of minus-strand DNA synthesis* (Figure 5A, 5B). As with any DNA polymerase, reverse transcriptase requires a DNA or RNA primer with a free 3'-OH group for the initiation of synthesis. In the case of HIV-1, $tRNA^{Lys3}$ serves as a primer. Its 3' end is annealed to the primer-binding site (PBS) of the viral RNA (Figure 5A). DNA synthesis starts from the 3' end of $tRNA^{Lys3}$ and proceeds until it reaches the 5' end of the template strand. The addition of the first 6 nucleotides is slow, probably due to some structural features of the primer, but then the polymerization rate increases (Lanchy et al., 1996, 1998). RNA in the formed RNA:DNA heteroduplex is destroyed by the RNase H activity of reverse transcriptase, while $tRNA^{Lys3}$ and the PBS region are left untouched (Figure 5B). The resulting short DNA fragment that is attached to $tRNA^{Lys3}$ is called minus-strand strong-stop DNA.
2. *First strand transfer* (Figure 5C). To continue minus-strand DNA synthesis, the minus-strand strong-stop DNA must be transferred to the 3' end of the genomic RNA (either the same or the other copy of genomic RNA) (van

Wamel and Berkhout, 1998). This is possible because each of the HIV-1 RNA molecules have two identical R regions at their 5' and 3' ends (Figure 2A). After the R region at the 5' end is copied and the template RNA of the copied region is destroyed, the minus-strand DNA fragment can complementarily bind the R region at the 3' end of the genomic RNA. Then, synthesis of the minus-strand DNA can proceed until the PBS region of the RNA template is reached. It is worth noting that PBS is the end of the template because the rest of the template was destroyed by RNase H.

3. *Initiation of plus-strand synthesis* (Figure 5D, 5E, 5F). During minus-strand synthesis, a heteroduplex of template RNA and new DNA is formed. The RNaseH activity of reverse transcriptase destroys the RNA in this heteroduplex, but not completely: some RNA regions, called PPTs (polypurine tracts), are resistant to RNaseH degradation (Figure 5D). HIV-1 has 2 PPTs: a 3' PPT and a central one. They both serve as primers for the initiation of plus-strand DNA synthesis (using nascent minus-strand DNA as a template) (Figure 5E). Plus-strand DNA synthesis continues with the copying of the PBS region of $tRNA^{Lys3}$ and pauses when it reaches a modified A base in the tRNA primer. At that point, reverse transcriptase cuts the tRNA off of the minus-strand DNA (leaving a single tRNA base attached to the 5' end of the minus-strand DNA) and therefore plus-strand strong-stop DNA is formed (Figure 5F).
4. *Second-strand transfer* (Figure 5G). Plus-strand strong-stop DNA has a 3' overhang (created as a result of the cutting off of the $tRNA^{Lys3}$ primer), which is complementary to the PBS at the 3' region of the minus-strand DNA. These regions interact with each other; usually, this is an intramolecular process, and a circular structure is formed (Yu et al., 1998). Note that Figures 5F and 5G are identical; the structure depicted in Figure 5G is a circular form of 5F.
5. *Synthesis of plus- and minus-strands proceeds* (Figure 5H and 5I). Nascent DNA strands are used as templates for each other. Subsequent DNA synthesis requires the strand displacement activity of reverse transcriptase. Synthesis is complete when reverse transcriptase reaches the end of the template. The resulting cDNA is longer than the genomic RNA (it has identical left and right LTRs) and has plus-strand overlap in its center.

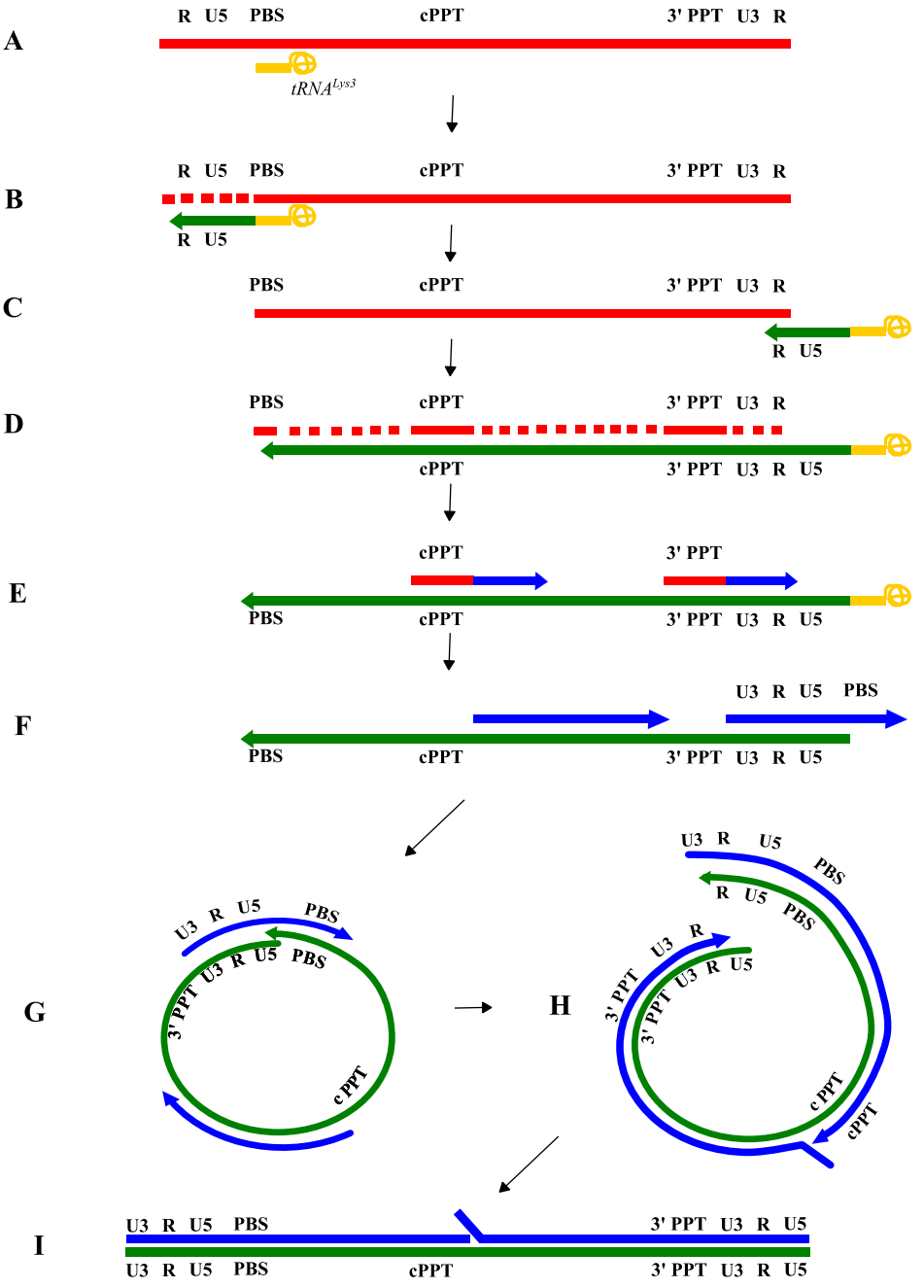


Figure 5. A schematic of the HIV-1 reverse transcription process (explanation in the text).

2.5 Molecular mechanisms used by reverse transcriptase and its polymerization reaction cycle

There are several steps in the polymerization reaction that are coupled with certain conformational changes in reverse transcriptase (these have been called "retroviral gymnastics" (Abbondanzieri and Le Grice, 2014; Abbondanzieri et al., 2008).

1. Unligated reverse transcriptase before the reaction has a "closed" conformation (the thumb subdomain touches the fingers subdomain).
2. In the first step, reverse transcriptase binds a template-primer duplex so that the 3'-OH group of the primer is located at the priming site (P site), which is located near the polymerase active site (Jacobo-Molina et al., 1991) (Huang et al., 1998). This binding is accompanied by the moving of the thumb subdomain to approximately 30º away from the fingers, enabling the enzyme to adopt an "open" conformation (Hsiou et al., 1996; Rodgers et al., 1995).
3. The next step is dNTP binding to the nucleotide-binding site (N site). This binding results in the formation of a ternary complex (Huang et al., 1998).
4. Then, part of the fingers subdomain moves to the thumb subdomain, forming an activated closed ternary complex. Thus, the fingers subdomain closes around the incoming dNTP, which helps to correctly align the α-phosphate of the dNTP and the 3'-OH of the primer relative to the polymerase active site. This is the rate-limiting step in single nucleotide incorporation reactions.
5. The 3'-OH of the primer attacks the α-phosphate of the incoming dNTP, and this leads to the formation of a 3'-5' phosphodiester bond and the release of pyrophosphate (PPi). Reverse transcriptase facilitates this reaction via a "two-metal-ion" mechanism (Steitz, 1998). Two magnesium ions coordinate the oxygen atoms of all three phosphates of the incoming dNTP and three catalytic aspartic-acid residues: one Mg^{2+} decreases the affinity of the 3'-OH of the primer for hydrogen and thus promotes 3' O^- attack on the α-phosphate; the other Mg^{2+} facilitates pyrophosphate release. Together, these two Mg^{2+} ions stabilize the charge and the structure of the transition state. After the formation of a 3'-5' phosphodiester bond, the fingers domain opens and allows PPi to leave the enzyme.
6. To bind the next incoming dNTP, a nucleic acid substrate must translocate relative to the reverse transcriptase so that the newly incorporated nucleotide moves from the N site to the P site.

In the case of efficient translocation, reverse transcriptase continues polymerization; this is called processive synthesis. However, the enzyme can also fall off of the nucleic acid substrate; in this case, polymerization starts again from the beginning (when the enzyme is in the "closed" conformation).

Overall, reverse transcriptase is a comparatively slow polymerase: its polymerization rate is 1–15 nucleotides per second, and it takes about 4 hours to

synthesize a full viral cDNA (Kim et al., 2010, 1989). It has been shown that reverse transcriptase can add a few hundred nucleotides in a single round of processive synthesis. However, it falls off of the substrate rather often, especially when it encounters difficult RNA secondary structures (pseudo-knots and so on) (Avidan et al., 2002). Thus, the processivity of reverse transcriptase (how many nucleotides are added before the enzyme falls off of the substrate) is quite low. Accordingly, the dissociation rate of the enzyme from the substrate limits the overall reaction rate (Avidan et al., 2002; Kati et al., 1992; Rittinger et al., 1995).

The fidelity of DNA synthesis (how accurately the template is copied) is also quite low, as reverse transcriptase lacks any proofreading activity: its mutation rate is $10^{-4} – 10^{-5}$ mutations per nucleotide (Menéndez-Arias, 2009; Preston et al., 1988; Roberts et al., 1988).

In addition to polymerase and RNaseH activity, HIV-1 reverse transcriptase also has strand transfer and strand displacement activities. Strand transfer activity is involved in both strand transfer events (the first being minus-strand DNA transfer and the second being plus-strand DNA transfer) (Basu et al., 2008). Strand displacement activity is necessary for plus-strand DNA synthesis; while copying minus-strand DNA, the remains of genomic RNA (RNaseH activity of reverse transcriptase cannot eliminate RNA completely) must be removed.

2.6 RNase H activity of HIV-1 reverse transcriptase

2.6.1 Structure

The structure of the RNase H domain of the p66 subunit is very similar to the RNase H enzymes of *Escherichia coli* and *Thermus thermophilus* (Davies et al., 1991; Schultz and Champoux, 2008). The active site of the enzyme consists of 4 highly conserved residues: Asp443, Glu478, Asp498, and Asp549. They coordinate two Mg^{2+} ions, which are important for RNase H activity (Nowotny et al., 2005).

RNase H primer grip is a region located close to the RNaseH active site, which consists of residues from the p66 and p51 subunits. It binds nucleic acid substrate and helps to position it correctly at both the RNase H and DNA polymerase active sites, and it also controls the cleavage specificity of RNase H (Sarafianos et al., 2001).

2.6.2 Enzyme activity

RNase H is an endonuclease that cleaves the RNA in RNA-DNA heteroduplexes: it hydrolyses the phosphodiester bond between two ribonucleotide residues and produces 3’-OH and 5’-phosphate ends (DeStefano et al., 1991; Krug and Berger, 1989). RNase H catalyzes this cleavage via a two-metal ion mechanism. As was mentioned above, there are two Mg^{2+} ions in the RNase H active

site. The first activates a nucleophilic water molecule, and the second (or both of them) stabilizes the transition state intermediate (Nowotny and Yang, 2006; Nowotny et al., 2005, 2007).

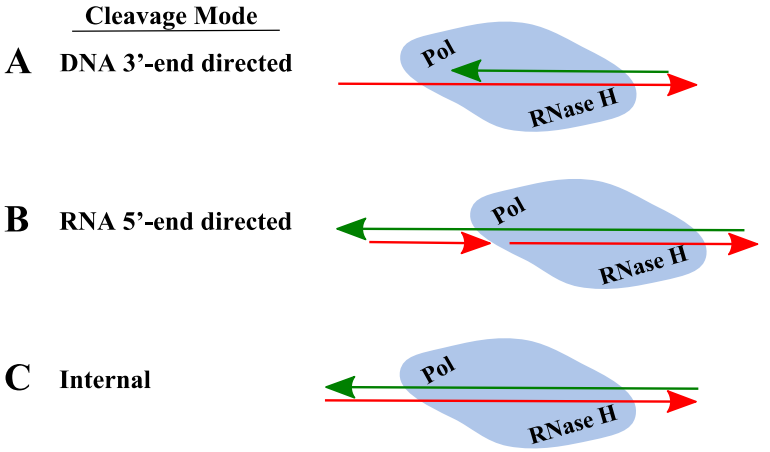


Figure 6. Modes of HIV-1 RNase H cleavage (from (Schultz and Champoux, 2008) with modifications). Reverse transcriptase is shown in blue-grey (pol: polymerase active site, RNase H: RNase H active site); DNA is shown in green; RNA is shown in red. The 3' ends of nucleic acids are indicated by arrowheads.

2.6.3 Modes of RNase H cleavage

1. DNA 3'-end-directed cleavage (can happen simultaneously with or independently from polymerization) (Figure 6A). The binding of reverse transcriptase to a nucleic acid substrate is identical to its binding during DNA synthesis. The polymerase domain is connected to the 3'-end of the nascent DNA chain, and the RNase H domain is located 15–20 nucleotides behind (towards the 5'-end of the nascent DNA chain) the polymerase active site. DNA 3'-end-directed cleavage leads to nicks and gaps in the RNA template.
2. RNA 5'-end-directed cleavage (polymerization-independent) (Figure 6B). Reverse transcriptase binds to an RNA-DNA heteroduplex in which the RNA template is nicked. The polymerase domain is connected to the 5' end of the nicked RNA, and the RNase H domain is 13–19 nucleotides ahead of it (towards the 3'-end of the RNA template strand).
3. Internal cleavage (polymerization-independent) (Figure 6C). Reverse transcriptase binds to an RNA-DNA heteroduplex irrespective of its nucleic acid ends.

Cleavage by the RNase H domain of HIV-1 reverse transcriptase is not sequence-specific. However, this reaction must produce PPT primers and the 5' end of the negative DNA strand very precisely.

2.7 Other proteins involved in the process of HIV-1 reverse transcription

In principle, HIV-1 reverse transcriptase is absolutely sufficient for viral cDNA synthesis. However, there are a number of viral and cellular proteins that take part (either directly or indirectly) in this process.

2.7.1 Viral proteins involved in reverse transcription

2.7.1.1 Nucleocapsid protein

Nucleocapsid protein binds nucleic acids mostly in non-specific manner. NC acts as a nucleic acid chaperone: it destabilizes secondary structures and promotes the formation of more stable nucleic acid duplexes (Levin et al., 2010). At the beginning of DNA synthesis, it helps to anneal the $tRNA^{Lys3}$ primer to the PBS site and facilitates the copying of a highly structured 5' region of HIV-1 RNA (Li et al., 1996; Sleiman et al., 2012). Nucleocapsid is important in strand-transfer reactions (especially in the first minus-strand transfer, when it unwinds the R region) (Rodríguez-Rodríguez et al., 1995). It also helps to remove the already-copied and fragmented (due to the activity of RNase H) pieces of RNA template from RNA-DNA heteroduplexes, thus ensuring that the initiation of positive-strand synthesis mostly occurs from PPT fragments (Jacob and DeStefano, 2008; Post et al., 2009).

2.7.1.2 Integrase

Integrase physically interacts with reverse transcriptase and promotes the early steps of DNA synthesis. It increases the processivity of reverse transcriptase probably by stabilizing it and enhancing its interactions with genomic RNA (Dobard et al., 2007; Wu et al., 1999; Zhu et al., 2004).

2.7.1.3 Viral protein R (Vpr)

Vpr is an accessory protein (not required for viral replication in some cell types), but it is abundant in the HIV-1 virion core, RTC and PIC. Like many viral proteins, it is a multifunctional protein, and it probably also influences reverse transcription. For example, it was shown that several peptides derived from Vpr could bind to reverse transcriptase and inhibit its activity (Gleenberg et al., 2007). Also, Vpr interacts with $tRNA^{Lys3}$-synthetase and probably inhibits the acetylation of $tRNA^{Lys3}$, thus facilitating the initiation of DNA synthesis ($tRNA^{Lys3}$ serves as a primer for viral DNA synthesis, and its 3'-terminus must not be bound to the amino acid) (Stark and Hay, 1998).

2.7.1.4 Negative factor (Nef), Trans-Activator of transcription (Tat) and matrix protein

Nef and Tat are accessory proteins. They increase the effectiveness of viral DNA synthesis, but the mechanisms of their actions are not completely understood; probably, their effects on reverse transcription are indirect (about nef: (Aiken and Trono, 1995; Schwartz et al., 1995), (about tat: (Harrich et al., 1997)). Matrix protein also influences some early stages of viral replication, although whether it affects reverse transcription has not been shown (Kiernan et al., 1998).

2.7.2 Cellular proteins involved in HIV-1 reverse transcription

2.7.2.1 Topoisomerase I

Topoisomerase I is an enzyme that helps resolve overwound DNA structures: it cuts a single DNA strand, allows DNA to unwind and then joins the phosphate backbone again. It physically interacts with HIV-1 NC and is a part of the virion core (Jardine et al., 1993). It stimulates the process of reverse transcription, but the exact mechanism of this stimulation is not clear (Shoya et al., 2003; Takahashi et al., 1995).

2.7.2.2 Proteins from the APOBEC group, uracil DNA glycosylase (UNG2) and the role of viral protein Vif (viral infectivity factor)

APOBEC (apolipoprotein B mRNA-editing enzyme catalytic polypeptide) is a family of RNA/DNA editing enzymes that deaminate cytidine into uridine residues. In the absence of Vif, APOBEC3 proteins inhibit HIV-1 replication (Moris et al., 2014; Sheehy et al., 2002) because they are packaged into assembling virions. This packaging occurs due to the interaction that occurs between APOBEC3, viral RNA and the NC part of the Gag polyprotein. During reverse transcription, APOBEC3 proteins recognize single-stranded DNA and change C to U; this editing eventually leads to hypermutation in the complementary DNA strand (a large number of G residues are changed to adenosine residues) (Goila-Gaur and Strebel, 2008). In some reports, it was found that hypermutation is not the only mechanism by which APOBEC proteins inhibit reverse transcription, but the alternative mechanism(s) of inhibition is not fully understood (Bishop et al., 2006; Holmes et al., 2007).

Vif, an accessory protein of HIV-1, inhibits APOBEC3G/F activity. Briefly, Vif interacts with APOBEC3 proteins, targets them for proteasomal degradation and thus reduces their packaging into HIV-1 virions (Feng et al., 2014). Another way the virus evades damage caused by dUTP incorporation into DNA is through its use of the cellular enzyme uracil DNA glycosylase (UNG2). UNG2 is packaged into assembling virions, and during reverse transcription it repairs incorrect G:U pairs with correct G:C pairs (Priet et al., 2003).

3. HIV-1 REVERSE TRANSCRIPTASE INHIBITORS

3.1 Nucleoside (nucleotide) reverse transcriptase inhibitors (NRTI)

3.1.1 General information

Nucleoside reverse transcriptase inhibitors (NRTIs) were the first compounds found to suppress HIV-1 replication. Correspondingly, a representative of this class of compounds, 3'-azidothymidine (AZT, zidovudine), was the first compound approved for clinical treatment of HIV-1-infected patients (Broder, 2010; Mitsuya et al., 1985). Currently, there are eight FDA-approved compounds from this class (see Table 1) (http://aidsinfo.nih.gov/drugs/search/searchterm/2/1). All NRTIs are nucleoside analogs; the common property of these compounds is that they lack 3'-hydroxyl groups. All of these compounds are prodrugs; after entering the cell, they are phosphorylated by cellular enzymes into their active triphosphate forms. They compete with normal nucleotides for binding with reverse transcriptase and can be incorporated into nascent viral cDNA. Due to the lack of the 3'-hydroxyl, a phosphodiester bond with the next incoming dNTP cannot be formed, and as a result a cDNA chain with NRTI at its 3' end cannot be elongated further (Cihlar and Ray, 2010; Menéndez-Arias, 2008; Sarafianos et al., 2009). NRTIs are, however, relatively toxic because in addition to HIV-1 reverse transcriptase they also inhibit cellular DNA polymerases, most prominently mitochondrial DNA-polymerase γ (Koczor and Lewis, 2010). As all modified nucleosides, NTRI-drugs are also potential mutagenic agents.

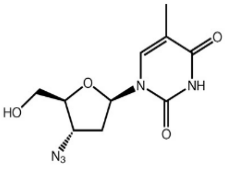
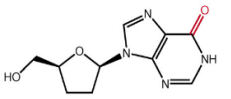
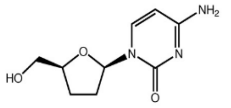
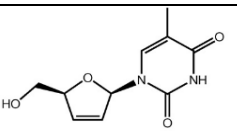
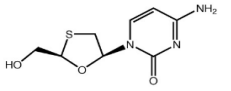
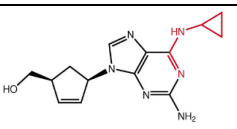
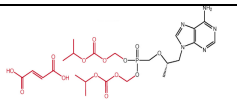
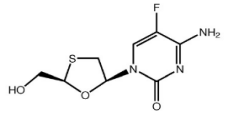
3.1.2 Life and fate of NRTI inside an infected cell

Cell entry. NRTIs are hydrophilic compounds and require carriers to cross the cellular membrane. For example, some nucleoside transporters influx NRTIs into the cell (Cass et al., 1999). In the opposite flow, other transporters, such as multi-drug resistance related proteins (MRP4, MRP5 and MRP8), efflux NRTIs out of the cell, thus reducing their activity (Guo et al., 2003; Schuetz et al., 1999).

Phosphorylation. NRTIs are converted into their active triphosphate forms by cellular enzymes. The efficiency of phosphorylation and the stability of NRTI-TP (triphosphates) inside of a cell influence NRTI activity. Phosphorylation is catalyzed by enzymes that usually phosphorylate normal nucleosides (Furman et al., 1986; Ho and Hitchcock, 1989; Van Rompay et al., 2000; Shewach et al., 1993); (addition of the second phosphate: (Bourdais et al., 1996)) (addition of the third phosphate: (Krishnan et al., 2002)). For most NRTIs, the rate-limiting step is the addition of the first phosphate (Perno et al., 1988). To overcome this problem, nucleotide inhibitors can be used. Thus, tenofovir already has the first phosphate and hence requires only the addition of the last two. For AZT, however, the rate-limiting step is the addition of the

second and third phosphates (Furman et al., 1986). AZT and d4T are phosphorylated by TK1, an S-phase specific enzyme, which is why they are not very active in resting cells (Gao et al., 1993).

Table 1. Structures of NRTIs approved by the FDA (structures taken from (Menéndez-Arias, 2008)

Name(s) (year of approval)	Structure	Modifications present	
		In sugar moiety	In nitrogenous base
Azidothymidine (AZT, zidovudine) (1987)		3'-hydroxyl group is replaced by 3'-azidofunctional group	thymidine analog; contains unmodified thymine base
Didanosine (ddI) (1991)		3'-hydroxyl group is absent	guanosine analog; contains hypoxanthine as a base
Zalcitabine (ddC, dideoxycytidine) (*rarely used*) (1992)		3'-hydroxyl group is absent	cytidine analog; contains unmodified cytosine base
Stavudine (d4T) (1994)		3'-hydroxyl group is absent; sugar ring is unsaturated	thymidine analog; contains unmodified thymine base
Lamivudine (3TC) (1995)		3'-hydroxyl group is absent; C3' is replaced with a sulfur; sugar is L-deoxyribose	cytidine analog; contains unmodified cytosine base
Abacavir (ABC) (1998)		3'-hydroxyl group is absent; sugar ring is unsaturated; O4' is replaced with a carbon	guanosine analog; contains 6-modified diaminopurine ring
Tenofovir disoproxil fumarate (TDF, tenofovir) (2001) prodrug of tenofovir (TFV)		has an acyclic linker instead of a sugar moiety	adenosine monophosphate analog; contains unmodified adenine base
Emtricitabine (FTC) (2003)		3'-hydroxyl group is absent; C3' is replaced with a sulfur; sugar is L-deoxyribose	cytidine analog; fluorine at the 5'-position

Inhibition of reverse transcription. NRTI-TPs compete with natural dNTPs for binding to HIV-1 reverse transcriptase. They occupy the nucleotide-binding site and become incorporated into the nascent cDNA chain instead of their natural prototypes (Huang et al., 1998; Ray et al., 2002). Their efficiency of incorporation depends upon the NRTI's capability to mimic the interactions of the natural substrate with the enzyme. For example, reverse transcriptase incorporates thymidine analogs (AZT-TP and d4T-TP) as efficiently as their normal counterparts (Kerr and Anderson, 1997; Vaccaro et al., 2000). On the contrary, the incorporation of cytidine analogs (ddCTP and 3TC-TP) is less efficient (Feng and Anderson, 1999; Krebs et al., 1997). After reverse transcriptase incorporates NRTI into a DNA chain, the end of this chain is translocated to the priming site. Next, dNTP can bind reverse transcriptase at the nucleotide-binding site, but it cannot be incorporated into the DNA chain because NRTI lacks a 3'-hydroxyl group and therefore cannot form a phosphodiester bond.

3.1.3 Resistance

There are two main mechanisms of resistance to NRTI: mutations that confer discrimination and those that confer excision; one reverse transcriptase can combine mutations of both types (Menéndez-Arias, 2008). Mutations that confer reverse transcriptase resistance to NRTI can also decrease its fidelity (when compared to wild type enzyme in the absence of inhibitor) (Menéndez-Arias et al., 2003). I will give a general overview of both mechanisms.

Discrimination mechanism. In this case, reverse transcriptase can "learn" to discriminate between normal dNTPs and their analogs without 3'-hydroxyl groups and does not incorporate the latter into the nascent cDNA chain. Mutations in the nucleotide-binding site confer this type of resistance. There are several examples of such mutations, which can be found either individually or as double-mutations:

1. *M184V/I* is associated with resistance to 3TC, FTC, ddC, ddI, and abacavir. Met 184 interacts with incoming dNTP at the 3' prime end. The substitution of Met with amino acid residues having branched side chains (Val, Ile) creates steric hindrance in the dNTP binding site and decreases the rate of inhibitor binding and incorporation by reverse transcriptase (Gao et al., 2000; Huang et al., 1998; Sarafianos et al., 1999). When combined with the K65R mutation, it makes reverse transcriptase susceptible to TFV (Feng et al., 2006).
2. The *L74V* (resistance to ddI, abacavir) and *Q151M complex* (resistance to AZT, ddI, ddC,d4T, abacavir and tenofovir) are mutations that change the hydrogen bonding network between reverse transcriptase and dNTP and make the presence of a 3'-OH in dNTP very important for binding to the enzyme, hence preventing the binding of NRTI-TPs (Deval et al., 2004a, 2004b; Sarafianos et al., 2004).
3. *K65R* is associated with resistance to TFV, ddC, d4T, 3TC, and ddI. Lys 65 directly interacts with the triphosphate group of the incoming dNTP (Gu et

al., 1995; Huang et al., 1998; White et al., 2006). This mutation, however, makes the reverse transcriptase very susceptible to AZT (Parikh et al., 2007; White et al., 2005).

Excision mechanism. In this case, NRTI can be incorporated into the cDNA chain, but it is then selectively removed by reverse transcriptase (Arion et al., 1998; Boyer et al., 2001; Meyer et al., 1998, 1999). As one could expect, mutations of amino acid residues that are located away from the nucleotide-binding site are involved in this type of resistance. There is also some selectivity, as this type of resistance occurs mainly against thymidine-analogs and therefore these mutations are called TAMs, or thymidine-analog mutations (resistance to AZT and d4T) (Hooker et al., 1996; Kellam et al., 1992; Larder and Kemp, 1989). Reverse transcriptase does not have the exonuclease activity that is required for true proofreading; instead, the excision reaction resembles the reaction of polymerization but run in reverse (Arion et al., 1998; Meyer et al., 1998, 1999). One of the mechanisms of excision is the following. First, NRTI is incorporated into the DNA chain. Then, elongated nascent DNA translocates and thus NRTI enters the priming site. Next, incoming dNTP binds the nucleotide-binding site of the enzyme, and the fingers close down, but the polymerization reaction does not occur, and the enzyme is stuck in the closed position. However, if the inhibitor is AZT, which has a long azido group, the binding rate of the next incoming dNTP is reduced, and the closed reverse transcriptase structure is destabilized. This allows the enzyme to translocate DNA in the reverse direction (thus, NRTI is again in the nucleotide binding site). Subsequently, reverse transcriptase excises NRTI using ATP as a donor of pyrophosphate (Boyer et al., 2001; Sarafianos et al., 2002).

There are also a number of mutations that can make reverse transcriptase multi-drug resistant; for example, insertions of two amino acids in the fingers subdomain between residues 69 and 70, as well as some others. These mutations probably destabilize reverse transcriptase complexes regardless of the type of NRTI that is bound to the enzyme (Boyer et al., 2002; Meyer et al., 2003).

3.2 Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

3.2.1 General information

NNRTIs are allosteric inhibitors of HIV-1 reverse transcriptase. They have very different chemical structures (Figure 7), but they all share the same mode of action: they bind non-covalently to a special site in the palm domain of the p66 subunit of reverse transcriptase. This binding leads to conformational changes in the enzyme and to an inhibition of its polymerase activity. NNRTIs are not prodrugs; they do not require cellular metabolism to become active, as they are

already present in their active form (de Béthune, 2010; Sluis-Cremer and Tachedjian, 2008).

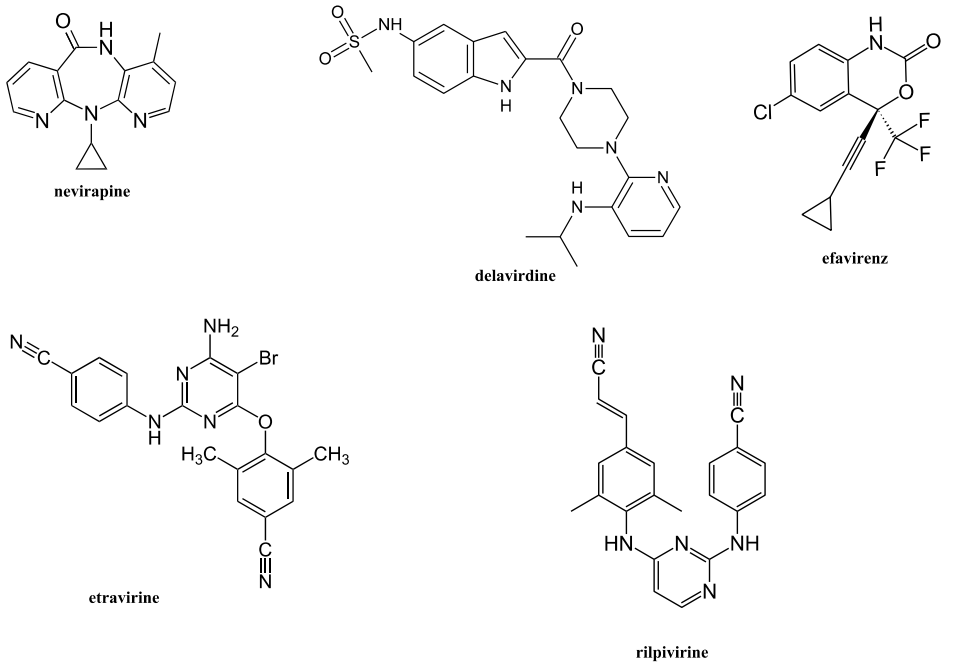


Figure 7. Structures of FDA-approved NNRTIs

Currently, 5 NNRTIs are approved by the FDA for clinical use: nevirapine (1996), delavirdine (1997), efavirenz (1998), etravirine (2008), and rilpivirine (2011) (http://aidsinfo.nih.gov/drugs/search/searchterm/3/1). The former three are first generation NNRTIs, while the latter two are second generation NNRTIs. NNRTIs are highly specific to HIV-1 reverse transcriptase and are unable to bind to and inhibit other retroviral reverse transcriptases (even that of HIV-2) or cellular enzymes. As such, they have comparatively low toxicity and very high therapeutic indexes; nevertheless, some side effects are still present (Balzarini, 2004).

3.2.2 Chemical structure

The NNRTIs are a group of small (<600 Da) compounds with diverse chemical structures and include more than 50 families of molecules (Balzarini, 2004; Zhan et al., 2013). In spite of their chemical diversity, NNRTIs contain common fragments, which participate in specific types of attractive noncovalent interactions. These fragments include the following:

1. Hydrocarbon-rich domain (involved in hydrophobic interactions, a type of interaction that occurs between nonpolar molecules).
2. Aromatic ring (involved in π-π stacking, a type of hydrophobic interaction that occurs between aromatic rings when they are arranged like a stack of coins)
3. Amide or thioamide moieties (involved in hydrogen bonding, a type of electrostatic interaction that occurs between an electronegative atom and a hydrogen atom (H) that is bound covalently to another electronegative atom (these electronegative atoms can be nitrogen (N), oxygen (O) or fluorine (F))

When NNTIs bind with reverse transcriptase, they can adopt two types of conformations:

- The "*Butterfly-like*" conformation is common to the first generation NNRTIs. These compounds have two hydrophobic residues (wings) connected by a linker group (body). Wing I lies next to the polymerase active site, and wing II is distal; the body lies at an angle of 110°–115° relative to the wings (Ding et al., 1995). Their structures are rigid, and they bind to reverse transcriptase in only one possible conformation; as such, only a single mutation in the NNRTI binding pocket is needed to make the virus resistant to the whole group of such inhibitors (Das et al., 2004).
- The "*Horseshoe-like*" or "*U-like*" conformation is common to the second generation NNRTIs. Unlike inhibitors with "butterfly-like" structures, these compounds are more flexible and can bind to reverse transcriptase in several possible conformations, thus inhibiting viruses with a mutation(s) in the NNRTI binding pocket (Das et al., 2004).

3.2.3 The NNRTI binding pocket

The NNRTI binding pocket is a special site to which all NNRTIs bind (Figure 5). It is located in the palm domain about 10 Å away from the polymerase active site (Ren et al., 1995; Smerdon et al., 1994). It is absent in the normal 3D-structure of reverse transcriptase and appears only when the enzyme and NNRTI interact (Kohlstaedt et al., 1992; Tantillo et al., 1994). The NNRTI binding pocket is flexible, and its conformation depends on the size and chemical nature of the NNRTI. It contains the hydrophobic sub-pocket (which is involved in hydrophobic interactions with NNRTIs and includes the aromatic residues Tyr181, Tyr188, Phe227, and Trp229) and hydrophilic amino acid residues (for example, Lys101 or Lys103), which can form hydrogen bonds with select NNRTIs. Interestingly, the reverse transcriptases of HIV-1 and HIV-2 have different amino acid residues at their 181 and 188 positions (HIV-1 has Tyr181 and Tyr188; HIV-2 has Ile181 and Leu188). This can partially explain why NNRTIs are not active against HIV-2 reverse transcriptase (Condra et al., 1992). In addition, the NNRTI binding pocket contains additional residues that

are contributed by the p66 subunit and even some residues from the p51 subunit.

3.2.4 Mechanism of inhibition

NNRTIs act as non-competitive inhibitors. They bind and inhibit the enzyme regardless of whether it is bound to a substrate or not (Debyser et al., 1992; Spence et al., 1995). The binding of an NNRTI to reverse transcriptase has several consequences:

1. Conformational changes in the polymerase active site, especially in the YMDD motif (Asp185 and Asp186 residues change in their positions) (Esnouf et al., 1995). This inhibits phosphodiester bond formation, while the rate of dNTP binding to the reverse transcriptase is increased (Rittinger et al., 1995; Spence et al., 1995; Xia et al., 2007).
2. Conformational changes in the "primer grip" (it moves about 5 Å upward), which makes it difficult to align the 3' end of the primer relative to an incoming dNTP (Hsiou et al., 1996; Xia et al., 2007).
3. Decreased mobility of the thumb subdomain (the "arthritic thumb" model), which inhibits the translocation of the primer-template duplex (Kohlstaedt et al., 1992; Tantillo et al., 1994). A slightly different hypothesis was proposed by Temiz and Bahar (Temiz and Bahar, 2002). For nevirapine, it was shown that the amplitude of the subdomain movements does not change but rather that they change in direction. For the second generation NNRTI efavirenz, it was shown that the amplitude decreases in addition to changes in the orientations of the subdomain movements.

3.2.5 Influence of NNRTI binding to particular steps of the reverse transcription reaction

Minus-strand DNA synthesis. The inhibitory effect of NNRTI depends on the type of primer/template (DNA/DNA or RNA/DNA and their sequences) and increases with the length of DNA product. For example, a large amount of nevirapine is required to inhibit minus-strong stop DNA synthesis, but further synthesis of minus-strand DNA is readily inhibited with lower nevirapine concentrations (Quan et al., 1998).

RNase H activity. The distance between the NNRTI-binding pocket and the RNase H active site is quite large, at approximately 60 Å. Nevertheless, NNRTIs influence the RNase H activity of reverse transcriptase according to the mode of RNase H cleavage; they can inhibit polymerase-independent or stimulate polymerase-dependent RNase H activity (Hang et al., 2007; Radzio and Sluis-Cremer, 2008; Shaw-Reid et al., 2005).

Initiation of plus-strand DNA synthesis. This process is also very efficiently inhibited by NNRTIs (Grobler et al., 2007). NNRTIs suppress binding of dNTP to the enzyme-primer/template complex if the primer/template is PPT RNA

primer/DNA template. In addition, NNRTIs disfavor reverse transcriptase binding to the PPT RNA primer/DNA template complex in a polymerase-dependent mode.

3.2.6 Inhibition of other stages of the viral life cycle by NNRTIs

Viral entry. The pyrimidinedione IQP-0410 acts as an NNRTI and also inhibits viral entry into the cell, as it also acts during the stage in which viral glycoproteins are already complexed with cellular receptors, but fusion with cellular membrane has not yet occurred (Buckheit et al., 2001).

Gag-pol processing and new viral particle formation. Efavirenz is also a potent enhancer of reverse transcriptase dimerization (Tachedjian et al., 2001) and is therefore able to enhance gag-pol dimerization in the cell before particle formation. Protease (as well as reverse transcriptase) is synthesized as a part of the gag-pol polyprotein and is active in a dimer form. For this reason, the enhanced gag-pol dimerization that occurs in producer cells induces premature protease activity. Activated protease cleaves gag-pol before particle budding, thus leading to impaired particle formation (Figueiredo et al., 2006; Tachedjian et al., 2005).

3.2.7 Resistance

The emergence of resistant variants of HIV-1 is the main obstacle to the successful application of NNRTIs in the clinic. This is especially the case for the first generation NNRTIs, as only a single mutation is needed to make the virus resistant to the whole class of compounds, whereas the second generation NNRTIs are active against these mutants. NNRTI resistant mutations mainly arise in the area adjacent to the NNRTI binding site (Ren and Stammers, 2008; Wensing et al., 2014). Here, I will provide a few examples of such resistance-associated mutations.

Mutations of Tyr181/Tyr188. Tyr181 can be mutated to Cys, and Tyr188 can be mutated to Cys/Ile/His. The Tyr181Cys mutation is very common in the clinic (Cheung et al., 2004). Tyrosines at positions 181 and 188 are involved in very important ring-stacking interactions with the first generation NNRTIs. Mutations of these residues to aliphatic amino acids without aromatic rings leads to the loss of such ring stacking interactions and decreases the abilities of NNRTIs to bind reverse transcriptase (Das et al., 1996, 2007; Ren et al., 2001). Contacts with Tyr181/Tyr188 are not very important for inhibitor binding if an inhibitor is flexible and can balance the loss of this important interaction by forming interactions with other amino acids (Tyr183, for example). In this case, this mutation will have a minor effect on the inhibitor activity of the compound. For example, nevirapine is 100 times less active against reverse transcriptase when it has the Y181C mutation, whereas efavirenz activity is less affected (Das et al., 2008; Ren et al., 2001).

The K103N mutation. The Lys103Asn mutation is the most common mutation that leads to resistance against NNRTIs (Cheung et al., 2004) and confers resistance to a wide range of NNRTIs. It is also frequently associated with transmitted resistance to NNRTIs. The exact molecular mechanism of resistance is not completely understood. Lys103 forms hydrogen bonds with only a few inhibitors (Esnouf et al., 1997) and does not interact with others. In the mutant enzyme, Lys is changed to Asn, and it was proposed that in unligated enzyme Asn can form a hydrogen bond with Tyr188, thus stabilizing the unligated enzyme's conformation and preventing the formation of the NNRTI binding pocket (Das et al., 2007; Hsiou et al., 2001; Lindberg et al., 2002; Maga et al., 1997; Ren et al., 2000; Rodríguez-Barrios and Gago, 2004). The second generation NNRTIs are active against the K103N mutant; however, the exact mechanism that allows them overcome its resistance is unknown. One possible explanation is that these NNRTIs can make additional contacts with Asn103, which has been shown for GW4511 (Chan et al., 2004; Ren et al., 2008). It has also been demonstrated that etravirine can destroy the Asn103-Tyr188 interaction (Das et al., 2004; Rodríguez-Barrios et al., 2005).

Mutations of L100I/V106A/V108I. These amino acids constitute the hydrophobic core of the NNRTI binding pocket. The main effect of these mutations is to change the Tyr181 (or Tyr188) position in reverse transcriptase and disrupt its aromatic ring-stacking interactions with NNRTIs (Ren et al., 2004, 2007).

STATE OF THE ART AND DEFINITION OF TASKS

Antiretroviral drug is a compound that inhibits replication cycle and propagation of a retrovirus (in almost all cases HIV-1). Two main approaches are used in antiretroviral drug discovery; both of them were applied in studies presented in this thesis.

The first one is a traditional *trial-and-error* method that relies on testing of many different (mostly random) compounds for their antiretroviral activity. This approach is time-consuming and not very fruitful. However, it holds a promise for discovery of inhibitors, which could have previously undiscovered mechanism of action. To increase the probability of such finding the library of compounds, that are analyzed, generally needs to be very large. At this case the method of choice is high-throughput screening by use of automated workstations. The required equipment and materials (including library of compounds) are often beyond the reach of academic research groups. Nevertheless the same approach can be used by screening of smaller sets of compounds, though one should expect that chances for discovery of hits, which could subsequently be optimized to efficient inhibitors, is accordingly much lower.

The second way to find novel inhibitors is a *rational drug design* (Menéndez-Arias and Gago, 2013). This approach requires knowledge of molecular mechanisms of a viral life cycle. Rational drug design relies on a defined drug target which is well studied and directly linked to a disease (or in case of viruses, essential for virus propagation). The initial library of compounds is, again, very large but the difference is that this needs not to be real library. Thus, in the beginning, thousands or millions of compounds are virtually screened for their ability to bind and change the function of the drug target. This results in shortlisted compounds. Only compounds, selected in this screen, should be verified for their antiretroviral activity experimentally. Theoretically, the hit discovery rate based on such method could be higher but the diversity of discovered compounds are limited to the known paradigm. Other limitations of this approach is that virtual screening is not always as reliable as a real screening and number of promising compounds could be missed (false negative results). Similarly, number of compounds with no activity are likely to occur at the top of the hit list (false positive results). Thus, virtual screening is as good as good is the algorithm. Too stringent algoritms, which may result in too many false negative results, have therefore their limitation. In contrast, it is relatively easy to eliminate false positive hits in subsequent assays. Thus, even preselected library will contain number of inactive compounds and the true hit (or lead) compound may even not be present in the hit list. Instead it may be hidden somewhere “close in chemical space”.

Currently, there are many classes of compounds targeting different stages of HIV-1 life cycle (viral entry, reverse transcription, integration into the host DNA, particle maturation and others) (Pau and George, 2014). Many of them are clinically approved compounds and thanks to them HIV-1 infection, once universally lethal, has been converted to the chronic infection. Surely, this has

somewhat decreased the need for new anti-HIV-1 drugs. Nevertheless, HIV-1 still represents exteremly important pathogen (and remains the most common killer among viruses infecting humans); thus it is likely short-sighted to conclude that fight with this virus is over.

The most part of this thesis is devoted to the development of new reverse transcriptase inhibitors – both NRTIs and NNRTIs. Reverse transcriptase inhibitors are very important in antiretroviral therapy as they proved to inhibit HIV-1 replication very efficiently. In addition, NNRTIs are very specific to HIV-1 and thus have comparably low toxicity. Reverse transcriptase itself and the reaction it catalyzes is very well studied: this makes the process of finding the candidates for testing more straightforward and reliable. Furthermore, several crystal structures of a wild-type and drug-resistant forms of reverse transcriptase are solved. This allows use of virtual screening for NNRTIs capable of inhibiting HIV-1 and its mutants resistant to the known NNRTIs. Hits from such screens may thus have higher genetic barrier to resistance and/or other propertis much needed for improvement of antiretrovirus therapy (long in vivo half-lifes, for example). Finally, even inhibitors which never reach clinical development (much less clinical use), teach us something new about virus and possibilities to inhibit it. Indeed, the 30 antiretroviral drugs represent just very small tip of iceberg compared to thousands compounds found to affect, at least to a certain extent, HIV-1 infection and millions of compounds lacking such property.

AIMS OF THE CURRENT STUDY

The main aim of this work was to find novel inhibitor(s) of HIV-1 replication from groups of compounds previously not tested for antiretroviral activities. Thus, such compounds would have novel (for HIV-1 inhibitor) folds. We were looking for compound(s) that would simultaneously be non-toxic to cells and highly active against wild-type and, preferably, mutant forms of the virus. However, this was not the absolute requirement; we were also looking for hit compounds with unique folds that could be subsequently modified to obtain more active and less toxic leads. To accomplish this aim we had to:

1. Develop and take into use a fast, safe and reliable system for screening compounds with possible antiretroviral activity;

2. Screen three different groups of compounds for their antiretroviral activity. The compounds belonged to:

I. acyclic thymine nucleoside analogues
II. bimorpholines and their derivatives
III. saccharide hydrazones;

3. Experimentally verify the results of the previous *in silico* screening (García-Sosa et al., 2011) and reveal whether the *in silico* selected compounds represent promising anti-HIV-1 hits. Upon finding such compound it became important to identify the active component of the original sample. By this we also aimed to provide experimental verification whether novel and potent HIV-1 inhibitors can be developed using this drug design strategy.

RESULTS AND DISCUSSION

1. Development of a new screening system for testing the antiretroviral activities of compounds (unpublished)

In the early stages of this study, the conditions needed for the use of infectious HIV-1 were not available in our institute. To overcome this obstacle, a simple, efficient and safe assay using HIV-1-based virus-like particles (VLPs) was designed. The VLPs were produced using a modified ViraPower Lentiviral Expression System (Invitrogen) (Figure 8). To produce such particles, 4 plasmids are required:

1. The pLenti6/V5-D-TOPO plasmid, which contains modified left and right LTRs and other HIV-1 regulatory sequences. These sequences enable the mRNA that is synthesized from this plasmid to be packaged into the VLPs and, importantly, to be reverse-transcribed and integrated into cells infected with such VLPs. This plasmid (and its mRNA, packaged into the VLP) contains sequences for the expression of both a blasticidin resistance marker and a reporter gene.
2. Three plasmids with helper functions. Two of them provide structural and regulatory proteins and HIV-1 enzymes in *trans*. The pLP1 plasmid encodes the gag/pol protein, and the pLP2 encodes the Rev protein. The third plasmid, pLP/VSV-G, encodes the envelope G glycoprotein from vesicular stomatitis virus, which was used to pseudotype the formed particles (it also serves as an antireceptor and a fusion protein for VLP entry into infected cells).

These four plasmids together are transfected into the 293FT cell line, resulting in the production of VLPs. These particles contain both HIV-1 enzymes (reverse transcriptase, integrase, protease) and mRNA produced from the modified pLenti6/V5-D-TOPO plasmid. When VLPs infect cells (generally we used U2OS cells for infection), this mRNA is reverse transcribed, and its corresponding cDNA is integrated into the cell genome, from which the antibiotic resistance and reporter protein genes are expressed (Figure 8).

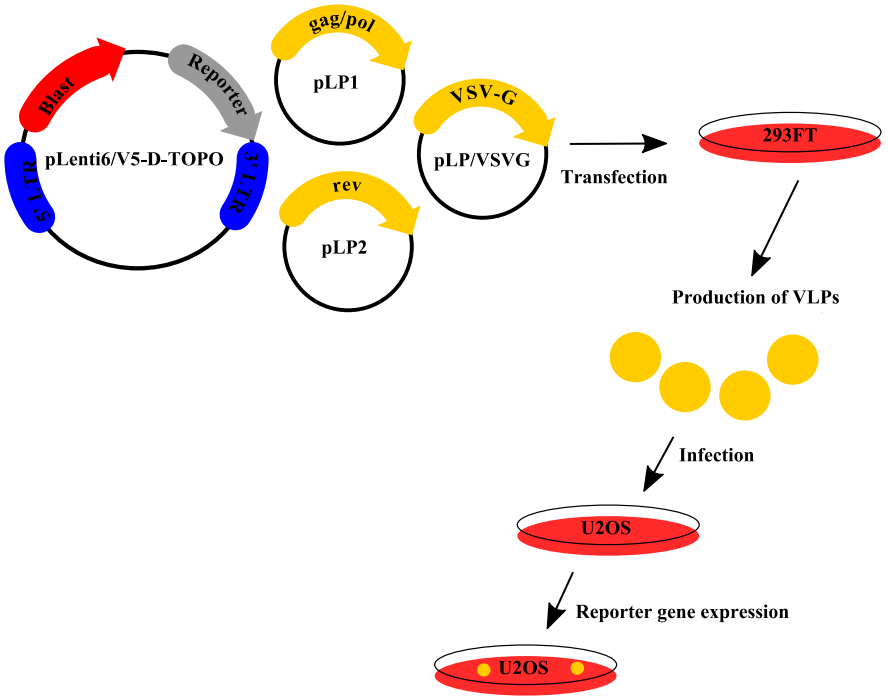


Figure 8. Schematic representation of ViraPower Lentiviral Expression System (Invitrogen)

We have introduced two main changes in ViraPower Lentiviral Expression System: changed the reporter gene in the pLenti6/V5-D-TOPO plasmid and introduced point mutations in the gag/pol gene in the pLP1 plasmid (Figure 9).

1. In our first experiments (article I and II), we used VLPs with EGFP reporter genes and performed colony-formation assays to evaluate the antiretroviral activities of the tested compounds. However, this method is time-consuming (10–14 days are required for colonies to form). Therefore, we replaced the EGFP-encoding sequence in the pLenti6/V5-D-TOPO-EGFP plasmid with a *Gaussia* luciferase-encoding sequence and obtained the pLenti6/V5-D-TOPO-GLuc plasmid (Figure 9A). *Gaussia* luciferase is produced only from synthesized (but not necessarily integrated) cDNA; thus, the activity of this reporter depends on the efficiency of reverse transcription. This assay is very sensitive and allowed us to determine the antiretroviral activity of each of the compounds by measuring *Gaussia* luciferase activity at 2 days post-infection, which was faster and also more accurate than the previous method.
2. One of benefits of using a VLP-based system is that this system is essentially free of reversions (mutations do occur during reverse transcription but because no progeny are made and the experiments are limited to a single round of reverse transcription, the mutations do not become dominant). This makes VLPs an ideal tool for the incorporation of reverse transcriptases

containing resistant-associated mutations. To do this, we introduced point mutations in the gag/pol gene in the pLP1 plasmid (Figure 9B). The mutations were selected from those known to produce resistance against the NNRTI nevirapine (and a number of other NNRTIs). In total, three mutant variants of reverse transcriptase were obtained and packed into VLPs: K103N, Y181C, and a double-mutant with both K103N and Y181C changed. These tools were used to analyze the specificity of each inhibitor and their individual abilities (or lack of it) to inhibit NNRTI-resistant reverse transcriptase.

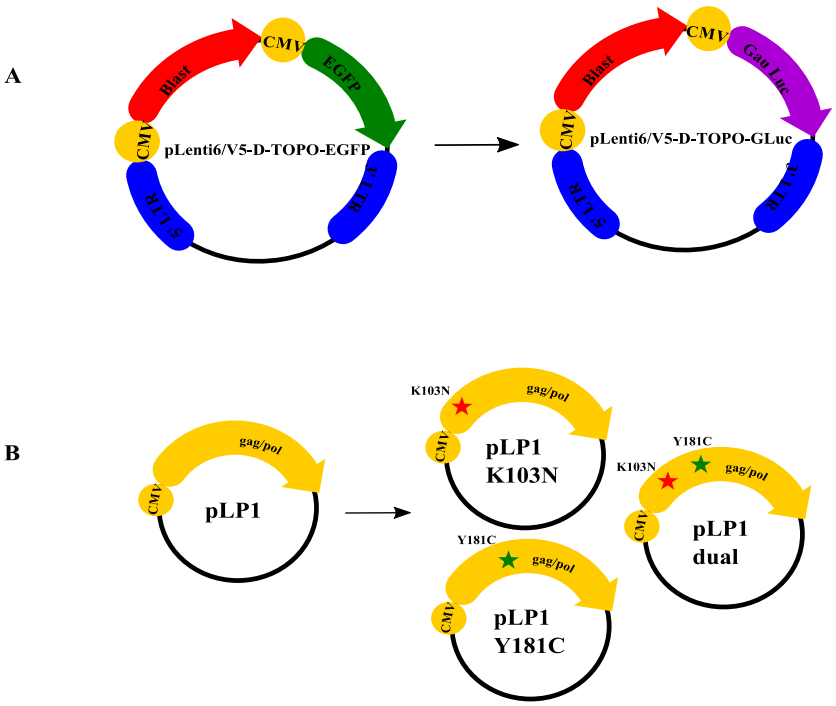


Figure 9. Schematic representation of plasmids used in the assay: (A) pLenti6/V5-D-TOPO-EGFP and pLenti6/V5-D-TOPO-GLuc plasmids, (B) pLP1 plasmids: wild type and with mutations (pLP1 K103N, pLP1 Y181C, pLP1 dual).

2. Antiretroviral activities of novel acyclic thymine nucleoside analogues (article I)

Acyclic nucleoside analogues are analogues of cellular nucleosides; they contain a normal (or, sometimes, modified) nitrogenous base and an acyclic sugar moiety. Such compounds can have antiviral activity. In the cell, they may become phosphorylated to their corresponding triphosphate forms and act as

chain-terminating inhibitors (in other words, they act as NRTIs for HIV-1). The most famous examples of acyclic nucleoside analogues include acyclovir and penciclovir (guanine derivatives, herpes simplex virus inhibitors) (Boyd et al., 1987; Elion et al., 1977), ganciclovir (guanine derivative, cytomegalovirus inhibitor) (Mar et al., 1983), tenofovir (adenosine monophosphate analogue, HIV-1 and hepatitis B virus inhibitor) (Balzarini et al., 1993) and several others (De Clercq and Field, 2006).

In this study, five acyclic thymine nucleoside analogues were synthesized (article I, schemes 1–4) and analyzed for their antiretroviral activities. First, the toxicity of each compound was measured using an MTT cell viability assay. This assay revealed that the synthesized compounds were not toxic at the highest concentration applied (50 μM) (article I, Table 1 and this thesis, Table 2). Then, the antiretroviral activity of each compound at 50 μM was analyzed using HIV-1 VLPs in a colony formation assay. All tested compounds had some inhibitory effect, reducing colony formation by 45 – 63% compared to DMSO, which was used as a vehicle control (article I, Table 2 and this thesis, Table 2).

Table 2. Biological properties of novel acyclic thymine nucleoside analogues

Compound	Toxicity assay, HeLa[a] 50 μM	Antiretroviral activity[b] 50 μM
DMSO (negative control)	100	100
6a	97	60
6b	104	48
6c	110	52
6d	108	45
6e	103	63
Lam	ND	9
AZT	ND	3

[a] cell viability, percentage from negative control, [b] efficiency of colony formation, percentage from negative control, ND – not determined.

As these compounds were not toxic, it served as indication that they had direct effect on infectivity of HIV-1 VLPs. The exact mechanism of their actions was not studied, but presumably they can act as chain-terminators (which is a common mechanism of acyclic nucleoside analogues). Another possibility would be that they inhibit the integration of proviral cDNA; in the absence of integration, colony formation would have been inhibited as well. However, this is much less likely, as the tested compounds share no similarities with known HIV-1 integrase inhibitors. It should be also noted that attempts at finding the rate-limiting step restricting the potency of these compounds were made, but they were not successful. Indeed, the lack of prominent inhibition of these compounds may result from poor cellular uptake, inefficient conversion of these prodrugs into active (triphosphate) forms or the inability of HIV-1 reverse transcriptase to use

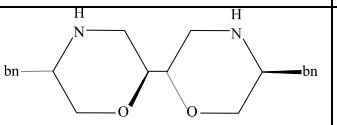
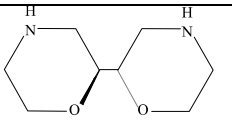
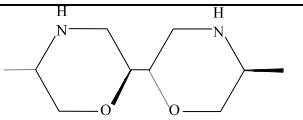
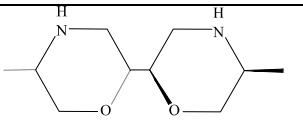
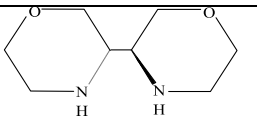
them. Obtaining these compounds in triphosphate forms was a complicated endeavor, which hampered their use in the cell-free replication assay. The expression of the thymidine-kinase of herpes simplex virus (an enzyme known to phosphorylate many acyclic nucleoside) had no effect on the potencies of these compounds. Thus, the rate-limiting step remained unknown. Regardless of their individual natures and the mechanisms of action of the members of this class of compounds, their detected antiviral effects were not prominent enough to warrant subsequent studies.

3. Antiretroviral activity of bimorpholines (article II and unpublished data)

Morpholine is a simple heterocycle. The morpholine moiety is a structural motif that is found in different natural products and therapeutically active compounds and has been extensively used in drug design (Wijtmans et al., 2004). Examples of morpholine-containing compounds include the antidepressant drug reboxetine (Wong et al., 2000), the neurokinin 1 receptor antagonist aprepitant (Patel and Lindley, 2003) and several others. Also, morpholine-containing compounds have been shown to have antiviral activity. They act as potent non-nucleoside allosteric inhibitors of HCV polymerase (Beaulieu et al., 2004; Hirashima et al., 2007) and HIV-1 integrase inhibitors (Gardelli et al., 2007).

In this study, six compounds representing bimorpholine and its derivatives were synthesized (article II, Figure 1, schemes 1–4 and this thesis, Table 3), and their biological activities were studied. I will focus on the toxicity profiles and antiretroviral activities of compounds **1** and **8**–**11**. Using an MTT cell viability assay, it was found that these compounds were generally not toxic at a 50 μM concentration. However, the results for compound **1** were somewhat controversial, as its toxicity depended on cell type and even on the amount of cells present (confluency) at the time of treatment (article II, Tables 1 and 2). This somewhat confusing data was one of the reasons why in subsequent studies the MTT method was substituted with the more sensitive and reliable xCELLigence assay. The controversy regarding the toxicities of the compounds did not, however, affect the outcome of the anti-retroviral activity assay. When applied at a 50 μM concentration, none of the compounds were active in the HIV-1 VLP-based colony formation assay (this thesis, Table 3). Thus, it should be concluded that the analyzed compounds were unable to inhibit HIV-1 reverse transcriptase or integrase.

Table 3. Biological properties of bimorpholines

Compound	Structure	Toxicity assay, HeLa[a] 50 μM	Antiretroviral activity[b] 50 μM
DMSO (negative control)	–	100	100
1		101	67
8		90	91
9		98	72
10		91	63
11		96	69
Lam	–	ND	9

[a] cell viability, percentage from negative control, [b] efficiency of colony formation, percentage from negative control, ND – not determined.

4. Antiretroviral activity of saccharide hydrazones (article III)

Hydrazones containing heterocycles and sugar moieties can be considered acyclic nucleoside analogues, and such types of compounds have been shown to have antiviral activity. For example, they can inhibit herpes simplex virus type-1 (HSV-1) and hepatitis A virus replication (Abdel-Aal et al., 2008; Rashad et al., 2009). Moreover, some hydrazone derivatives have antiretroviral activity: they inhibit HIV-1 reverse transcriptase RNase H and/or DNA polymerase activities (Arion et al., 2002; Distinto et al., 2012; El-Sayed et al., 2009; Himmel et al., 2006).

Table 4. Biological properties of saccharide hydrazones

Compound	Monosaccharide	Toxicity assay, U2OS[a] 1 mM	Antiretroviral activity[b] 1 mM
DMSO (negative control)	–	100	100
4	L-arabinose	108	131
5		106	96
6		108	11
7	D-galactose	102	117
8		106	126
9		109	136
10	D-mannose	100	101
11		94	93
12		100	122
13	D-ribose	112	93
14		115	115
15		113	101
16	L-rhamnose	110	95
17		115	133
18		108	117
19	2-deoxy-D-ribose	105	121
20		102	150
21		104	64
nevirapine	–	ND	0,03

[a] cell viability, percentage from negative control, [b] Gaussia luciferase activity, percentage from negative control, ND – not determined.

A rather large set of novel compounds (18 in total) with different sugar moieties was synthesized, and their biological activities were tested (article III, Table 1 and this thesis, Table 4). The xCELLigence cell viability test revealed that these compounds were very well tolerated by cells, and no toxicity was observed even at 1 mM concentrations. The antiretroviral activities of these compounds were tested using HIV-1 VLPs, which carried RNA molecules capable (when reverse-transcribed) of expressing the *Gaussia* luciferase reporter. It was found that only two compounds (**6** and **21**) inhibited the infectivity of HIV-1 VLPs. Of these, compound **21** was a very weak HIV-1 inhibitor: when applied at 1 mM, it reduced the *Gaussia* luciferase signal to 64 % relative to the vehicle control and thus has an IC50 > 1 mM. In contrast, when compound **6** was applied at a 1 mM concentration, it reduced the *Gaussia* luciferase signal to 11 % of that from the samples treated with vehicle control (this thesis, Table 4). However, when this compound was used at lower concentrations, its inhibitory effect was dramatically reduced. The IC50 for this compound was ≈ 400 μM, which is much higher than that of the typical acyclic nucleosides (article I) or non-nucleoside inhibitors that are described in article IV. Therefore, the anti-HIV-1 properties of these compounds were not studied further, and their mechanisms of action

remain unknown. However, as these compounds were absolutely not toxic, it could be assumed that their effects on the infectivity of HIV-1 VLP were direct.

5. Non-nucleoside reverse transcriptase inhibitors and *in silico* screening (article iv)

5.1 Cytotoxicity and antiretroviral activity of 16 compounds selected using *in silico* screening

The starting point of the work was to develop novel NNRTIs using a previously described *in silico* screening technique (García-Sosa et al., 2011). Two approaches were used in this *in silico* screen: i) The compounds were ranked according to their abilities to bind both wild-type and Y181C drug-resistant HIV-1 reverse transcriptases, and ii) the compounds were screened for their abilities to bind several antitargets (i.e., biomolecules, the binding to which can cause undesired side-effects). This combined approach allowed us to find possible inhibitors of HIV-1 reverse transcription and to simultaneously roughly predict their metabolic interactions. Not surprisingly, the top hits resulting from this screen were known antiretroviral compounds, but the hit list also contained a large number of compounds antiretroviral properties of which were not known. To analyze whether any of these compounds had the ability to suppress HIV-1 infection (article IV, Figure 1), 16 compounds with diverse chemical structures were experimentally tested for their antiretroviral activities. These compounds were purchased, and their biological properties were determined. Each of the 16 compounds was screened for toxicity (using the xCELLigence cell viability test), antiretroviral activity (using HIV-1 VLPs with *Gaussia* luciferase reporter gene) and the ability to inhibit a cell-free reverse transcription reaction (enzymatic reverse transcription assay).

Cell viability analysis revealed that at 50 μM compounds **1**, **14** and **15** were highly toxic; moderate toxicity was detected for compounds **4**, **8**, **11** and **16** (article IV, Figure 2A, Table 1). For these compounds, an analysis was also performed using a 5 μM concentration, revealing no detectable toxicity at that concentration (article IV, Figure 2B, Table 1). Accordingly, in subsequent experiments using HIV-1 VLPs, non-toxic concentrations of each compound were applied, except for the moderately toxic compounds, which were tested at both analyzed concentrations (thus, 50 μM was used for compounds **2**–**13** and **16**, and 5 μM was used for **1**, **4**, **8**, **11**, **14**, **15**, and **16**).

When tested against HIV-1 VLPs, compounds **11**, **13**, and **16** showed moderate activities, and compound **8** was highly active: compound **8** decreased *Gaussia* luciferase signal to 5.2% of that of the vehicle control when it was applied at a 50 μM concentration and to 20% of the control when it was applied at a 5 μM concentration (article IV, Figures 2C and 2D, Table 1). Further analysis revealed that the IC50 for compound **8** was 0.33 ± 0.07 μM (article IV, figure 3A, table 2), which is well below any of the compounds that were ana-

lyzed in studies I–III and only 10 times higher than the activity of the licensed NNRTI nevirapine, which was used as a positive control (its IC50 in the same test was 0.043 ± 0.007 μM) (article IV, Table 2). Thus, compound **8** was considered to represent a potential NNRTI with relatively high activity.

The efficacies of compounds are often seriously affected by their abilities to enter cells. To determine whether any of the compounds were more active in the absence of this obstacle, all of the compounds were tested in a cell-free enzymatic reaction. As NNRTIs are not particularly efficient in inhibiting the type of reaction that was used in this study (see above), each compound was tested at high concentrations (200 μM and 50 μM). It was found that at 200 μM only compounds **1**, **2**, **8**, and **12** were able to inhibit the activity of HIV-1 reverse transcriptase (article IV, Figure 4A, Table 1), whereas at 50 μM only compound **8** was active (article IV, Figure 4B, Table 1). The IC50 for compound **8** in this cell-free reaction was much higher than in the cell-based assay; it was 105.7 ± 30 μM (article IV, Figure 3B, Table 2), which is about 100 times higher than the IC50 of nevirapine (0.92 ± 0.15 μM) in the same assay (article IV, Table 2). Nevertheless, this assay clearly confirmed that compound **8** acts as an NNRTI, as only compounds belonging to this class of inhibitors should be active in this cell-free assay. Compounds **1, 2** and **12** probably possess some NNRTI activity as well, but such activity was clearly lower than that of compound **8**. None of these compounds showed detectable activities in a cell-based assay, indicating that their anti-HIV-1 activities were hampered by intrinsic cytotoxicity (compound **1**) and/or by an inability to enter cells (the most likely explanation for the lack of activity in the cases of compounds **2** and **12**).

Thus, three performed tests revealed that only compound **8** represents a potential HIV-1 inhibitor.

5.2 Chemical characteristics of compound 8. Antiretroviral activity of compound 8 (purified from the commercial sample) and its separated isomers

Chemically, compound **8** is a diaryltriazine derivative. It is a symmetrical molecule, and it binds to HIV-1 reverse transcriptase the same way after a 180° flip. It has a chiral center and exists in two stereo-isomeric forms. Diaryltriazine derivatives can have anticancer activities (Menicagli et al., 2004), antibacterial activities (Kumar and Menon, 2009; Lübbers et al., 2000; McKay et al., 2006; Srinivas et al., 2005), and antifungal activities (Ghaib et al., 2002). Furthermore, some diaryltriazine derivatives act as potent NNRTIs (Ludovici et al., 2001), although for compound **8**, or even compounds with similar structures, antiretroviral activity has not been previously shown.

To determine which stereoisomer of compound **8** is responsible for the inhibition of HIV-1 replication, a large sample of compound **8** was purchased. First, its purity was tested using HPLC (article IV, Figure 5). It turned out that commercial samples of compound **8** (both the one used in the first screen and the

one purchased later) were mixtures of at least four different chemicals, although compound **8** was indeed the main component of the mixture (about 89% of the total mass of the sample). Three additional compounds (designated initially as **x**, **y** and **z)** together constituted about 11% of the mass of the sample. To get rid of the additional compounds, compound **8** was purified by HPLC to homogeneity; the fractions containing the **x**, **y** and **z** compounds were also collected (note that it was impossible to separate compounds **x** and **y** from each other, so they were collected as a mixture of compounds designated **x**/**y**). Then, compound **8** was separated into two stereo-isomers using an HPLC chiral column.

Next, the antiretroviral activities of compound **8** (purified from the commercial sample) and its two separated stereo-isomers were analyzed. To our surprise, the activity of purified compound **8** was drastically lower (detectable only at 50 μM, article IV, Figure 6) than that of the original (non-purified) mixture. It was impossible to detect whether this residual activity was due to the true anti-HIV-1 activity of the purified compound or whether it was caused by residual impurities and/or toxic effects of the compound (article IV, Figure 2A). Correspondingly, neither stereo-isomers of compound **8** had clear anti-HIV-1 activity (data not shown). Thus, it become clear that compound **8,** a compound revealed by an *in silico* screen and the major component of the purchased sample, could not be the true HIV-1 inhibitor; instead, the antiretroviral activity of the sample belonged to some additional component(s). It was also clear that, as the additional components together consisted of approximately 11% of the mass of sample, the anti-HIV-1 activity of an active component(s) should be much higher than that of the mixture. In light of these data, it would have been interesting to re-visit the data produced by compounds **1, 2** and **12,** all of which demonstrated some NNRTI activity in the cell-free assay. If these compounds are mixtures (which is quite possible) then their anti-HIV-1 activities may also belong to minor component(s); these may be more potent and/or less toxic as initial compounds. However, taking into account the amount of work that was required to identify and synthesize the active component of compound **8** (see below), such studies were not performed.

5.3 Identification of the active component(s) from the initial sample of compound 8

To test the hypothesis that the antiretroviral activity of compound **8** belongs to some other minor component(s) of the initial mixture, we analyzed the antiretroviral activities of: i). the compound **8** before purification (initial sample together with impurities), ii) compound **8** after purification, iii). impurity **z**, and iv). impurity **x**/**y** from the initial sample. For this, each compound was applied at its maximum possible concentration (note that the exact concentrations of **x/y** and **z** could not be calculated, as neither their structures nor the ratio of **x**/**y** in the mixture were not known). First of all, toxicity testing was performed; it revealed that only the **x/y** mixture was non-toxic, as the other compounds

showed moderate toxicities (data not shown). In VLP-based activity assay, the **x**/**y** mixture had the highest antiretroviral activity; this activity was even higher than the activity of the compound **8** sample before purification. At the same time, both purified compound **8** and the **z** impurity had moderate antiretroviral activities (article IV, Figure 6).

This experiment confirmed that compound **8** itself was not the active component of the initial sample. Instead, most (if not all) of the sample's antiretroviral activity belonged to components **x** or/and **y**. Hence, our next goal was to determine the active compound(s) present in the initial sample.

To do this, two approaches were used:

1. An analysis of the methods that were possibly used for the synthesis of compound **8**.

2. An analysis of the **x**/**y** fraction using combined high-resolution MS and NMR approach.

5.4 The analysis of the methods that were possibly used for the synthesis of compound 8

5.4.1 Identification of compounds 17, 18, and 19

The molar masses of compounds **x**, **y**, and **z** were estimated using HPLC analysis. It was assumed that compounds **x**, **y**, and **z** could be "side" products or intermediates of compound **8** synthesis. As there was no information on how exactly compound **8** was produced, all possible synthetic routes were analyzed. Based on this, possible candidates for **x**, **y**, and **z** were proposed. To maintain consistent numbering, they were correspondingly designated as compounds **17**, **18**, and **19** (article IV, Table 3). All of these compounds were similar to compound **8**; they each shared the same diaryltriazine core but had somewhat different functional groups. When compounds **17**, **18**, and **19** were docked into the NNRTI binding pocket of HIV-1 reverse transcriptase (similarly to what was done in the previous article (García-Sosa et al., 2011)), it was predicted that compound **18** would be the most potent inhibitor, that compound **19** would be less potent and that compound **17** would be the least potent (article IV, Table 3).

5.4.1 Toxicity and antiretroviral activity of compound 8 synthesized in-house

The synthesis route that was most likely used for the synthesis of compound **8** was applied to obtain a larger amount of the compound and larger amounts (as was hoped) of the impurities **x, y** and **z** (note that by this time we had exhausted the world supply of compound **8**, and no more could be purchased from any known source). It should be noted that while the synthesis of compound **8** was highly efficient, the final product was very clean and did not contain impurities similar to **x**, **y**, or **z** (thus, synthesizing a dirty compound is sometimes a problem). As could be expected, synthesized compound **8** and its two stereo-isomers

behaved similarly to purchased and purified compound **8** and its purified stereoisomers. Again, the activities against HIV-1-based VLPs of both compound **8** and its isomers were rather low (IC50 (comp **8**) = 16.0 ± 3.5 μM; IC50 (**isomer 1**) = 18.2 ± 5.2 μM; IC50 (**isomer 2**) = 32.0 ± 12.0 μM) (article IV, Figure 7, Table 2 and data not shown). In addition, synthesized compound **8** and its isomers did not inhibit an *in vitro*, cell-free reverse transcription reaction (data not shown). These data indicate that compound **8** has some anti-HIV-1 activity of its own, but it was too low to be detected in the cell-free assay.

5.4.2 Toxicity and antiretroviral activity of compounds 17, 18, and 19 synthesized in-house

For the next step, compounds **17**, **18**, and **19** were synthesized. Compounds **17** and **19** were not active in the HIV-1-based VLP assay (article IV, Table 2). As such, our attention was focused on compound **18.** It was determined that this compound was rather toxic (a first indication that it is not identical to impurity **y**), but it was certainly the only active compound synthesized in-house (which was consistent with *in silico* predictions). Using HIV-1-based VLPs, its IC50 was determined to be 2.5 ± 0.3 μM, and its CC50 (in these experiments, the U2OS cell line was used) was 42.2 ± 5.6 μM. Hence, its SI (selectivity index, the ratio CC50/IC50) was about 17 (article IV, Figure 8A, Table 2). When the experiment was performed using full infectious virus, compound **18** was also found to be active, with IC50 = 5.6 ± 1.1 μM, CC50 = 57 ± 22 μM (determined for TZM-bl cells) and SI = 10 (article IV, Figure 8B, Table 2). Finally, it inhibited reverse transcription in a cell-free reaction (IC50 = 116 ± 12 μM) (article IV, Figure 8C, Table 2) but lacked activity against HIV-1 VLPs carrying mutant forms of reverse transcriptase resistant to the known NNRTI nevirapine (3 types of mutants were used: with K103N mutation, with Y181C mutation or with both of these mutations together).

Taken together, all of these data confirmed that compound **18** is a directly acting antiviral and NNRTI and that its binding to its target is affected by known anti-NNRTI resistance mutations.

5.4.3 Analysis of compound 18 binding mode

Computational analysis showed that compound **18** binds to reverse transcriptase similar to the binding modes of approved NNRTIs (etravirine (Das et al., 2004) and rilpivirine (Das et al., 2005; Pauwels, 2004) (article IV, figure 9). It binds deeply into the tight, closed NNRTI binding pocket. It has a flexible structure, which allows it to adopt different conformations and therefore it can bind reverse transcriptase in several modes. Compound **18** forms the following interactions with the NNRTI binding pocket:

1. π-π stacking interactions with the aromatic residues Tyr 181, Tyr 188, Trp 229, and Phe 227;

2. hydrophobic interactions with the non-polar residues Leu 234, and Val 179;
3. hydrogen bonds with the charged residues Lys 101 and Glu 138.

The important features that differentiate compound **18** from other diaryltriazine derivatives with NNRTI activities (Ludovici et al., 2001) include the following:
1. its charged ammonium group, which strongly interacts with the Glu 138 carboxylic group;
2. its molecular symmetry, which allows it to bind to the NNRTI binding pocket tightly after a 180° flip.

However, despite the flexible structure of compound **18**, mutations at positions 103 and/or 181 still completely inhibited its anti-HIV-1 activity, presumably by preventing (or weakening) its binding to its target.

5.4.4 Compound y was predicted incorrectly

Although compound **18** was the most potent of the compounds synthesized in-house, it was also obvious that it is not identical to impurity **y**; it is more toxic, and most importantly its activity (IC50 = 2.5 ± 0.3 μM) was not sufficient to be responsible for the high antiretroviral activity of the initial compound **8** sample (IC50 = 0.33 ± 0.07 μM). As the latter contained only a small fraction of the **x**/**y** impurity, the structure of one of the compounds (**x** or **y**) must have been predicted incorrectly. To test this hypothesis, we compared HPLC chromatograms for the **x**/**y** fraction from the initial compound **8** sample (two compounds in one peak, impossible to separate) and for an artificial **17**/**18** mixture (two clearly separated peaks). At the same time, the peak of compound **x** was identical to the peak of compound **17**, whereas the peaks of compound **y** and compound **18** were different (data not shown). Thus, we concluded that the structure of compound **x** (**17**) was predicted correctly, whereas the structure of compound **y** (**18**) was predicted incorrectly. It was also clear that all antiviral activity of the **x/y** mixture belonged to compound **y** because compound **17** (corresponding to **x**) lacked antiretroviral activity. This indicates that the true compound **y** should have an IC50 that is considerably lower than 0.33 μM (the IC50 of the initial sample of compound **8**).

5.5 The analysis of the x/y fraction using combined high-resolution MS and NMR approach

5.5.1 Identification of compound 20

Because the amount of the **x**/**y** impurity in the initial sample of compound **8** was very small (only ≈ 0.2 mg of material was collected), a combined high-resolution MS and NMR approach was applied to resolve the structure of compound **y**. In this experiment, synthesized compound **17** was used as a reference for the

x component of the **x**/**y** mixture. First, the exact molar mass of **y** was measured using LC-ESI-QTOF-MS, and from this its molecular formula, $C_{17}H_{18}N_5O$, was determined. Then, the structures of compounds from the **x**/**y** mixture were analyzed by NMR, which confirmed the presence of oxygen instead of nitrogen (as in compound **18**). Based on this, a structure for **y**, now designated compound **20**, was proposed (article IV, Table 3). Compound **20** is very similar to compound **18**, and they have identical molar masses; the only difference is that the $-NH_2$ group of compound **18** is substituted with an –OH group in compound **20**. According to computational analysis, compounds **18** and **20** bind to the NNRTI binding pocket in the same way, with the only difference being that compound **20** binds to the NNRTI binding pocket more strongly (article IV, Figure 10). In addition, compound **20** exists in several tautomeric forms and protonation states, which helps the compound to adapt to the protein.

5.5.2 Toxicity and antiretroviral activity of compound 20 synthesized in-house

Compound **20** was synthesized in-house in quantities sufficient for the analysis of its biological properties. The analysis was, however, partly hampered by the fact that compound **20** was poorly soluble in DMSO (its highest possible concentration was 2.5 mM, compared to >10 mM for compound **18** and the other compounds tested in this study). Thus, the highest concentration we could apply in our cell culture experiments was 12.5 μM (in order not to exceed a 0.5% concentration of DMSO in culture media; it was found that DMSO above this concentration affects cell viability). At 12.5 μM, compound **20** was clearly non-toxic, and it is likely that it would also be tolerated at considerably higher concentrations. Most importantly, it was highly active in the HIV-1-based VLPs assay (IC50 = 0.067 ± 0.009 μM) and also against full HIV-1 (IC50 = 0.16 ± 0.05 μM) (article IV, Figures 11A and B, Table 2). Taking into account that compound **20** was not toxic at the highest tested concentration (12.5 μM), its selectivity index values should be no less than 186 (for VLPs and U2OS cells) and 78 (for HIV-1 and TZM-bl cells). Compound **20** also inhibited reverse transcription in cell-free conditions (IC50 = 55.1 ± 7.5 μM) (article IV, Figure 11C, Table 2). Despite its high activity, compound **20** was not active against HIV-1 VLPs carrying a reverse transcriptase that was resistant to nevirapine (which had a K103N or Y181C mutation) (data not shown); however, surprisingly, it showed some activity against the dual mutant (with both K103N and Y181C mutations). This activity was, however, rather limited (IC50 = 8.0 ± 4.0 μM), indicating that the combination of mutations reduces the activity of compound **20** by more than 100-fold (article IV, Figure 11D).

CONCLUSIONS

This thesis summarizes results obtained during several studies dedicated to a search for new compounds capable of inhibiting HIV-1 infection.

1. The developed HIV-1 VLP based assay was found to be fast and sensitive. The assay is also safe-to-use and can be used outside of BSL3 facilities needed for studies involving infectious HIV. The assay allows screening compounds for their ability to inhibit some particular stages of HIV-1 life cycle: reverse transcription reaction and entry into the nucleus; also it can be modified for screening for integrase inhibitors. Another benefit of this system is that it allows testing the activity of compounds against HIV-1 VLPs carrying mutant forms of reverse transcriptase. Such VLPs enable the confirmation of the mechanism of action of the inhibitors and allow us to analyze whether NNRTI-resistant replicases are sensitive to new compounds. Finally and most importantly, in all cases where HIV-1 VLP based assay and the assay based on the use of infectious HIV-1 were used in parallel the results of these assays correlated with each other. If anything, the HIV-1 VLP based assay was somewhat more sensitive than assay based on use of wt virus.

Currently, our laboratory provides the opportunity for different Estonian chemical research groups to test their compounds for toxicity and antiretroviral activity. Furthermore, simple and sensitive HIV-1 VLP based assay can be used by laboratories lacking BSL3 facilities. I believe this promotes the field of the development of antiretroviral compounds in Estonia.

2. During these studies, several sets of compounds with presumable anti-HIV-1 activities were analyzed. The experimental tests of these hypothesis yielded in different results.

I. Each of the five analyzed acyclic thymine nucleoside analogues had minor antiretroviral activity. Thus, acyclic nucleoside analogues can act as antiretroviral compounds and probably share mechanism of action described for NRTIs.
II. The least active group of compounds, analyzed in the presented studies, was the group of bimorpholines and their derivatives. None of these compounds had any detectable direct antiretroviral activity. In addition, these compounds were rather toxic, and their minor effects on the infectivity of HIV-1 VLPs can be attributed to their toxic effects on cells.
III. None of eighteen studied novel saccharide hydrazones was toxic at a 1 mM concentration, and two of them had antiretroviral activity. This activity, however, was far lower than the activities found for the most potent compounds selected in studies I and IV (and more so compared to licensed anti-HIV-1 drugs).

3. Antiretroviral activities of compounds selected in the previous *in silico* screening (García-Sosa et al., 2011) were tested. None among 16 selected and tested compounds was a potent HIV-1 inhibitor indicating that most of these represented false-positive hits of *in silico* screen. One compound (number **8**), however had a low antiretroviral activity. Search for compounds with structures similar to that of compound **8** resulted in several new compounds, 4 of which (compounds **17**, **18**, **19**, and **20**) were synthesized and experimentally tested. Out of these, compound **18** was moderately active while the activity of compound **20** was comparable with the activity of the known NNRTI nevirapine. Unfortunately, neither of these compounds was able to efficiently inhibit HIV-1 VLPs carrying certain forms of reverse transcriptase resistant against NNRTI drugs. This limits possible practical value of the compounds themselves. However, the set consisting from several compounds with similar structures but very different anti-HIV-1 activities, offers possibilities of *in silico* hit-to-lead optimization. Indeed, compounds **17**, **18**, **19**, and **20** were also ranked according to their relative abilities to bind wild-type reverse transcriptase using *in silico* methods (the same approach was used when the initial set from 16 compounds were selected). The results of prediction fitted perfectly with the experimental data which additionally proves that we have developed a working algorithm for searching for HIV-1 NNRTIs which can be altered for purpose of lead-to-hit optimization.

Thus, *in silico* prediction indeed revealed a structure that had some anti-HIV-1 potency but that was clearly not optimal for this activity. However, *in silico* analysis clearly allowed for the narrowing down of chemical space when searching for compounds with high activities.

Every year hundreds of articles describing new *in silico* methods and predictions based on them are published. Such searches have not been limited to the prediction of novel HIV-1 inhibitors; large variety of pathogens and the diseases have been targeted. However, results of only very few of such screens have been verified experimentally. In this study we performed such verification and describe several problems that may be characteristic for this type of approach. Verification of theoretical data is important and is the only way to improve existing *in silico* methods and to finally find really potent inhibitor(s) (or other compounds with desired properties).

SUMMARY IN ESTONIAN

Kui bioloog kohtab keemikut: HIV-1 inhibiitorite otsingul

HIV on ohtlik virus, mis avastati eelmise sajandi 80-ndate alguses. Tollel ajal tähendas diagnoos "HIV-positiivne" nakatunutele sisuliselt surmaotsust. Nüüd, veidi rohkem kui 30 aastat hiljem, elavad HIV-positiivsed inimesed oluliselt kauem ja palju täisväärtuslikumat elu. Kõik see on saanud võimalikuks tänu ennenägematult ulatuslikele HIV-alastele uurimistöödele. Erakordselt põhjalikult on uuritud viiruse bioloogia eripärasid; välja on töötatud süsteemid viiruse tuvastamiseks organismis. Alates 1987-ndast aastast on loa kliiniliseks kasutamiseks saanud ligi 30 erinevat ühendit, mis jagunevad toimemehhanismide alusel viide erinevasse klassi. Erinevatesse klassidesse kuuluvate inhibiitorite kombineeritud kasutamine on kujunenud HIV-ga nakatunud patsientide ravimise aluseks. Samas, seoses viiruse suure muteerumisvõime ja osade (eriti vanemate) ravimite kõrge toksilisusega, on säilinud vajadus leida uusi ja paremaid (aktiivsemaid ja vähem toksilisi) toimeaineid. Seetõttu tegeleb meie töörühm, nagu ka sajad teised laborid üle maailma, uute retroviirus-vastaste ühendite otsinguga.

Antud väitekirja põhieesmärgiks oli leida efektiivseid HIV-1 replikatsiooni inhibiitoreid, mis kuulvad kas nukleosiidsete (NRTI) või mittenukleosiidsete (NNRTI) pöördtranskriptaasi inhibiitorite hulka. Enne käesolevat uuringut ei olnud Eestis süstemaatiliselt tegeletud keemiliste ainete HIV-vastaste omaduste uurimisega ning puudus ka võimalus infektsioonilise HIV-1-ga koekultuuris töötada.

Töö esimeseks eesmärgiks oli HIV-1 inhibiitorite analüüsi tarvis kiire, ohutu ja usaldusväärse süsteemi väljatöötamine. Selleks valisime HIV viirus-laadsetel osakestel (VLP) põhineva test-süsteemi, mida kasutasime alternatiivina tõelisele (infektsioonilisele) HIV-1 viirusele. Selle süsteemi loomiseks modifitseerisime lentiviirusel põhinevat ekspressioonivektorit nii, et see ekspresseeriks kergesti määratavat markervalku – *Gaussia* lutsiferaasi. See iseenesest lihtne täiendus tegi test-süsteemi palju kiiremaks ja tundlikumaks ning muutis selle kasutamise kergemaks. Lisaks sisestasime HIV-1 *pol*-geeni sisaldavasse ekspressioonikonstrukti NNRTI vastase resistentsusega seotud mutatsioone; see võimaldas meil analüüsida uuritavate keemiliste ainete toimet ravimiresistentsete replikaaside suhtes. Tulemused, mis saadi selliseid HIV VLP-sid kasutades, korreleerusid infektsioonilist HIV-d kasutades saadud tulemustega; reeglina oli HIV VLP-de põhine süsteem infektsioonilisel viirusel põhinevast süsteemist isegi tundlikum.

Järgmiseks eesmärgiks oli neid süsteeme kasutades uurida kolme erineva ühendite komplekti HIV vastast aktiivsust. Esiteks analüüsiti viie uudse mittetsüklilise tümidiini nukleosiidi analoogi omadusi. Igal selle grupi ühendil tuvastati madal retroviirus-vastane toime. Kõige aktiivsem aine sellest rühmast (**6d**) vähendas (50 μM kontsentratsioonil) VLP-de infektsioonilisust kuni 55%. Lähtudes ühendite struktuurist oletati, et need ained toimivad NRTI-dena; selle oletuse kinnitamist takistas aga ühendite madal aktiivsus. Teine grupp ühendeid

koosnes kuuest bimorfoliinist ja nende derivaatidest. Ühelgi neist ühenditest olulist retroviirus-vastast aktiivsust ei tuvastatud. Selline tulemus oli võimalikest ka kõige tõenäoliseim, sest sellise struktuuriga ühendid ei saa HIV-d inhibeerida seni teadaolevate mehhanismide kaudu. Seega on antud töö näiteks "katse ja eksimuse" meetodi rakendamisest inhibiitorite otsingul. Sellised eksperimendid võivad küll viia põhimõtteliselt uudsete toimeainete ja mehhanismide avastamiseni, kuid edu saavutamise tõenäosus on madal. Kolmanda ainete grupi moodustasid kaheksateistkümmend uudset sahariidhüdrasooni. Neid aineid võib pidada mitte-tsükliliste nukleosiidide analoogideks. Üsna ootuspäraselt olid need ühendid madalama HIV-1 vastase aktiivsusega, kui esimesse gruppi kuuluvad ained: kõige aktiivsema aine (ühend number **6**) inhibeeriv kontsentratsioon 50 (IC50) oli ≈400 μM. Ehkki neil ühenditel puudusid toksilised kõrvalmõjud muutis väga madal aktiivsus nende põhjalikuma uurimise ebapraktiliseks.

Kolmandaks eesmärgiks oli uurida alternatiivse lähenemise – ratsionaalse ravimidisaini – kasutamise võimalusi. Eelneva töö (García-Sosa *et al*., 2011) käigus *in silico* meetodil uuritud ühenditest valiti järgnevateks analüüsideks välja kuusteist keemilist ühendit. Meie ülesandeks oli eksperimentaalselt kindlaks teha, kas need ained võiksid toimida NNRTI-dena. Läbiviidud katsed näitasid, et vaid üks ühend (number **8**) kuueteistkümnest omas retroviirus-vastast toimet. Ehkki selle ühendi aktiivsus oli madal võimaldas selline tulemus algatada ühendile **8** sarnaste ühendite uurimise. Selle töö käigus leidsime kaks uut HIV-1 inhibiitorit: ühendid number **18** ja **20**. Ühend **20** osutus kõikidest meie laboris uuritud ainetest kõige tugevamaks HIV-1 inhibiitoriks: selle aktiivsus metsik-tüüpi viiruse suhtes oli võrreldav kliinilisel kasutusel oleva nevirapiini (NNRTI) aktiivsusega. Samas polnud ühelgi uuritud ühendil võimet oluliselt maha suruda NNRTI resistentsete pöördtranskriptaaside aktiivsust. Seega võimaldas ravimite *in silico* ennustamine tõepoolest leida struktuuri, mis omas HIV-1-vastast toimet. Ehkki esialgsel kujul polnud see struktuur kaugeltki optimaalne võimaldas järgnev *in silico* analüüs kõrgema aktiivsusega ühendite kavandamist. Antud töö tõestab *in silico* eeluuringute rakendatavust akadeemilises uurimistöö (kus pole reeglina võimalik läbi viia kõrge läbilaskevõimega skriiningut); samuti tõstab see esile *in silico* analüüsis saadud tulemuste eksperimentaalse kontrollimise tähtsust.

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