Ph.D. Thesis

Modulation of HIV-1 Protease Activity

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Contents

Abstract

HIV-1 protease plays a crucial role in the late state of the life cycle of HIV virus. It is responsible for the cleavage of the viral polyprotein precursors during virion maturation into the structural and functional proteins. If it is effectively inhibited, HIV particles remain immature and noninfectious. The application of highly active antiretroviral therapy (HAART) including protease inhibitors can reduce plasma HIV-1 levels below the detection limit in adherent patients and thus dramatically change their life expectancy. However, the unusual and unexpected plasticity of the protease together with high replication error rate leads to rapid development of viral resistance under the selection pressure of HIV protease inhibitors. According to the Stanford HIV Drug Resistance Database, almost one-half of amino acids in HIV protease can mutate and substitutions at one-third of all residues have clinical relevance to drug resistance to the current protease inhibitors. The clinical utility of the first inhibitors was limited by severe side effects, low bioavailability and high pill burdens, which ultimately reduced adherence of patients to the recommended therapy. These problems often lead to therapy failure and development of drug resistance. To overcome these difficulties, second-generation inhibitors were developed. Despite an indisputable improvement they brought to antiretroviral therapy, the development of new highly active HIV-1 protease inhibitors with optimal pharmacokinetic properties, higher metabolic stability, little off-target activity, and particularly, more favorable resistance profiles is still of high importance.

This thesis provides an overview of anti-HIV- drugs including development of substituted metallacarboranes, a new class of potent, unusual, nonpeptidic HIV protease inhibitors with therapeutic potential.

Next, the impact of protease background on the development of resistance of maturation inhibitor bevirimat is analyzed. Our data suggest that the mutations in the protease influence the level of antiviral resistance towards bevirimat. The viruses with mutated proteases show more diverse resistance profiles compared to those with wild-type protease. These observations can be explained by the different efficiencies of the Gag substrate cleavage by the different proteases.

Finally, regulation of enzymatic activity by small alkali cations has an important role in many biological processes. Their specific effects on the HIV protease activity were studied by a combination of experimental and computational techniques. Our molecular dynamic simulations confirm that the affinity of alkali cations to the HIV protease surface follows the Hofmeister series, mostly due to interactions with carboxylate side chain groups of aspartates and glutamates. Accordingly, our experimental data also showed that the initial velocity of peptide substrate hydrolysis in the presence of different alkali cations generally follows the Hofmeister series, with the exception of caesium. The higher catalytic efficiencies (k_{cat}/K_M) in the presence of K^+ ions in comparison to other alkali cations were observed at corresponding salt concentrations. Furthermore, we observed an unexpected increase in the hydrolysis of a specific substrate at very low salt concentration.

Abbreviations

AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ARV	antiretroviral drugs
AZT	zidovudine
BVM	bevirimat
CA	capsid
CA	carbonic anhydrase
CCR2	C-C chemokine receptor type 2
CCR5	C-C chemokine receptor type 5
CD4	receptor found on the surface of immune cells
CD8	co-receptor, predominantly expressed on the surface of cytotoxic T cells
CDC	Centers for Disease Control and Prevention
CTD	C-terminal domain
CXCR4	alpha-chemokine receptor type 4
DNA	deoxyribonucleic acid
FDA	Food and Drug Administration
FTC	emtricitabine
HAART	highly active antiretroviral therapy
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
HS	Hofmeister series
IDV	indinavir
IN	integrase
INSTI	integrase strand transfer inhibitor
IOCB	Institute of Organic Chemistry and Biochemistry
k_{cat}	unimolecular rate constant, turnover number
K_i	inhibition constant
K_M	Michaelis constant
LEDGF	lens epithelial derived growth factor

LPV	lopinavir
MA	matrix
MES	2-(*N*-morpholino)ethanesulfonic acid
MSM	men who have sex with men
MUT	mutant
NC	nucleocapsid
NFV	nelfinavir
NNRTI	non-nucleoside reverse-transcriptase inhibitor
NRTI	nucleoside analog reverse-transcriptase inhibitor
NTD	N-terminal domain
NtRTI	nucleotide analog reverse-transcriptase inhibitor
PDB	Protein Data Bank
PI	protease inhibitor
PR	protease
RNA	ribonucleic acid
RT	reverse transcriptase
RTI	reverse transcriptase inhibitor
RTV	ritonavir
SIV	simian immunodeficiency virus
SQV	saquinavir
TAF	tenofovir alafenamide
TDF	tenofovir disoproxil fumarate
TFV	tenofovir
TPV	tipranavir
UNAIDS	Joint United Nations Programme on HIV and AIDS
WHO	World Health Organization
WT	wild-type

1. Introduction

1.1 The evolving epidemic of HIV/AIDS

Since its recognition in 1981, the human immunodeficiency virus (HIV) epidemic has evolved in the most severe pandemic in modern times with estimated 34 million people living with HIV and 2.5 million newly infected worldwide in 2011 (*1*). Although the first patients with AIDS (Acquired Immune Deficiency Syndrome) were in MSM (Men who have sex with men) in the United States and Western Europe, today almost half of infected people are women and two third of all new cases come from sub-Saharan Africa, mostly via heterosexual transmission (Fig. 1). More than 25 million people died of AIDS-related illnesses since the beginning of the epidemic, over 60 million people have been affected.

Intensive prevention campaigns, HIV screening of donated blood and successful use of antiretroviral therapy (ART) to prevent mother-to-child transmission resulted in the decline of new infections by more than one fifth compared to 1997, when the pandemic culminated. Prevention of sexual transmission has turned out to be more difficult. Despite the optimism about expanded access to treatment and prevention programs, several western countries face rising incidence of new HIV diagnoses again; particularly in the community of MSM the number has been increasing steadily in recent years (*2*) and the HIV has no longer appropriate collective attention there.

Although the Czech Republic still belongs to the countries with the lowest HIV prevalence, in recent years the number of newly infected rises three times faster than it did a decade ago. At the end of 2012, the total number of 1,858 HIV-positive was registered in the Czech Republic with 363 cases that have progressed to AIDS, 186 people died (*3*). With substantially reduced government spending on HIV/AIDS prevention, education, and therapy, experts are afraid of further acceleration of the HIV spread.

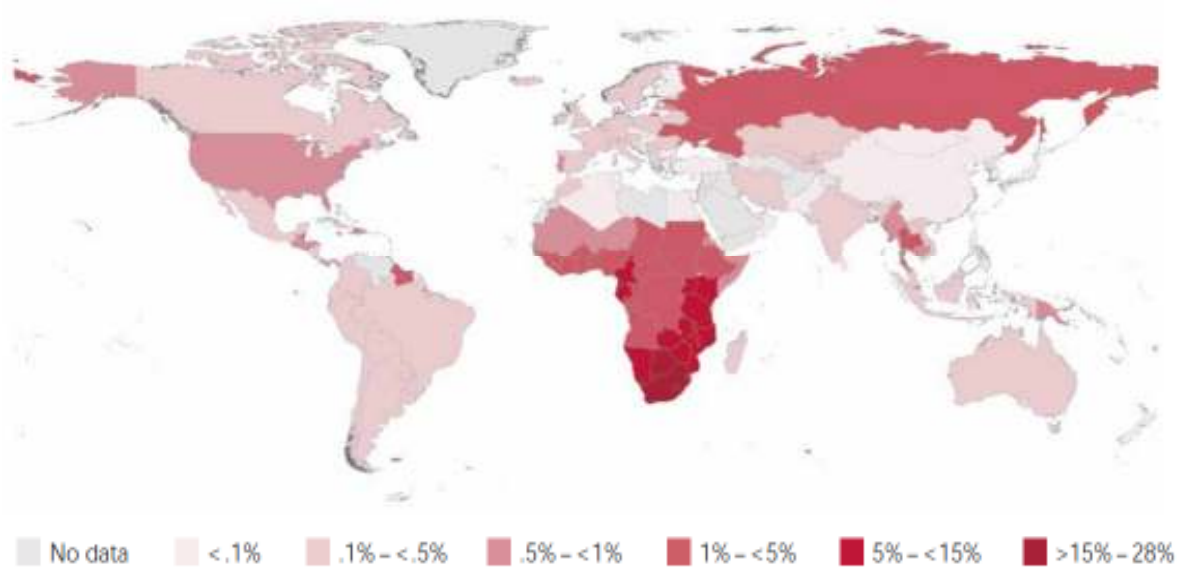


Figure 1. Estimated global prevalence of HIV, 2009 (percentage of infected persons to total population)

Source: Epidemic update, UNAIDS 2010 GLOBAL REPORT (*4*)

1.2 HIV virus

1.2.1 Discovery of human immunodeficiency virus

The causative agent of AIDS, lately termed as HIV-1, was isolated and described independently and almost simultaneously by two virological teams led by Prof. Luc Montagnier in Pasteur Institute (*5*) and Robert Gallo from the National Institute of Health in the USA (*6*) in 1983. L. Montagnier and his colleague Francois Barre-Sinoussi shared 2008 Nobel Prize in Physiology and Medicine for this discovery. A virus with approximately 50% homology to HIV-1 was isolated from patients with AIDS in Central Africa in 1985 and was termed as HIV-2 (*7*). It has been only rarely seen outside of Africa and its infectivity is lower compared to HIV-1; the clinical course can be slow, in some cases even nonpathogenic. HIV-1 is related to chimpanzee simian immunodeficiency virus (SIV) strain and HIV-2 mostly to SIV endemic in sooty mangabey monkey (*8-9*). However, retrospective studies reported that HIV-infected blood samples have been found as early as 1959 in Africa, appointing thus the outbreak of infection to this region.

1.2.2 The HIV genome and structure

The HIV virus is a member of the viral family Retroviridae, subgroup of Lentivirus, which is typically responsible for long-duration illnesses with a long incubation period. The HIV genome is composed of two copies of single-stranded, positive-sense RNA molecules, encoding nine different genes (*10*). The *gag*, *pol*, and *env* genes are characteristic for all retroviruses. The *gag* gene (from "group specific antigen") contains the sequence information for structural proteins matrix, capsid, nucleocapsid, C-terminal p6 protein and two small spacer peptides (p1 and p2). The *pol* gene (from "polymerase") is translated as a part of the fusion GagPol polyprotein after a -1 nucleotide translational frameshift event00 which occurs with a frequency of 5-10% (*11*). It carries the information for the viral enzymes reverse transcriptase, protease, RNase H and integrase. The *env* gene ("envelope") encodes surface glycoprotein gp160, a precursor of glycoproteins gp120 and gp41. The six remaining auxiliary genes, *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* (or *vpx* in the case of HIV-2), are regulatory genes for proteins involved in viral maturation, replication, and infectivity.

HIV structure is quite complex and rather different from other retroviruses. The mature HIV virion is a roughly spherical particle with diameter about 110 nm (*12*). It is coated by two layers of phospholipids originating from the membrane of a human cell. In this envelope, proteins from the host cell are embedded, as well as on average 72 copies of a HIV protein complex known as Env. This protein is composed of a stem consisting of three molecules of protein gp41 and a cap made of three molecules of highly glycosylated protein gp120. They form the spikes that protrude from the viral particle (*13-14*). Cone-shaped core (or capsid) is formed by the major core protein, p24, and encloses RNA genome, nucleocapsid proteins p6, p7, p9, and enzymes needed for the development of the virion such as reverse transcriptase, ribonuclease and integrase. The localization of protease is unclear. The layer between the viral core and the viral envelope is formed by the matrix protein p17.

1.2.3 *In vivo* evolution of HIV

HIV-1 displays a greater degree of diversity and size of the population within a host than any other virus studied. This variability is a direct result of a high mutation rate of the

virus, large replication capacity (it can generates as many as 10^{10} virions every day in a single patient), the influence of innate and adaptive immune pressures (*15-17*), and a high tolerance of variation while maintaining reproductive capacity. The underlying biochemical mechanism of a high mutation rate, a common property of lentiviruses such as HIV, is the low fidelity of viral reverse transcriptase, which lacks proof-reading mechanism that revises incorrect base-pairing. The error-rate is approximately 100,000 times higher than that during *in vivo* DNA transcription in eukaryotic cells and is estimated to be 3.4×10^{-5} per base pair per replication cycle (*18*). Considering the HIV-1 genome of about 10 kb, one third of newly produced virus particles harbor a mutation.

Based on the differences in the envelope (*env*) region – surface glycoproteins, three groups of HIV-1 have been identified: M, N, and O (*19*). The most prevalent group M is divided into eight clades (subtypes) A- H, among them the most widespread is subtype B (occurring mainly in North America and Europe), A and D (both found mainly in Africa), and C (Asia and Africa).

The great HIV genetic and antigenic variability presents the main challenge to maintaining the effectiveness of antiretroviral therapy and to the introduction of new preventive strategies such as vaccines or microbicides.

1.2.4 Current antiretroviral therapy and drug resistance

Immediately after the identification of HIV as the etiological agent for AIDS, many research teams both from academia and industry started to study possibilities of anti-HIV therapy. Only four years later, dideoxynucleoside reverse transcriptase inhibitor (RTI) azidothymidine (AZT) was approved by the U.S. Food and Drug Administration (FDA) for the use in patients with advanced HIV. Unfortunately, optimal clinical benefits were observed only in first 24 weeks of the treatment (*20-21*). Drug resistance developed readily and quickly. Moreover, the poor toxicity profile was a serious problem. It was realized that monotherapy was not sufficient to combat HIV infection effectively. Discovery of other RTIs and later protease inhibitors (PIs) and their introduction into clinical practice in 1995 enabled simultaneous blocking of two viral enzymes, reverse transcriptase and protease, using a mixture of antiretrovirals. This combination therapy known as a highly active antiretroviral therapy (HAART) has resulted in effective lowering of "viral load"

(concentration of HIV in plasma) and delayed progression of HIV, which translated into improved life expectancy of HIV positive patients. The fatal HIV infection was turned into a chronic, controllable though not yet curable disease. Currently, three decades after the first description of AIDS, there are twenty six antiretroviral drugs (ARV) and six fixed-dose combinations of drugs on the market used for the treatment of HIV infection, divided into five different classes according to their viral target. The current attitude is to treat early and thoroughly to keep ahead of the virus. Generally recommended management of HIV/AIDS typically includes the use of several drugs from different classes, at least two or three, taken in combination, in order to suppress viral replication and thus to slow the emergence of multiply resistant viruses.

Despite the success in the high-income countries, the situation in resource-limited settings is still dramatic because of high average therapy cost (of up to US$ 20 000 per person per year) and thus the treatment coverage. However, thanks to WHO (The World Health Organization), number of private charities as well as drug availability programs of various pharmaceutical companies and their effort to provide universal access to treatment for all those who need it using standardized and simplified ARV regimens, today, 6.65 million HIV infected people in low and middle-income countries have access to ARV treatment (*22*) (Fig. 2, 3).

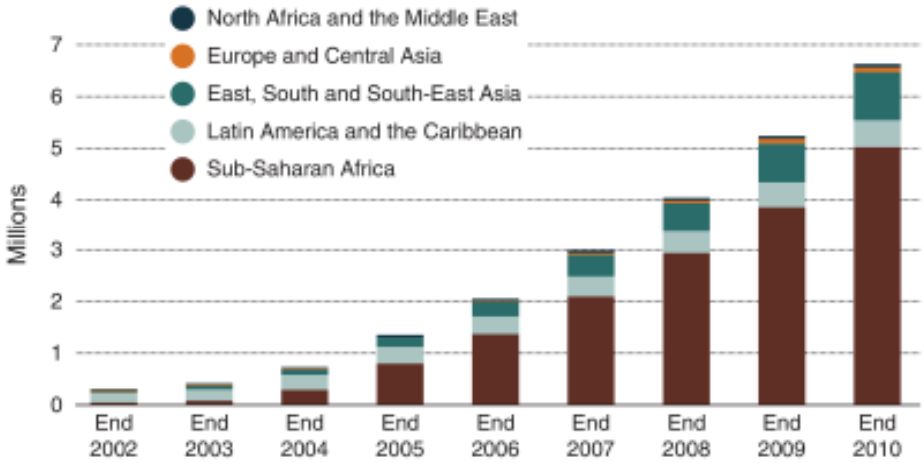


Figure 2. Number of people receiving antiretroviral therapy in low and middle-income countries, by region (2002–2010)

Source: Global HIV/AIDS Response. Epidemic update and health sector progress towards Universal Access. Progress Report 2011. WHO, UNAIDS, UNICEF, Geneva (*22-23*)

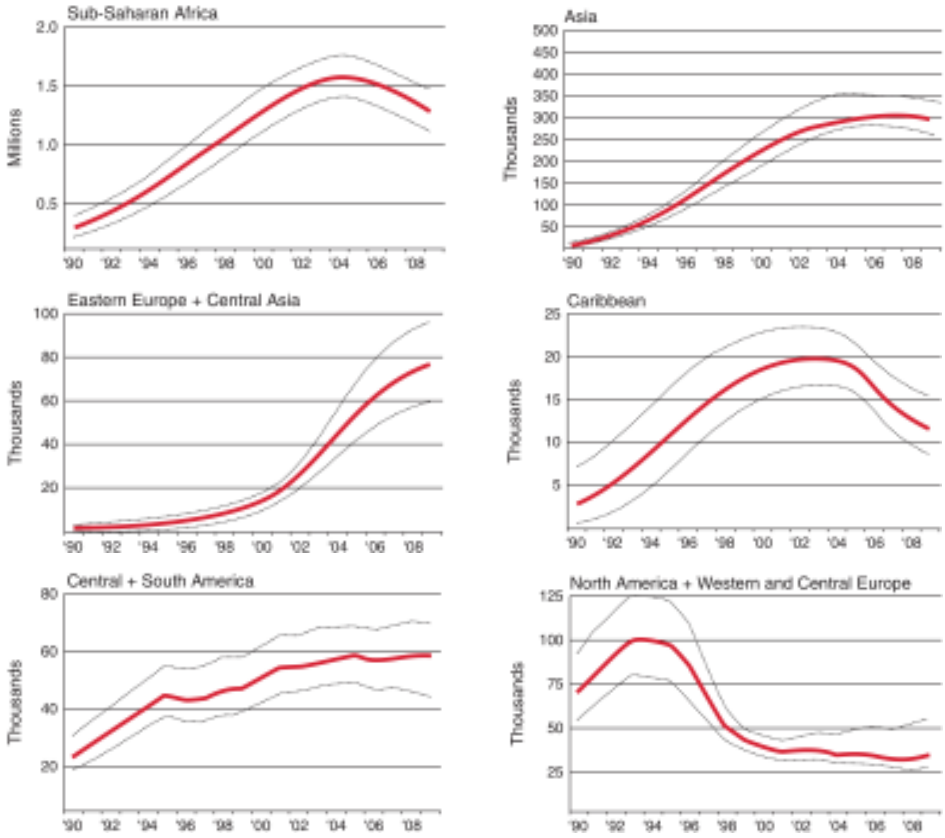


Figure 3. Course of annual AIDS-related death, by region, with the progressive introduction of antiretroviral therapy (see Figure 2). In North America and in Western and Central Europe, the number of people dying from AIDS-related cause began to decline soon after antiretroviral therapy had been introduced in 1996. The grey curves indicate the uncertainty range around the estimate.

Source: Global HIV/AIDS Response. Epidemic update and health sector progress towards Universal Access (*22-23*)

1.3 Five antiviral drug classes according to their viral target in HIV replication cycle and drug resistance

Currently used ARVs can be divided into five different classes according to the phase of the HIV life-cycle that the drugs block: inhibitors of entry/attachment, reverse transcriptase, integrase, protease, and maturation (Fig. 4).

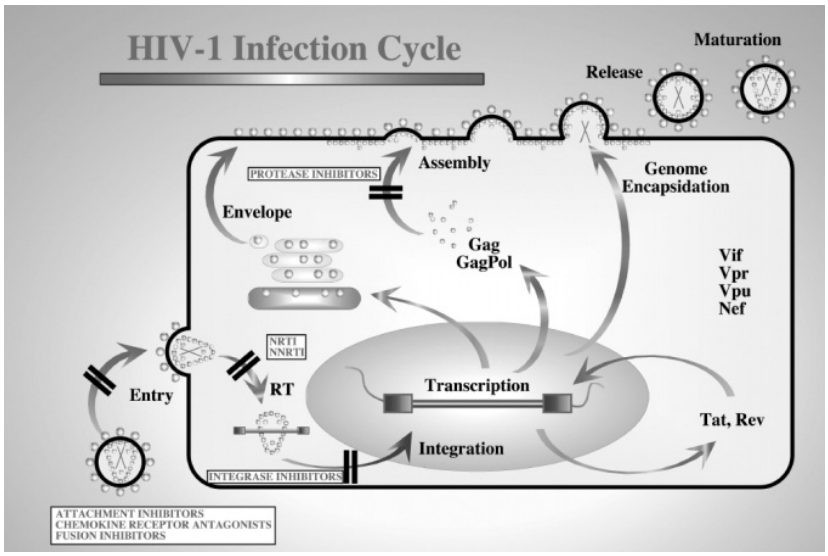


Figure 4. Therapeutic targets for antiretroviral drugs in the human immunodeficiency virus 1 (HIV-1) life cycle

Source: Fields: Virology, fifth edition (*24*)

However, a number of resistance associated mutations have been described for all currently approved antiretrovirals. They are divided into "major" vs "minor" or "primary" vs "secondary" mutations depending on their effect in antiviral therapy. Major mutations are usually selected early under the selection pressure of the drug and are much more inhibitor specific. In the second step, minor mutations, mostly considered accessory, are selected to restore impaired viral replication and/or enhance drug resistance. This pathway of resistance development is common for reverse transcriptase, integrase, and protease inhibitors. Furthermore, apart from amino acid substitution, deletions (*25-28*) and insertions in the HIV reverse transcriptase (*28-32*) or protease region (*33-34*) or mutations in the Gag precursor cleavage sites, NC/p1 and p1/p6 (*35-37*), have also been observed during antiretroviral therapy. In addition, the number of primary infections that involve transmission of drug resistant strains steadily increases. The selection of the optimum therapy has become a complex issue, namely in salvage regimens.

1.3.1 Entry/attachment inhibitors

HIV infects a variety of immune cells such as $CD4^+$T cells, macrophages, and microglial cells. The first step in the viral replication cycle is the entry into the cells, which primarily involves the high-affinity attachment of gp120 to cellular receptors CD4. This binding triggers a structural change allowing gp120 interact with the target chemokine coreceptors CCR5 or CXCR4. This causes gp41 to undergo a conformational change resulting in the insertion of a hydrophobic fusion peptide region into the cell membrane. Viruses using CCR5 alone, CXCR4 alone, or both coreceptors, are labeled R5-tropic, X4-tropic, and RX54 or dual-tropic viruses, respectively. The viral envelope then fuses with the cell membrane, entry pores are created and the viral capsid is released into the cell (*38-39*).

The fusion inhibitors (FIs) are a new class of antiretrovirals, which are used in combination with other inhibitors of viral enzymes to prevent the virus from spreading in the body and to reduce the amount of virus in the blood. They block several important target proteins involved in virus penetration into the cell. Other group of drugs is represented by cell surface receptor antagonists. They interact with receptors and lock them in a conformation that prohibits their function.

Enfuvirtide (formerly known as T-20 and DP-178), the first approved fusion inhibitor, is a peptide drug that mimics a fragment of gp41, blocking the conformational switch needed for the fusion event (*40*). Anti-CCR5 antibody **PRO 140** potently inhibits CCR5-mediated HIV entry with no dose-limiting toxicity being observed (*41*). It acts through competitive binding mode and presents a high genetic barrier to viral resistance. Now undergoing Phase II clinical trial, the FDA designated this product as a fast-track candidate based on its properties. The post-attachment anti-CD4 monoclonal antibody **ibalizumab** (TNX-355) (Fig. 5) has shown activity, safety, and efficacy in Phase I and II trials for treatment of HIV-1 infection and now is undergoing a Phase I clinical trial in HIV-1 uninfected individuals for prevention (*42-43*).

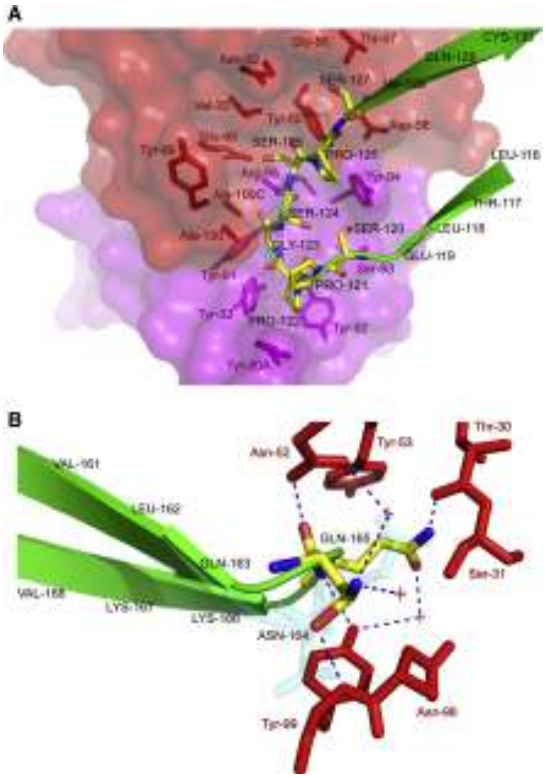
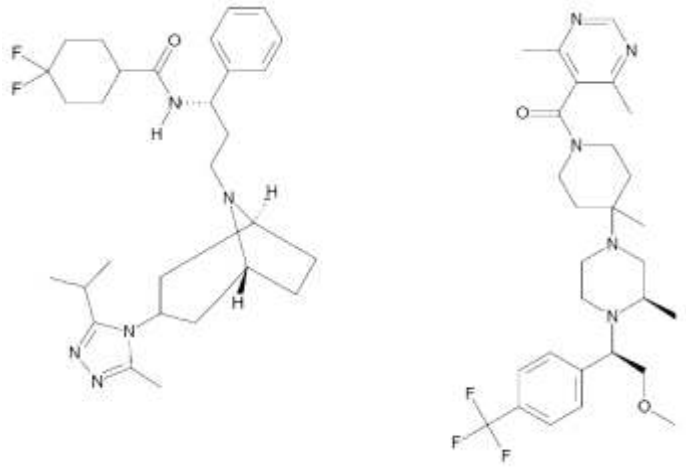


Figure 5. Closeup of major contacts between CD4 receptor and ibalizumab
(A) Residues making direct contacts between the heavy and light chains of ibalizumab and CD4. The heavy and light chains of ibalizumab are shown by surface representation in red and magenta, respectively, and the residues that make contacts with CD4 are also shown by stick model in the same color scheme. The BC-loop of CD4 is in green in ribbon diagram and the residues interacting with ibalizumab are shown by stick model with carbon atoms in yellow, nitrogen in blue and oxygen in red.
(B) Contacting residues between CD4 and the ibalizumab heavy chain. The residues that make direct contacts with CD4 in the heavy chain are shown by stick model in red. The FG loop of CD4 is shown in ribbon diagram in green with residues 164 and 165 in stick model in the same color scheme as in (A). The conformation of the two residues in the unbound CD4 is also shown in cyan; a flip of the loop upon binding to ibalizumab is evident.

Source: Michael M. Freeman et al.: Crystal Structure of HIV-1 Primary Receptor CD4 in Complex with a Potent Antiviral Antibody (*44*).

FDA-approved **maraviroc** (Selzentry) (*45-46*) and **vicriviroc (**previously named SCH 417690 and SCH-D) (*47*) are small molecule allosteric CCR5 antagonists preventing its use during HIV-1-cell fusion (Fig. 6). **Cenicriviroc** (TBR-652) is an experimental small-molecule entry inhibitor blocking CCR5 and CCR2 receptors (*48*). CCR2 receptors have been linked to inflammation in the body, therefore cenicriviroc may also reduce the risk of certain non-AIDS health conditions that are becoming increasingly common among HIV patients, including metabolic complications, neurologic problems, cardiovascular disease, and some cancers. Entering to Phase IIb clinical trial was announced in June 2011.



a) maraviroc (MVC) b) vicriviroc (VVC)

Figure 6. Chemical structures of (a) maraviroc (MVC) and (b) vicriviroc (VVC)

Source: Kevin C. Brown et al.: Drug Interactions with New and Investigational Antiretrovirals (*49*)

The observation that native chemokines can inhibit HIV-1 replication started the development of chemokine receptor antagonists (*50*). The first antagonists-based entry inhibitor was modulated endogenous chemokine RANTES (CCL5), a natural HIV-suppressive factor secreted by activated $CD8^+$ T cells and other immune cells. Modifications of the N-terminus resulted in compounds with increased affinity for CCR5, **met-RANTES** and **AOP-RANTES** (*51-52*). Another advantage and motivation for the development of chemokine-like inhibitors is their ability to induce ligand-mediated internalization of their receptor through a clathrin-dependent mechanism and thus inhibition of HIV spread via receptor downregulation (*53*). Further design of the RANTES N-terminus resulted in a third generation compound, **PSC-RANTES**, a promising candidate in the development of a vaginal microbicide to prevent sexual transmission of HIV-1 (*54*).

Resistance to this new class of antiretrovirals has not yet been well described. Several possible mechanisms have been considered: change in tropism (utilization of CXCR4 instead of CCR5 for entry), increased virus entry efficiency into host cells, and utilization of inhibitor-bound receptor for entry (*55*).

The fact that some strains of HIV can use only the CCR5 coreceptors could be utilized for total eradication of all HIV-infected cells from any cellular or anatomical compartment in an infected individual, as was observed on the case of Timothy Brown, also known as

"the Berlin patient". He was diagnosed with HIV in 1995 and for the next 11 years he was treated by the ART. Then he developed acute myelogenous leukemia and received two stem cell transplants from a donor harboring a homozygous *ccr5*Δ32 (CCR5 mutation that makes cells immune to the R5-tropic HIV strain). Now he is off therapy for 4 years with no evidence of leukemia and, though some of his cells might still carry latent virus, his viral load has remained undetectable in tissues including the peripheral blood, gut, bone marrow, and brain (*56*). This success has provided hope that a cure might be achievable. However, the stem cell transplantation is so difficult, risky, and expensive that it is unlikely that it will be used as a general treatment option. Nevertheless, it has prompted attempts to simulate the genetic knockout of CCR5 that was achieved in this case (*57*).

1.3.2 Reverse transcriptase inhibitors

Reverse-transcriptase inhibitors (RTIs) inhibit HIV reverse transcriptase (RT), RNA-dependent DNA polymerase. HIV-1 RT is an asymmetric heterodimer with subunits of 66 kDa (p66) and 51 kDa (p51). The p66 subunit resembles the human right hand, so its sub domains are labeled thumb, palm, and fingers (*58*). In the palm domain there is the polymerase active site, the RNase H sub domain is located near the finger domain. This enzyme is used by retroviruses for reverse-transcription of their RNA genomes into DNA which is then incorporated into the host genome. If it is effectively inhibited, the viral genome is not able to integrate into the host cell and to replicate. There are three different families of RTIs: a) nucleoside analog reverse-transcriptase inhibitors (NARTIs or NRTIs), b) nucleotide analog reverse-transcriptase inhibitors (NtARTIs or NtRTIs), and c) non-nucleoside reverse-transcriptase inhibitors (NNRTIs). NRTIs and NtRTIs are analogues of the naturally occurring deoxynucleotides with the same mode of action – they compete with them for incorporation into the nascent viral DNA chain. Since they lack 3'- hydroxyl group required for the phosphodiester bond formation, they arrest cDNA synthesis after being integrated and they act as chain terminators (*59-60*) (Fig. 7). In contrast, non-nucleoside compounds, which have been found by routine screening, inhibit reverse transcriptase by binding at a different site on the enzyme. They are not incorporated into the growing viral DNA but instead they bind directly to reverse transcriptase and inhibit the movement of its protein domains (*61-62*). Thus they stop the ability of the reverse transcriptase to add new nucleotides to DNA chain.

a) Nucleoside analogs (NRTIs)

They must be activated by cellular enzymes and converted into their active NRTI triphosphate form; for recent review see (*60, 63*). **Zidovudine** (AZT, ZDV), developed by scientists at the Burroughs-Wellcome Company (now GlaxoSmithKline), was the first antiretroviral drug approved by the FDA for the treatment of HIV in 1987. Pro-drug of **didanosine** (ddI), 2'3'-dideoxyadenosine (ddA) (first synthesized by J.M. Robins and R.K. Robins in 1964), was identified as a reverse transcriptase inhibitor by researchers from the National Cancer Institute (NCI). It was then licensed to Bristol-Myers Squibb because the NCI may not market or sell drugs. Its FDA approval in 1991 helped bring down the price of AZT, the first anti-HIV drug. **Zalcitabine** (ddC) has similar history. It was licensed to Hoffmann-La Roche, after developed in the NCI. It is now removed from the market in some countries due to serious adverse events and inconvenient administration three-times daily. **Stavudine** (d4T), **lamivudine** (3TC), **abacavir** (ABC), and **emtricitabine** (FTC) later followed. Experimental drug **apricitabine** (**AVX754**, **SPD754**, **ATC**) now being developed by Avexa, an Australian pharmaceutical company, is undergoing Phase-III evaluation.

There are two different mechanisms conferring multi-drug resistance to NRTIs. Mutations Q151M, M184V and other alter discrimination between nucleoside RT inhibitors and natural substrates, whereas M41L, D67N, K70R, T215Y/F, and K219E/Q mutations increase the RT's phosphorolytic activity, which in the presence of a pyrophosphate donor (usually ATP) enhance the removal of chain-terminating inhibitors from the 3′ end of the primer. This model was firstly proposed for zidovudin resistance (*28, 64*).

b) Nucleotide analog (NtRTI)

Tenofovir (TFV or PMPA**)** was discovered by Antonín Holý at the Institute of Organic Chemistry and Biochemistry (IOCB) in Prague in collaboration with Eric De Clercq from Riga Institute in Leuven, Belgium (*65-66*). The current prodrug, marketed by Gilead Sciences, is tenofovir disoproxil fumarate (TDF, Viread) that is metabolized intracellularly to its active form TFV-diphosphate. It is available alone or in a fixed-dose combination with emtricitabine under the brand name **Truvada** or in **Atripla** - combination with

emtricitabine and efavirenz. Recently, a fixed-dose drug combining tenofovir, emtricitabine, and rilpivirine has been licensed under the brand name **Coplera**. These products provide once-a-day dosing. Furthermore, combining the two or three agents into one tablet reduces the pill burden and increases adherence to antiretroviral therapy. Truvada was approved by the FDA for prevention on 16.7. 2012, the day A. Holý died.

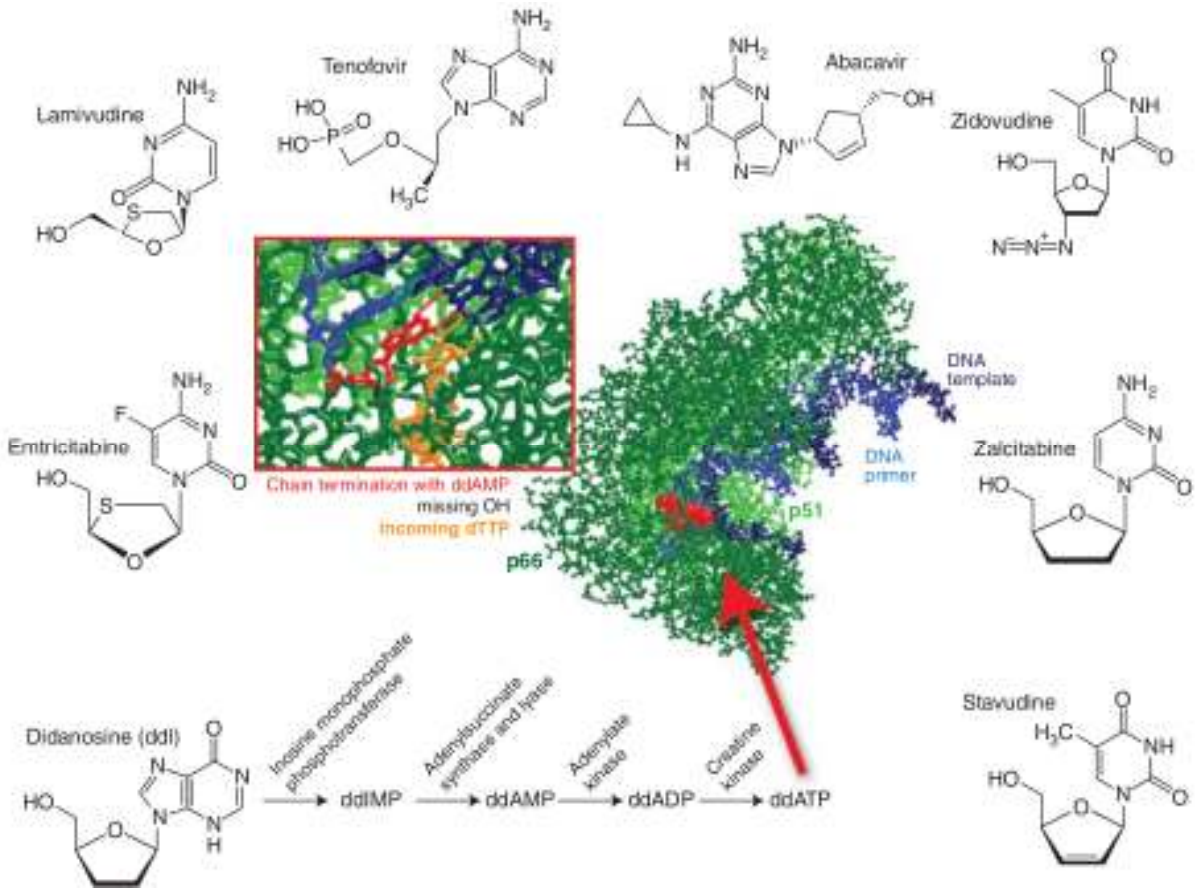


Figure 7. Nucleos(t)ide reverse transcriptase inhibitors and X-ray crystal structure of HIV-1 RT in complex with DNA primer/template chain terminated with ddAMP and with an incoming dTTP

Source: Eric J. Arts et al.: HIV-1 Antiretroviral Drug Therapy (*60*)

Scientists from Gilead Sciences reported a next-generation oral prodrug of TFV, tenofovir alafenamide (TAF), on HIV DART Conference 2012 (*67*). TAF demonstrates an improved antiviral activity against both wild-type HIV isolates from all types/groups/subtypes and HIV-1 NRTI-resistant isolates at lower doses than TDF. This improvement is attributed to a higher plasma stability and unique metabolic activation pathway, where lysosomal serine protease cathepsin A plays a critical role.

Mutation K65R, conferring broad cross-resistance to NRTIs was highly associated with the use of tenofovir and to a lesser extent with abacavir and didanosine, mainly with the inclusion of an NNRTI to the therapy. However, a decline in K65R selection rate of tenofovir has been observed recently due to changed treatment recommendations and the availability of tenofovir in co-formulation with emtricitabine (*68*).

c) Non-nucleoside reverse-transcriptase inhibitors (NNRTIs)

They are highly specific allosteric inhibitors of HIV-1 transcriptase with a very low cytotoxicity. For this class of inhibitors a high chemical diversity is typical, with more than 50 families of molecules (*69-71*) (Fig. 8, 9). Majority of them were identified by screening campaigns using cell-based assays. Despite their heterogeneity, they all bind at the same site in the RT, so called palm domain, at a distance of approximately 10 Å from the catalytic site. This binding results in conformation change that is responsible for their inhibitory effect. The first-generation NNRTIs are generally safe and well tolerated, however typically with a low genetic barrier to the development of resistance. They have to be administered with at least two other fully active non-NNRTI antiretroviral drugs. The first approved NNRTI was **nevirapine** (NVP), discovered at Boehringer Ingelheim Pharmaceuticals, Inc., followed by **delavirdine** (DLV), which is barely used nowadays, and **efavirenz** (EFV).

The second-generation inhibitors of this class were designed to be active against HIV with mutations that confer resistance to the two most commonly prescribed first-generation NNRTIs – efavirenz and nevirapine. **Etravirine** (ETR, formerly known as TMC125) and **rilpivirine** (RPV, TMC278) indeed show high resistance genetic barrier, the latter allows also convenient dosing schedule. These properties were utilized for co-formulation with other antiretroviral drugs and a fixed-dose drug combining rilpivirine, emtricitabine and tenofovir was approved by the FDA under the brand name Coplera in 2011. **RDEA806** (*72*), **IDX899** (*73*), and **lersivirine** (UK-453061) (*74*) are other next generation NNRTIs being studied in Phase II clinical trials.

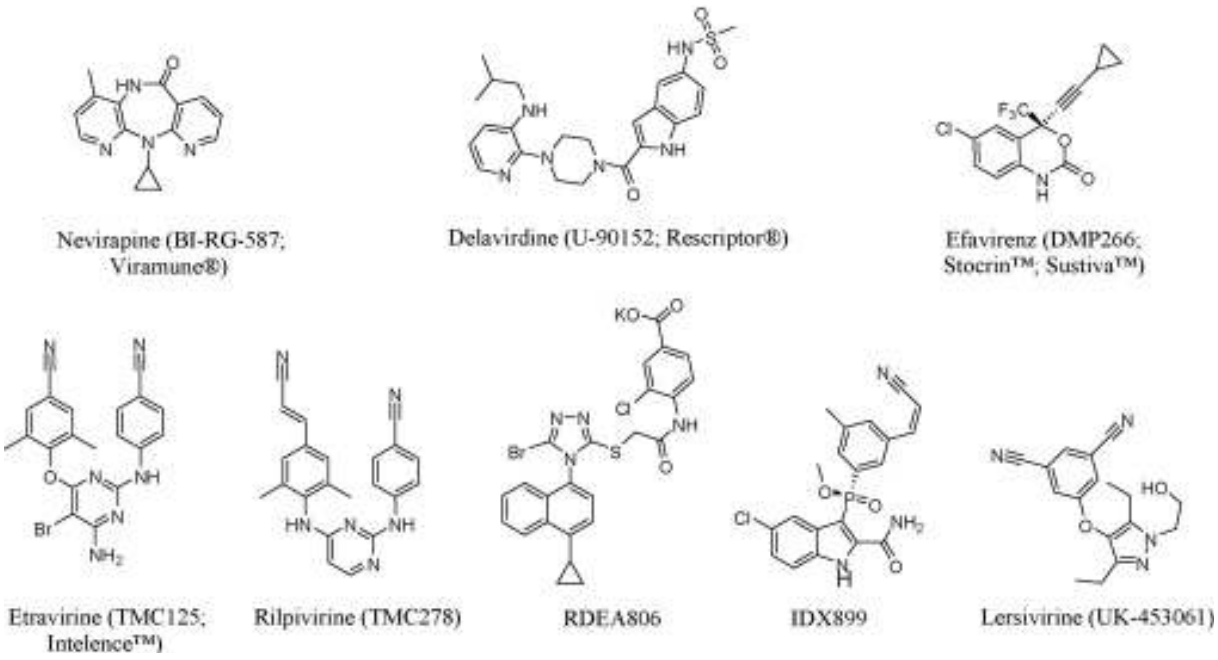


Figure 8. Chemical structures of the first generation NNRTIs (nevirapine, delavirdine, and efavirenz) and next generation NNRTIs (etravirine, rilpivirine, RDEA806, IDX899, and lersivirine)

Source: Marie-Pierre de Béthune: Non-nucleoside reverse transcriptase inhibitors NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: A review of the last 20 years (1989–2009) (*71*)

First described mutations related to the resistance towards NNRTIs were all located in the NNRTI binding pocket of the p66 subunit (*75*). The prevalent mutations connected with NNRTI-treatment failure are K103N and Y181C (*76-77*). Other substitutions, Y318F and E138, outside the originally identified domains have also been observed (*78-80*). So far, over 40 amino acid substitutions have been reported to be associated with NNRTI resistance, *in vitro* and *in vivo (77, 81)*. Recently, other substitutions in the connection domain of the p66 subunit between the thumb and the RNase H domains, outside the NNRTI binding pocket, have been reported in NRTI-treated patients (*82-84*). The N348I mutation causes resistance to both AZT and nevirapine (*85*). Interestingly, the V106A and P236L mutations have impact rather on the slowing RNase H cleavage activities than on DNA polymerization (*86-87*), whereas the Y181C mutation is reported to accelerate cleavage (*87*).

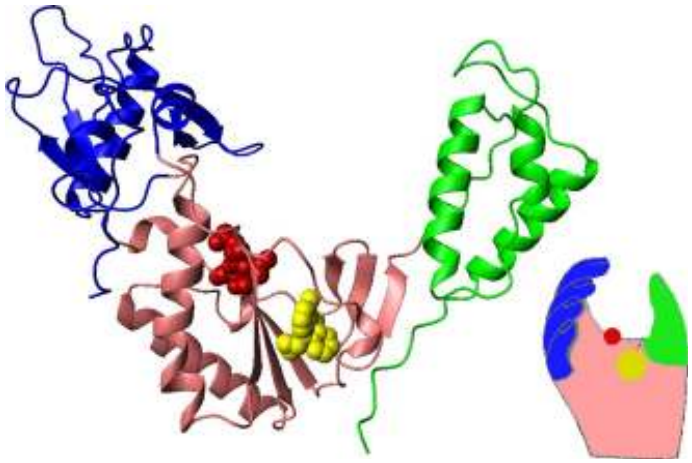


Figure 9. Ribbon representation of the active domain of RT. It illustrates its hand-like structure, showing fingers (blue), palm (pink) and thumb (green). The active site (red atoms), where DNA is elongated, is located in the palm region. Also shown is an RT-inhibitor drug (yellow) in the pocket where it binds.

Source: Marie-Pierre de Béthune: Non-nucleoside reverse transcriptase inhibitors NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: A review of the last 20 years (1989–2009) (*71*)

1.3.3 Integrase inhibitors

The integration of the reverse transcribed viral genome into a chromosome of the host cell is essential for retroviral replication. It is a two-step reaction, catalyzed by HIV-1 integrase (IN) (*88-89*). The first step is known as 3'-processing of proviral DNA, which includes the endonucleolytic cleavage to expose the invariant CA dinucleotides at both 3'-ends of the viral DNA. This reaction takes place in the cytoplasmic pre-integration complex. Following translocation to the nucleus, human chromatin-associated protein LEDGF/p75 promotes viral integration by tethering the preintegration complex to the chromatin. The processed 3' ends of the viral DNA are then covalently ligated to the host chromosomal DNA in the second step referred to as strand transfer. All currently used integrase inhibitors and most of those in development block this step of integration (integrase strand transfer inhibitors – INSTIs) (*90-91*). When the whole process is finished, there is no return for the cell, it becomes a permanent carrier of the provirus.

IN consists of three domains – an N-terminal domain and a less conserved C-terminal domain connected by a flexible linker, and a central catalytic core carrying the catalytic D, D-35, E motif. The N-terminal domain promotes IN multimerization through zinc coordination (HHCC motif) and enhances integration of the two viral cDNA ends together

into a host cell genome. The C-terminal domain is responsible for non-specific DNA binding.

IN has become an attractive target, because no human enzyme is known to possess similar activity (*92*). Therefore IN inhibitors have been expected to improve the outcome of therapy without adverse effects. The first of INSTIs, **raltegravir** (RAL, MK-0518) (*93-94*) was approved by FDA in 2007 (Fig. 10). **Elvitegravir** (EVG) (*95*) should be approved soon and second-generation experimental inhibitor **dolutegravir** (DTG, S/GSK1349572 or "572") (*96*) has so far shown promising outcomes in advanced clinical studies. As a part of pre-exposure prophylaxis with substantially prolonged half-time, compound **MK-2048** is now developed**.** These compounds have been reported to inhibit effectively viral strains that are resistant to other drug classes (*97*). Moreover, they are useful against both B HIV and non-B HIV subtypes (*98-99*).

Mutations associated with resistance to the first INSTIs involve primary mutations at positions Y143R/C, Q148K/R/H, and N155H and a number of secondary mutations. Second-generation inhibitors including dolutegravir and MK-2048 demonstrate a more robust resistance profile (*100*). Although integrase inhibitors were initially developed to target one of key HIV enzymes, they could be applied also to other retroviruses.

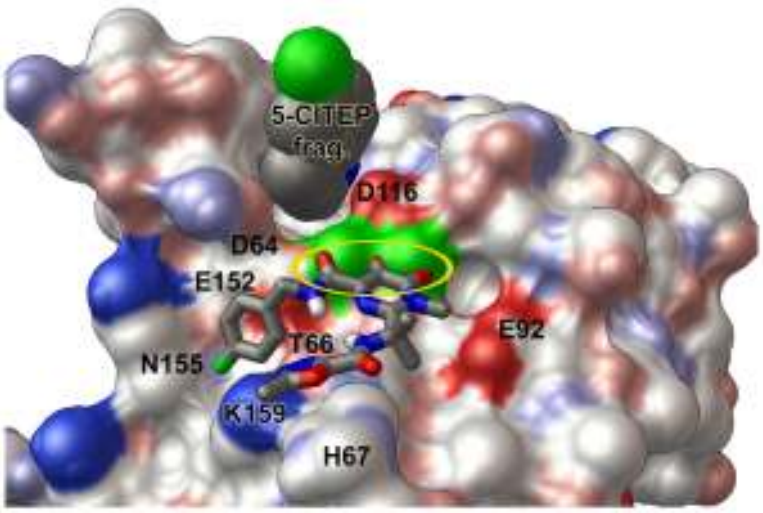


Figure 10. Predicted binding mode of raltegravir with the wild type HIV integrase catalytic core domain. A solvent-accessible surface is shown with labels for key residues. In this binding mode, three coplanar oxygen atoms of raltegravir exhibit four optimal Mg-O distance values (highlighted by the golden oval), and the non-coordinating end of raltegravir has two oxygen atoms forming favorable electrostatic interactions with the side-chain of the critical H67 residue. The fluorobenzene and oxadiazole moieties in the two ends of raltegravir make other important interactions in this binding mode with N155, T66, and K159.

Source: Alex L. Perryman et al.: A Dynamic Model of HIV Integrase Inhibition and Drug Resistance (*101*)

Another promising approach is inhibition of integrase interaction with its cellular co-factor LEDGF/p75 (lens epithelial derived growth factor). Knockdown or knockout of LEDGF/p75 and overexpression of its IN-binding domain without the chromatin association domain caused delayed or defective replication of HIV virus (*102*). Moreover, this study showed that the recently described IN-LEDGF/p75 inhibitors (**LEDGINs**), that compete with LEDGF/p75 for the binding to integrase, remained active even in the absence of LEDGF/p75 and blocked the interaction between IN and HRP-2, LEDGF/p75-related protein.

1.3.4 Protease inhibitors

1.3.4.1 Biological function of HIV protease and its structural features

Protease (PR) is a retroviral aspartic protease which plays an indispensable role in HIV maturation. It cleaves newly synthesized viral polyprotein precursors Gag and Gag-Pol at nine distinct sites in a defined order into structural proteins and functional viral enzymes. If this step is effectively blocked, HIV virions remain noninfectious (*103-104*). PR is coded by Gag-Pol fusion product and it is released from it by an autoactivation via a mechanism still not fully understood (*105*). However, it is believed that it is an autocatalytic process - the initial cleavage is performed by the immature PR dimer in an intramolecular transient event when two still embedded protease domains dimerize (*106-107*). PR was the first HIV-1 protein to be structurally characterized using X-ray crystallography (*108-110*). It functions as a symmetric homodimer, with each subunit composed of 99 amino acids. In the middle of the enzyme there is a cavity that forms the substrate-binding cleft. Each monomer contributes one of the two triplets Asp^{25}-Thr^{26}-Gly^{27} joined by conserved network of hydrogen bonds ("fireman's grip"). The PR structure is predominantly β-sheet with the two catalytic aspartic acids Asp25 (25´) located in the bottom of the active site. These residues are expected to be in opposite states of protonation to be active (*111-112*) and to induce general acid/base protein hydrolysis.

The top of the cleft is "covered" by two β-hairpin loops known as flaps. Consistent structural differences are observed between the liganded and unbound enzyme. Upon a substrate binding the flaps are pulled toward the bottom of the active site ("closed" form), whereas the free enzyme adopts a "semiopen" conformation (*113-114*). This movement of up to 7 Å is essential for substrate binding and release.

The dimer interface is composed of several features: the β-hairpins of the two flaps, the catalytic triad, helices (residues 84-93) interacting with residues 4-10, and N- and C-terminal β –strands creating a four-stranded antiparallel β-sheet (Fig. 11).

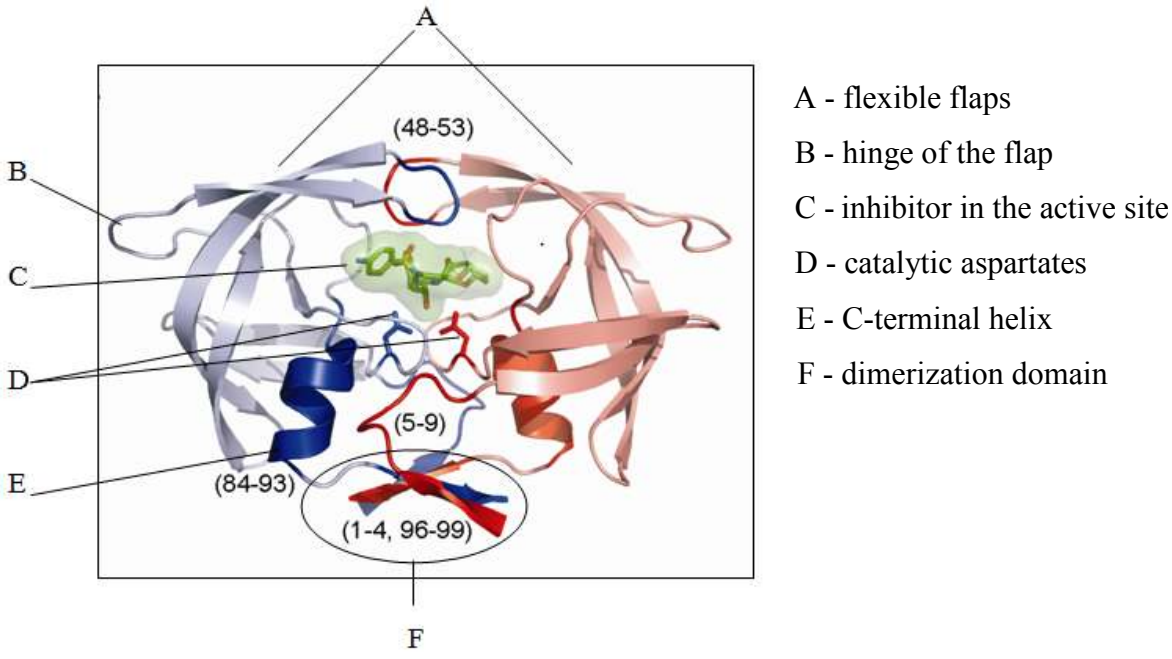


Figure 11. The structure of the HIV protease with inhibitor darunavir bound in the active site. The PR dimer is in ribbon representation and darunavir is represented by stick model. Monomers are colored blue and red, respectively, and regions involved in dimeric interface are indicated by residue numbers. The figure was prepared by P. Řezáčová from IOCB AS CR using a patient derived protease (PDB 3GGU (*115*)) and software PyMol (*116*).

1.3.4.2 Ion specific effects on the activity of HIV protease

Number of biological processes such as protein folding/misfolding, solubility, thermodynamic and kinetic stability or enzyme catalysis are significantly affected by salts that are integral components of biological systems (*117-121*). The first attempt to order ions according to their effects on proteins was done by Franz Hofmeister at the German Medical School in Prague more than 100 years ago (*122*). According to their ability to precipitate egg-whites proteins and to affect surface tension he ranked them in a series, noting that anions have a larger effect then cations. This sequence soon became a fundamental basis for the study of biochemical systems often involving saline solutions. About twenty years later, Traube found twenty different properties, including solubility of

gases and colloids, irritability of nerve cells, or catalysis of chemical reactions to follow the same Hofmeister series, suggesting the same principle for these various phenomena (*123*). Explanation of the Hofmeister effects broadly excepted for nearly 100 years was based on the idea that ions have long-range influence on water's hydrogen-bond networks, acting as "structure breakers" or "weakers". However, infrared and Raman spectroscopy studies together with thermodynamic studies performed in the first decade of 21st century showed that the ions had no effect on water structure beyond the first hydration shell of surrounding water molecules (*124-125*). The attention of researchers turned to surfaces and macromolecular interfaces. Now it is known that ion-specific protein precipitation or denaturation is caused by ions repelling from or adsorbing to hydrophobic interfaces. Interestingly, the ion ordering strongly depends not only on the surface charge but also on the level of polarity. Original Hofmeister series (HS) was studied using the precipitation of ovalbumin, at neutral pH a negatively charged protein. For cationic proteins, the ordering of ions was found to be reversed (*126-127*). When going from hydrophobic surfaces to very polar colloids, the ordering is even more complicated. Cationic polar colloids obey the direct HS, anionic the reverse HS, and for intermediate situations, common in biological systems, partial reversal is observed (*128*). In addition, because binding of ions involves water replacement, osmotic effects should be considered as well (*117, 129*).

The dependence of the kinetics of HIV protease enzymatic activity on ionic strenght/salt concentration has been studied by several groups. Analyses have shown that the higher salt concentration influences in particular the substrate binding (K_M), although an effect on the catalytic rate (k_{cat}) is also described. The effect on K_M is usually assigned to the "salting out" of the substrate into the enzyme binding cavity and Wondrak et al. showed that it correlates with the Hofmeister series (*130*). On the contrary, Szeltner and Polgar suggest that the increasing salt concentration facilitates the peptide hydrolysis via an entropic effect by stabilizing the protein structure (*131*). Different effects of salts on enzyme kinetics have been reported in the literature. While the activity of Human T-cell Leukemia Virus 1 Protease (HTLV-1 PR), an enzyme homologous to HIV-1 PR, is not influenced by NaCl concentration in a range from 0 to 1.5 M, thermolysin is significantly activated in the presence of high concentration of NaCl (*132*). Some enzymes even require the presence of a specific cation for full enzymatic activity, as thrombin and other serine proteases involved in the blood coagulation cascade (*133-134*).

Recently, the higher affinity of Na^+ over K^+ to protein surfaces has been quantified by means of molecular dynamic simulations and conductivity measurements (*135*). Further kinetic studies showed that the enzymatic activity of the HIV protease is enhanced by increasing salt concentration (*136-137*). The analysis of this phenomenon is a part of this thesis.

1.3.4.3 The use of HIV protease inhibitors in clinical practice

Due to the protease key role in HIV replication, it has become a prime target for drug therapy. There are other examples of inhibiting aspartic proteases with therapeutic success, e.g. renin (*138*) or memapsin, now in advanced clinical trials for the treatment of Alzheimer´s disease (*139*). Since the first reports on HIV PR it took only ten years to the introduction of its first inhibitor on the market. Most protease inhibitors (PIs) were designed to mimic the tetrahedral intermediate of the hydrolysis of PR natural peptide substrates. The peptide bond of the substrate was replaced by the non-hydrolyzable reduced peptide group or hydroxyethylene isostere (*140-141*). All PIs approved so far contain hydroxyethylene or hydroxyethylamine group as a core peptidomimetic motif with the exception of non-peptide drug tipranavir, with coumarin in the center of the molecule (*142*).

Despite the critical role in viral maturation and infectivity, the HIV protease has displayed great variability. More mutations are selected by PIs than by any other class of antiretrovirals. According to the Stanford HIV Drug Resistance Database, 46 amino acid substitutions from the total number of 99 residues have been identified in protease isolates.

1.3.4.4 The first generation HIV protease inhibitors

The first HIV protease inhibitor and the sixth antiretroviral drug approved for treatment in 1995 was **saquinavir** (SQV, RO 31-8959, marketed by Hoffman-La Roche). Its design was based on a rather uncommon cleavage site in Gag-Pol in the sequence Asn-Phe-↓-Pro (*143-144*). Primary mutations G48V or L90M emerged after one year of monotherapy in 50% of patients (*145*).

Other two protease inhibitors, **ritonavir** (RTV, ABT-538, designed by Abbot Laboratories) (*146*) and, after a complicated development, **indinavir** (IDV, MK-639, L-

735,524, manufactured by Merck & Co) (*147*), were approved four months later. Ritonavir design originally started on C-2 symmetric molecules because of the C-2 symmetry of HIV protease. Surprisingly, the structural analysis of the enzyme/inhibitor complexes revealed asymmetric manner of binding into the protease even for symmetrical compounds. This finding eventually led to the asymmetric inhibitor structure. Due to frequent occurrence of severe side effects, ritonavir is currently used almost solely as a pharmacokinetic booster owing to its potent inhibiting of cytochrome P-450 3A4, the microsomal enzyme responsible for the metabolism of numerous drugs including several PIs. The resistancy profile of ritonavir and indinavir is very similar: it involves major mutations M46I, V82/A/F/T, and I84V and many minor mutations (*148-151*).

The first compound which could be used for the therapy of children and also the first non-peptidic inhibitor was **nelfinavir** (NFV, AG-1343, Agouron Pharmaceuticals) (*152*). Two possible pathways lead to the NFV resistance. The first includes D30N mutation, often accompanied by N88D, the second involves the selection for L90M mutation (*153-155*).

Higher oral bioavailability and easier synthesis was achieved in the case of **amprenavir** (APV, 141W94 or VX-478, Vertex Pharmaceuticals and GlaxoSmithKline). It contains tetrahydrofurancarbamate as the P2 end group and sulfonamide derivative at the P1′-P2′ position (*156*). These structural features were later successfully used for development of other inhibitors. The production of amprenavir was discontinued by the manufacturer in 2004, its prodrug version **fosamprenavir** (FPV, fAPV, GW 433908, VX-175, Vertex Laboratories and GlaxoSmithKline) (*157*), is still available. Mutations that confer resistance to amprenavir and fosamprenavir are identical and typically include I50V, I54L/V/M and several minor mutations (*158*).

The inclusion of PIs in antiviral therapy resulted in decrease of the HIV viral load and recovery of the immune system. Unfortunately, the effectiveness of treatment by this group of drugs (Fig. 12) was compromised by rather fast emergence of drug resistance, insufficient bioavailability, and poor drug tolerance (*139, 159*).

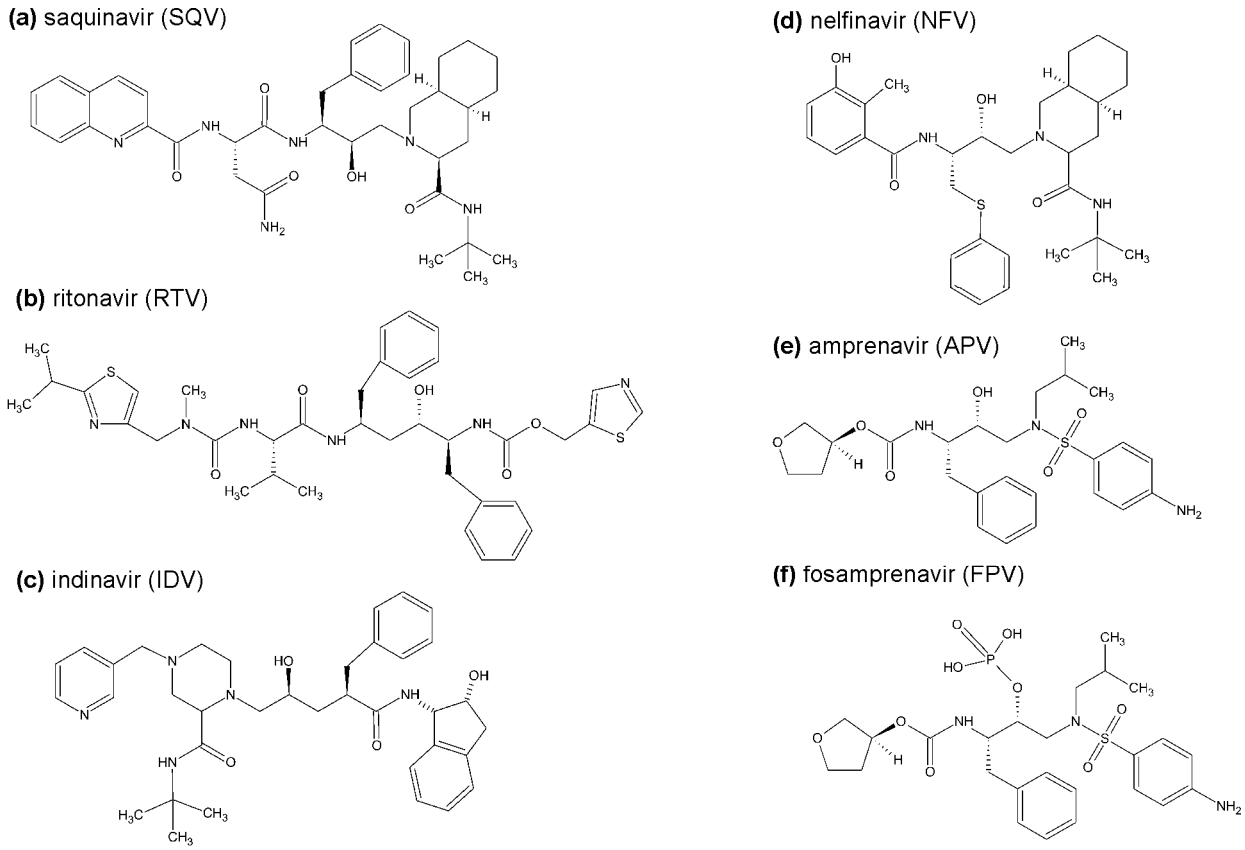


Figure 12. Chemical structures of the first generation HIV protease inhibitors

Source: Pokorná, J. et al.: Current and Novel Inhibitors of HIV Protease (*160*)

1.3.4.5 The second generation HIV protease inhibitors

Further progress was achieved by the development of second-generation inhibitors (Fig. 13). They have been designed to ensure greater resilience to resistance, high inhibition potency, and low incidence of side effects.

The molecular design of **lopinavir** (LPV, ABT-378, Abbott) was based on the structure of ritonavir but was aimed to inhibit resistant PR mutants that contain the common mutation V82A (*161*). Fixed-dose combination drug **Kaletra** (lopinavir/ritonavir) is one of the first choice PIs for an initial antiretroviral regimen. The mutations associated with the development of lopinavir resistance occur at nine to eleven positions ("lopinavir mutation score") (*162*). Single mutation I47A, contributing to the reduced sensitivity of HIV-1 and HIV-2 protease to the LPV, namely in combination with V32I and/or I54V, was described

five years after LPV had entered clinical practice. Substitutions in the p1/p6 and NC/p1 cleavage sites of Gag substrate were also identified (*163*).

The first PI allowing convenient once-daily dosing, with good tolerability and safety profile, was **atazanavir** (ATV, CGP 73547, BMS-232632), based on azadipeptide analogue (*164*). It was originally developed by Ciba-Geigy and marketed by Bristol-Myers Squibb since 2003. Typical primary resistance-conferring mutations selected under the pressure of ATV are unusual substitution I50L, I84V, and N88S. Interestingly, I50L mutation accounts for increased susceptibility to other protease inhibitors (*165-166*). To reduce peptidic backbone character, in later PIs the peptide-like carbonyl group was replaced by sulfonamide moiety, forming strong hydrogen bonds with the active site cavity residues. The second nonpeptidic inhibitor **tipranavir** (TPV, PNU-140690) contains 4-hydroxy-benzopyran-2-one moiety in addition. This unique compound was designed at Pharmacia & Upjohn and later manufactured by Boehringer-Ingelheim. Its hydroxyl group is hydrogen-bounded to the catalytic aspartates whereas carbonyl substitutes the flap water and forms hydrogen bonds with Ile-50 in flaps (*167*). Tipranavir maintains efficacy against numerous PI-resistant HIV strains (*168*). Resistance towards TPV is associated with high accumulation of mutations occurring at as many as 16-20 positions. Its main limitation is hepatotoxicity (*169*).

The most successful designed strategy to date yielded **darunavir** (DRV, TMC-114, UIC-94017, Tibotec BVBA, formerly Tibotec-Virgo NV). It is a structural analog of amprenavir, but it differs by the presence of a bis-tetrahydrofuranyl moiety which allows to form additional hydrogen bonds with conserved regions of HIV protease (*170*). These interactions with backbone are important since there is a minimal change in conformation between wild type and resistant strains. Twenty one mutations in the protease gene are needed for significant decrease of the protease susceptibility (*115*). Surprisingly, ultra-high resolution crystal structure revealed a second binding site of DRV in a surface pocket formed by one of the flaps (*171*). In addition, fluorescent resonance energy transfer assay suggested that DRV might also act as PR dimerization inhibitor (*172*). DRV resilience to the development of resistance, once-daily dosing, and tolerability make DRV one of the preferred therapeutic options both for drug-naïve and for treatment-experienced patients.

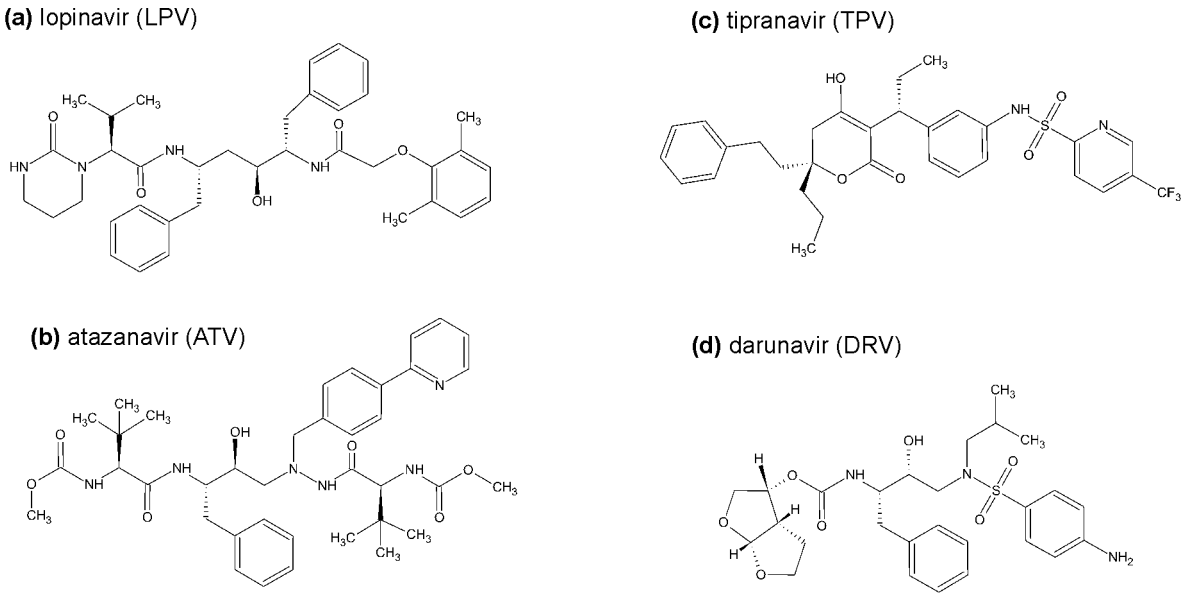


Figure 13. Chemical structures of the second generation HIV protease inhibitors

Source: Pokorná, J. et al.: Current and Novel Inhibitors of HIV Protease (*160*)

So far, ten competitive protease inhibitors were introduced into clinical practice, one withdrawn (*142, 160, 173*). First-line therapy for HIV infection now usually consists of two nucleoside reverse-transcriptase inhibitors (NRTIs) plus either the nonnucleoside reverse-transcriptase inhibitor (NNRTI) or a ritonavir-boosted protease inhibitor such as lopinavir/r, atazanavir/r, fosamprenavir/r, or darunavir/r, recently approved for first-line HAART (*174-176*). Unboosted nelfinavir, atazanavir, and fosamprenavir are suboptimal alternative choices in case of resistance or intolerance. Boosted lopinavir/r, tipranavir/r and darunavir/r are used for salvage therapy when treatment failure is caused by resistant mutants.

PIs development, most common side effects, and mutation profiles in the protease to this class of antiretrovirals are reviewed in detail in Paper I.

1.3.4.6 Inhibitors of HIV protease "in the pipeline"

The success of rational design of HIV PIs inspires other labs to continue in the effort to develop active novel PIs with simplified dosing regimens, little off-target activity, and, particularly, more favorable resistance profiles.

Peptidic HIV protease active site inhibitors

Ambrilia Biopharma Inc. (formerly Procyon) identified a highly active inhibitor against HIV PR and numerous resistant mutants in a screening of a compound library based on L-lysine scaffold. **PL-100,** later licensed by Merck Co. as Mk 8122 (*177*), showed very good inhibition activity against both wild type and multi-drug resistant viral strains. It selects a novel pattern of mutations, different from those described for other PIs. Two new active site mutations T80I and P81S and two flap mutations K45R and M46I were selected *in vitro* after 25 weeks (*178*). Interestingly, T80I mutation causes hypersensitivity to saquinavir and nelfinavir, whereas P81S mutation was shown to be lethal to the virus due to defect in the Gag processing. In addition, PL-100 also decreases activity of CYP450 3A4/5, which could allow a once-daily, un-boosted regime. Substantial improvement in solubility and pharmacokinetics was achieved by phosphorylation of hydroxyl group in **PPL-100** compound.

GS-8374 is a novel bis-tetrahydrofuran PI with a unique diethylphosphonate moiety showing picomolar inhibition potency in term of K_i. Researchers from Gilead Sciences based its design on TMC 126 (darunavir derivative) scaffold (*179*). The crystal structure analysis of the complex inhibitor / wild-type enzyme showed the phosphonate group to be exposed to the solvent, without any noticeable interactions with protease bringing favorable contributions to entropy upon inhibitor binding to the mutant enzymes (*179*). *In vitro* tests against a panel of highly resistant patient-derived viruses bearing mutations in the protease region showed potent antiretroviral activity, higher than any of the clinically approved PIs. Only a low level of cross-resistance to darunavir and atazanavir was described. A unique mechanism and pathway of moderate resistance development for GS-8374 have been recently observed *in vitro* after prolonged passaging (*180*). The isolates contained a single R41K mutation in PR combined with multiple genotypic changes in the Gag matrix, capsid, nucleocapsid, SP2 domains, and also in the transframe peptide and p6* domain of the Gag-Pol polyprotein. Analysis of viral particles revealed that a combination of substrate mutations without typical resistance mutations in PR rendered Gag more susceptible to proteolytic cleavage in the presence of GS-8374. Favorable features of GS-8374 together with low cytotoxicity in multiple human cell types support its further exploration as a potential antiretroviral agent (*181*).

When comparing the structures of wild-type and multi-drug resistant HIV proteases, scientists from Sequoia Pharmaceuticals identified conserved regions in PR active site that could not be changed by mutations. They developed a series of compounds and resulting **SP-256** and **SPI-256** make optimal interactions with such a conserved substructure and maintain high inhibition efficacy against wild-type and multidrug-resistant HIV PRs, better than any of the current FDA-approved inhibitors (*182*).

Potent PI **DG35** based on saquinavir was developed by Narhex Life Sciences Limited. The center of the molecule was derivatized by a hydroxyethylhydrazide group (*183*). Its poor solubility was successfully increased by phosphorylation yileding pro-drug **DG17** (*184*).

1.3.4.7 Examples of non-peptidic HIV protease active site inhibitors

Multidrug combination therapy dramatically improved life quality and expectancy of HIV infected patients. However, the development of resistance, high cost of therapy and adverse effects are severely limiting the long-term treatment options. Many studies suggest that cross-resistance remain a major obstacle in antiviral therapy with PIs. Therefore, in effort to develop new stably efficient anti-HIV-1 therapeutics, scientists search for novel non-peptidic templates to design the next generation of HIV PIs.

a) Cyclic urea derivatives

Rational structure-based design was employed in development of symmetrically substituted cyclic urea inhibitors **DMP 323** (*185*) and **DMP 450** (*186*). The urea oxygen mimicking the water molecule in the flap-proximal part of the enzyme active site allows to form two hydrogen bonds with main-chain NH groups of the Ile-50 (50´) and other four hydrogen bonds are formed between the hydroxyl groups of heterocyclic ring and carboxylates of the catalytic aspartates (*187*). Compound **SD 146**, more potent against resistant proteases, was reported later (*188*). It was modified by a benzimidazolylbenzamide group at P2 and P2´ positions and X-ray analysis showed that it forms fourteen hydrogen bonds and approximately 200 van der Waals interactions inside the binding cleft. Unfortunately, this most potent cyclic urea inhibitor (inhibition constant of 20 pM), is not a development candidate because of its poor pharmacokinetic properties.

b) Nb-containing polyoxometalates

These easily accessible inorganic compounds were reported to specifically inhibit HIV PR with submicromolar potency in tissue cultures (*189*). Computational studies suggest that they function via binding not to the active site of the protease but to the cationic pocket on the outer surface of the flaps (two compounds per active homodimer of HIV-1 PR). Inhibition kinetics also confirmed a non-competitive binding mode of inhibition. However, no direct structural evidence of their presumed binding mode has been provided and no follow-up reports on the activity of this class of compounds appeared in the literature ever since.

c) Fullerene derivatives

The ability of C_{60} fullerene derivatives to inhibit HIV-1 PR was suggested by means of molecular modeling and physico-chemical analysis. The prediction that these compounds should bind to the PR active site was based on their hydrophobic character, shape, and diameter, corresponding to that of the PR binding cavity (*190-191*). Kinetic analysis of water-soluble bis(phenethylamino-succinate) derivative showed a competitive binding mode of inhibition and modest selective activity against HIV-1.

d) Pyrrolidine-based inhibitors of HIV protease

These compounds represent inhibitors with novel scaffolds and deviating modes of binding targeting the open-flap conformation of the protease. They contain key structural elements from classical peptidomimetics and non-peptidic heterocyclic core with endocyclic amino group, which can interact with catalytic dyad (*192*). This basic structure was further modified by side chains already optimized for the recognition pockets of HIV protease or cathepsin D as methylendiamines or sulfonamides. They inhibit activity of HIV-1 protease with K_i value in submicromolar range. X-ray structure analysis of symmetric pyrrolidine esters and protease with active site mutation I84V revealed a unique binding mode (*193*). Two ligand molecules were detected in the protease binding cavity. One forms hydrogen bonds with catalytic aspartates through an amino group and a number

of hydrophobic interactions via naphtyl residues. The second molecule creates a water mediated hydrogen bond with G51 at the flap and van der Waals contacts with other flap residues. These two molecules of pyrrolidine esters and two HIV protease dimers form tetrameric structure, as observed with metallacarboranes (see bellow). It is not obvious whether this arrangement corresponds to the real interactions in solution or it is only the artifact of crystallization.

e) Icosahedral metallacarboranes as a novel type of HIV protease inhibitors

In broad screening of nonconventional compounds able to bind to the HIV protease active site and to possess favorable pharmacologic properties, icosahedral metallacarboranes were identified as novel nonpeptidic, potent, and specific HIV protease inhibitors (*194*). Carbon/boron (carborane) cluster complexes are composed of two clusters tightly sandwiching the central metal ion. Their extraordinary chemical and thermal stability comes from delocalized bonding within their triangular boron facets and over the whole cage, a phenomenon called three-dimensional aromaticity (*195*). In addition, due to the fact that hydrogen electronegativity is slightly larger than boron electronegativity, the boron-bound hydrogens carry a small partial negative charge. This facilitates interactions with positive counterions or positively-charged hydrogens of biomolecules via dihydrogen bonding (*196*). Moreover, they show low nucleophilicity and possibility of *exo*-skeletal substitutions.

The parental compound cobalt bisdicarbollide anion (**GB-18**) was i*n vitro* assays shown to be a competitive inhibitor with inhibition constant of 66 nM towards HIV protease. The crystal structure of GB-18 in complex with wild-type HIV-1 PR was the first carborane-protein structure ever reported. It was determined at 2.15 Å and revealed a unique binding mode (Fig. 14). Two molecules were bound asymmetrically in the symmetric hydrophobic pockets in the flap-proximal region of the S3 and S3' subsites of the enzyme active site, "above" the site usually occupied by currently used clinical inhibitors. This could explain the broad inhibition activity of metallacarboranes against highly PI-resistant HIV PR strains (*197*). Interestingly, tetrameric complex consisting of two protease dimers with metallacarboranes bound together "head to head" was observed in the crystal. It is unclear whether this structure exists in solution or it is a crystallization artifact. Electron density at the bottom of the active site was attributed to the short peptide, common in the structures

of free HIV protease or protease crystallized in the presence of weak inhibitors (*194*). Further development led to the design of double-cluster species connected with amino group containing linker as the most efficient inhibitors from the whole series. The X-ray structure of **GB-80** inhibitor was determined at the resolution of 1.7 Å. Although the linker part was disordered, cluster positions were supported by well defined electron density maps. Comparison with previous structure showed that PR binding cavity can accommodate the metallacarborane clusters in various positions.

Potential application in medicinal chemistry is however limited by the aggregation of metallacarboranes in aqueous solutions (*198-199*). It influences both the inhibition efficiency and the mode of inhibition (*200*). Nevertheless, the broad inhibition activity of metallacarboranes against wild type and multi resistant HIV PR variants, unique chemistry and binding mode make them attractive pharmacophores (*201*).

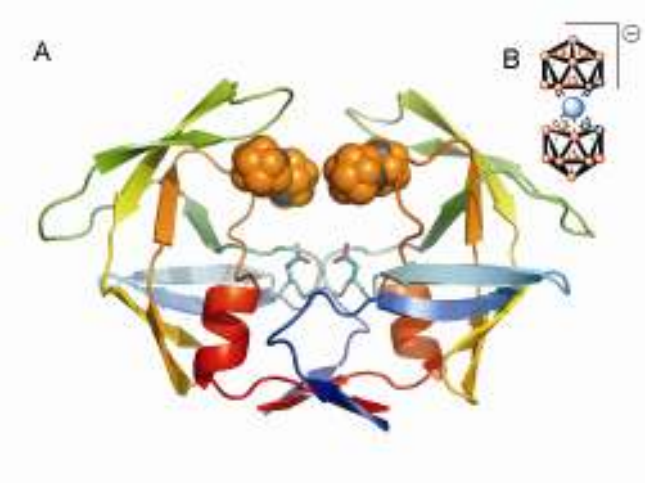


Figure 14: Crystal structure of metallacarborane inhibitor GB-18 bound to HIV PR.
A. Two metallacarborane clusters bind to the flap-proximal part of the active site. The HIV PR is represented by a ribbon diagram and colored by rainbow from blue to red (N- to C- terminus), the atoms of metallacarborane cluster are represented by spheres and colored orange for boron atoms, gray for carbon atoms, blue for cobalt. The structural formula is depicted in **B**. The figure was generated using the structure of HIV-1 PR – metallacarborane complex (PDB code 1ZTZ (*194*)) and program PyMol (*116*). Hydrogens are omitted for clarity.

Source: Pokorná, J. et al.: Current and Novel Inhibitors of HIV Protease (*160*)

f) Dibenzodiazepinone analogues as a potential new class of HIV protease inhibitors

More recently, through a screening of libraries of non-peptide low molecular weight compounds, dibenzo[b,e][1,4]diazepinone derivatives were identified as a micromolar inhibitors of HIV PR (*202*). 1,4-Benzodiazepines have been broadly used as a short term,

well-tolerated, and safe psychoactive compounds since the 1960s. X-ray structure of the parental compound in complex with the protease revealed two molecules of inhibitor in the binding cleft of the enzyme. Covalent linkage of two molecules of such a compound resulted in inhibitor showing an inhibition constant decreased by 2 orders of magnitude.

1.3.5 Maturation inhibitors

Maturation inhibitors are an experimental class of anti-HIV drugs that (similarly to PIs) prevent viral replication by blocking maturation of immature virions, the structural rearrangement essential to form infectious particles. During this process Gag polyprotein is cleaved by the viral protease into matrix (MA), capsid (CA), and nucleocapsid (NC) domains, p6 and two small spacer peptides (p1 and p2). MA remains membrane associated, CA creates the conical shell of the mature viral core (capsid), and NC domain captures the viral genome. Instead of inhibiting the viral enzyme responsible for Gag cleavage, maturation inhibitors target the structural proteins themselves. Among this class, the CA assembly inhibitors and CA/p2 inhibitors are the most promising.

The **CA assembly inhibitors** are believed to interfere competitively with interactions between capsid proteins which consist of two domains, the N-terminal and C-terminal domains (NTD and CTD). The mature capsid is built as a lattice of CA hexamers. Within each hexamer, each CA domain interacts with another one through NTD-NTD interfaces and NTD-CTD interfaces; each hexamer is associated with the six neighboring hexamers through CTD-CTD dimerization interfaces. By combinatorial approaches based on random libraries screening of small organic molecules the following compounds have been identified recently: **CAP-1** (*203*), **PF-3450074** from Pfizer (*204-205*), dodecapeptide **CAI** (*206*) and its derivative **NYAD-1** (*207*). CAP-1 and PF-3450074 interact with different sites in NTD. CAI and NYAD-1 associate with a hydrophobic pocket in CTD (*208-210*), in addition CAI also facilitates capsid disassembly (*211*).

Capsid assembly interfacial peptide inhibitors **CAC1**, its derivative **CAC1M**, and **H8** were discovered by the rational design of peptides mimicking capsid structural elements (representing CA helix 8) (*212*). Boehringer-Ingelheim scientists presented potent capsid assembly inhibitors **BI-257**, **BI-627,** and **BI-720** on conference in San Francisco in 2010 (*213*).

CA/p2 inhibitors block the final and rate-limiting steps in Gag processing, the separation of CA from p2. Incomplete cleavage of CA from CA-p2 interferes with core formation and results in release of an immature, non-infectious particle. CA/p2 inhibitors include **bevirimat** (BVM, PA-457 or MPC-4326) (*214*), a second generation maturation inhibitor **PA1050040** from Panacos based on bevirimat (*215*), two maturation inhibitors from Myriad Pharmaceuticals **Vivecon** (MPC-9055) (*216-217*) and **MPI-461359** (*218*), and **PF-46396** (*219*) from Pfizer.

Bevirimat (Fig. 15) is the first and best described maturation inhibitor, the only one which reached the Phase IIb trial. The study showed that bevirimat was well tolerated. However, the participants' virologic response depended greatly on the occurrence of natural HIV polymorphisms in the QVT motif of p2 (aminoacids 369-371) (*220*). About 50% of BVM-treated patients did not effectively respond to the drug. Further analyses of clinical isolates found at least one mutation associated with a reduced susceptibility to bevirimat (H358Y, L363M, Q369H, V370A/M/del, and T371del) at one third of treatment-naïve patient samples. Bevirimat resistance mutations were detected with significantly higher frequency, about 45%, in isolates with protease inhibitors resistance, especially in bevirimat target region, S368C, Q369H, V370A, and S373P (*221*). During *in vitro* selection studies in the presence of bevirimat, one of four mutations was identified: Gag V362I, A364V, S368N or V370A. Viruses with a mutated protease demonstrated more diverse resistant profile (*222*). It seems that coevolution of protease and its substrate Gag during prolonged PI therapy (*223-225*) may have consequences for development of HIV-1 resistance to bevirimat.

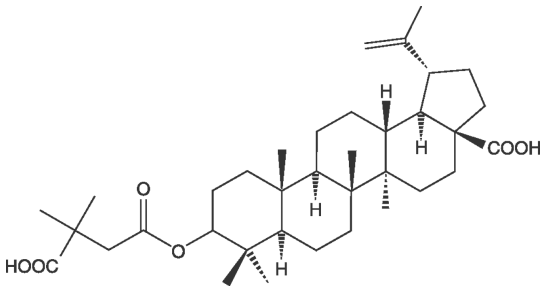
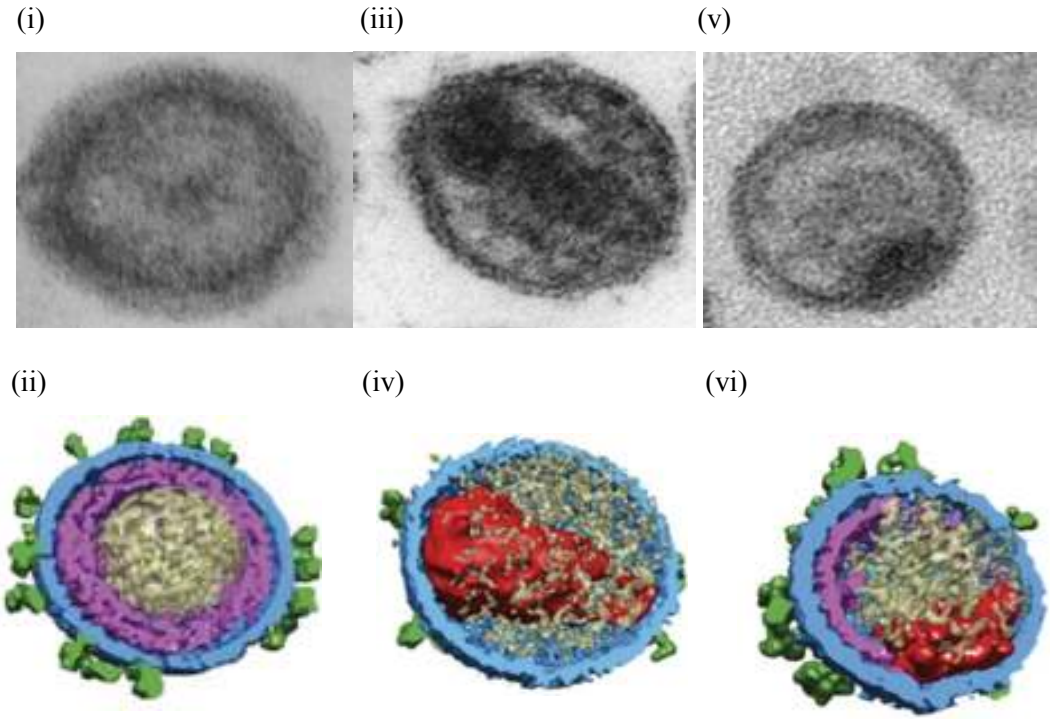


Figure 15. Chemical structure of bevirimat (BVM, PA-457)

Source: Martin, DE. et al.: Safety and pharmacokinetics of Bevirimat (PA-457), a novel inhibitor of human immunodeficiency virus maturation, in healthy volunteers (*226*)

Cryo-electron tomography of bevirimat treated virions (Fig. 16) showed that bevirimat has also a stabilizing effect on the immature Gag lattice, which further suppresses particle infectivity (*227*).



(A)

(B)

Figure 16. Proteolytic maturation of HIV-1 and its inhibition by bevirimat (BVM). (A) Virion morphology visualized by transmission electron microscopy (i, iii, v) and cryoelectron tomography models generated by segmented surface rendering. The glycoprotein spikes are colored green, the membrane and MA layer in blue, Gag related shells in magenta, core structures in red, and other internal density in beige (ii, iv, vi). Immature particles (i and ii), mature (iii and iv), and BVM-treated (v and vi). (B) Amino acid sequence at the CA-p2 junction region; amino acids highlighted in green indicate the highly polymorphic residues to which reduced susceptibility to BVM in clinical trials has been mapped and amino acids highlighted in red indicate those that at which BVM resistance arises *in vitro*.

Source: Catherine S. Adamson: Protease-Mediated Maturation of HIV: Inhibitors of Protease and the Maturation Process (*228*)

The location of bevirimat binding site to Gag within immature virus-like particles was identified using photoaffinity analogs of bevirimat and mass spectrometry because crystal structure of full length HIV-1 Gag is not yet available (*229*). A twenty-four amino acid residue peptide fragment (ALGPGATLEEMMTACQGVGGPGHK) of the CA-p2 cleavage region as a target within Gag was identified for the bevirimat. Its binding to the CA-p2 junction probably stabilizes the six helix bundle of CA-p2 and therefore blocks cleavage by the viral protease. Unexpectedly, a second site for analog crosslinking in the Major Homology Region (MHR, amino acid 295-299, in the context of assembled Gag in close proximity to CA-p2) was found.

Despite the fact that Myriad Genetics announced termination of bevirimat development in 2010, the data acquired during work on this inhibitor could provide help in the development of second generation maturation inhibitors.

1.3.6 Drug resistance and adverse effect of long-term therapy

Second-generation antiretroviral drugs of all classes have been designed with aim to increase their efficacy and avoid cross resistance with first-generation antivirals. Indeed, later inhibitors show exceptionally high genetic barrier to the development of resistance. Their benefit for HIV infected people is indisputable. However, current ART alone cannot completely eradicate HIV from the body. This means the need for life-long therapy; and viral resistance increases with increasing time on therapy. Other problems that arise from long-term therapy are adherence issues, chronic immune activation, and additive effects of drug toxicities. Many HIV patients are in increased risk of a number of "non-AIDS" complications, consistent with immunologic defects typically associated with aging, including cardiovascular and liver disease, renal problems, decreased bone mineral density, neurocognitive decline, and cancer (*230*). Therefore, maybe not only new compounds but entirely novel therapeutic approaches are needed to prevent both resistance emergence and immunologic abnormalities in this unique population of aging patients. Several new strategies in development focus on virus assembly, inhibition of RNase H (*231-232*), suppressing Vif function (*233*) and host cell factors critical for the virus replication (gene therapy associated with TRIM5α (*234*), down-regulation of cellular CD4 receptors, knocking down CCR5 and CXCR4 receptors (*235-236*), activating of the p53 pathway, inhibition of cyclin dependent kinase cdk9) (*237*) and others. However, the ultimate goal

of medicinal chemistry is still the complete eradication of the infectious agent from the patient; some sophisticated approaches towards this ambitious goal have been already suggested (*238*).

1.4 Progress in HIV prevention research

Huge effort of the scientific community and unprecedented investment in HIV prevention led to new HIV prevention option becoming available, including antiretroviral treatment as prevention with TDF/FTC (Truvada) as daily oral pre-exposure prophylaxis, voluntary medical male circumcision, and female condoms.

Phase IIb Caprisa 004 microbicide trial found 1% tenofovir gel to be 39% effective in reducing a woman's risk of becoming infected with HIV during sex. If other studies confirm these results, widespread use of the gel could prevent over half a million new HIV infections in South Africa alone over the next decade. The first successful vaccine candidate trial RV144, although modest in efficacy, along with the recently discovered broadly neutralizing antibodies (human antibodies capable of inhibiting over 90% of natural HIV-1 isolates) brought up a renewed optimism that the effective AIDS vaccine and protection by passive immunization is attainable (*239*). Recent positive results allowed Mitchell Warren, Executive Director of AVAC (AIDS Vaccine Advocacy Coalition) to say that: "For the first time, the end of the AIDS epidemic is within reach", (XIX International AIDS Conference in Washington, DC, 23 July 2012).

2. Results

2.1 The aims of the thesis

The aim of this thesis was to analyze the available literature on the protease from HIV and its inhibitors and characterize the structure-activity relationship, mechanism of action, and resistance profile of two new types of anti HIV virostatics: novel structural type of HIV PR inhibitors based on metallacarborane scaffold and maturation inhibitors blocking a particular HIV protease processing site.

The second aim of the thesis was to analyze the influence of small alkali ions on the activity and inhibition of HIV PR.

The results are summarized in five full papers that are included in this thesis as enclosures.

The first paper included in the thesis provides an overview of the protease inhibitor class of anti-HIV- drugs, from their initial introduction in 1995 to the current compounds in clinical development. It describes their mechanisms of action, structures, patterns of HIV resistance development, pharmacokinetic properties, side-effects, and off-target activities.

Screening of a variety of organic and inorganic compounds led to identification of metallacarboranes as promising frameworks for nonpeptide protease inhibitors. The second part of the thesis aimed to explore structure-activity relationship of these compounds as inhibitors of HIV protease and its resistant mutants.

Bevirimat is an inhibitor of a new experimental class of antiretrovirals that impede an essential step in the life-cycle of HIV-1, viral particle maturation. The third aim of the thesis was to study the impact of protease inhibitor resistance mutations on the development of bevirimat resistance and shed light on the complicated interactions between the viral protease and its polyprotein substrate.

The interactions of ions with biomolecules play an important role in many natural processes. These effects are traditionally described by lyotropic or Hofmeister series.

However, the molecular origin of these effects is still not fully understood. Small molecules including ions might play regulatory role in the unexplored mechanism of the activation of HIV PR within retroviral particle. The cation-specific effects on the enzymatic activity of the HIV protease were investigated by a combination of experimental and computational techniques.

2.2 Publications included in the thesis

1. Pokorná, J., Machala, L., Řezáčová, P. and Konvalinka, J. (2009) Current and Novel Inhibitors of HIV Protease, *Viruses* 1, 1209-1239.

2. Rezácová, P., **Pokorná, J**., Brynda, J., Kozísek, M., Cígler, P., Lepsík, M., Fanfrlík, J., Rezác, J., Grantz Sasková, K., Sieglová, I., Plesek, J., Sícha, V., Grüner, B., Oberwinkler, H., Sedlácek, J., Kräusslich, HG, Hobza, P., Král, V., Konvalinka, J. (2009) Design of HIV protease inhibitors based on inorganic polyhedral metallacarboranes, *J Med Chem* 52(22), 7132-41.

3. Fun, A., van Maarseveen, NM., **Pokorná, J.**, Maas, EM, Schipper, PJ., Konvalinka, J. and Nijhuis, M. (2011) HIV-1 protease inhibitor mutations affect the development of HIV-1 resistance to the maturation inhibitor bevirimat, *Retrovirology* 8:70.

4. Heyda, J.*, **Pokorná, J.***, Vrbka, L., Vácha, R., Jagoda-Cwiklik, B., Konvalinka, J., Jungwirth, P. and Vondrášek, J. (2009) Ion specific effects of sodium and potassium on the catalytic activity of HIV-1 protease, *Phys Chem Chem Phys* 11(35), 7599-604.

* These authors contributed equally to this work.

5. Pokorná, J.*, Heyda, J.* and Konvalinka, J. (2013) Ion specific effects of alkali cations on the catalytic activity of HIV protease, *Faraday Disc* 160 (1), 359 - 370

* These authors contributed equally to this work.

2.3 Publications not included in the thesis

1. Urban, J., Konvalinka, J., **Stehlíková, J.**, Gregorová, E., Majer, P., Soucek, M., Andreánsky, M., Fábry, M., Strop, P. (1992) Reduced-bond tight-binding inhibitors of HIV-1 protease. Fine tuning of the enzyme subsite specificity, *FEBS Lett* 298(1), 9-13.

2. Majer, P., Urban, J., Gregorová, E., Konvalinka, J., Novek, P., **Stehlíková, J.**, Andreánsky, M., Sedlácek, J., Strop, P. (1993) Specificity mapping of HIV-1 protease by reduced bond inhibitors, *Arch Biochem Biophys* 304(1), 1-8.

3. Cígler, P., Kozísek, M., Rezácová, P., Brynda, J., Otwinowski, Z., **Pokorná, J.**, Plesek, J., Grüner, B., Dolecková-Maresová, L., Másа, M., Sedlácek, J., Bodem, J., Kräusslich, HG, Král, V., Konvalinka, J. (2005) From nonpeptide toward noncarbon protease inhibitors: metallacarboranes as specific and potent inhibitors of HIV protease, *Proc Natl Acad Sci U S A* 102(43), 15394-9.

4. Kozísek, M., Bray, J., Rezácová, P., Sasková, K., Brynda, J., **Pokorná, J.**, Mammano, F., Rulísek, L., Konvalinka, J. (2007) Molecular analysis of the HIV-1 resistance development: enzymatic activities, crystal structures, and thermodynamics of nelfinavir-resistant HIV protease mutants, *J Mol Biol* 374(4), 1005-16.

5. Kozísek, M., Cígler, P., Lepsík, M., Fanfrlík, J., Rezácová, P., Brynda, J., **Pokorná, J.**, Plesek, J., Grüner, B., Grantz Sasková, K., Václavíková, J., Král, V., Konvalinka, J. (2008) Inorganic polyhedral metallacarborane inhibitors of HIV protease: a new approach to overcoming antiviral resistance, *J Med Chem* 51(15), 4839-43.

6. Řezáčová, P., Cígler, P., Matějíček, P., Lepšík, M., **Pokorná, J.**, Grüner, B., and Konvalinka, J. (2011) Medicinal Application of Carboranes, Inhibition of HIV Protease. In *Boron Science: New Technologies and Applications*; Narayan S. Hosmane; CRC Press: *Northern Illinois University, Dekalb, USA, 2011, 41-70.*

7. Grüner, B., Svec, P., Hajkova, Z., Cisarova, I., **Pokorna, J.**, Konvalinka, J. (2012) Derivatization chemistry of the double-decker dicobalt sandwich ion targeted to design biologically active substances, *Pure Appl Chem* 84 (11), 2243-2262.

National and international patents:

1. Král, V., Cígler, P., Konvalinka, J., Kožíšek, M., Prejdová, J., **Pokorná, J.**, Kräusslich, H.-G., Bodem J., Grüner B., Plešek J., Lepšík M.: Novel inhibitors of HIV protease. Czech patent CZ295245.

2. Král, V., Cígler, P., Konvalinka, J., Kožíšek, M., Prejdová, J., **Pokorná, J.**, Kräusslich, H.-G., Bodem, J., Grüner, B., Plešek, J., Lepšík, M.: II. Novel inhibitors of HIV protease. Czech patent CZ303046.

3. Král, V., Cígler, P., Konvalinka, J., Kožíšek, M., Prejdová, J., **Pokorná, J.**, Kräusslich, H.-G., Bodem, J., Grüner, B., Plešek, J., Lepšík, M.: Novel HIV protease inhibitors. International patent WO2005073240.

Conference presentations:

1. Pokorná, J., Cígler, P., Kožíšek, M., Řezáčová, P., Brynda, J., Plešek, J., Grüner, B., Sedláček, J., Bodem, J., Kraeusslich, H.-G., Král, V. and Konvalinka, J. (2006) Metallacarboranes as specific and potent inhibitors of HIV protease and its resistant mutants, 15th International Drug Resistance Workshop, Sitges, Spain.

2. Pokorná, J., Brynda, J., Kožíšek, M., Cígler, P., Lepšík, M., Fanfrlík, J., Řezáč, J., Grantz Šašková, K., Sieglová, I., Plešek, J., Šícha, V., Grüner, B., Oberwinkler, H., Sedláček, J., Kräusslich, H.-G., Hobza, P., Král, V., Konvalinka, J., Řezáčová, P. (2009) Design, structure and activity of potent HIV protease inhibitors based on inorganic polyhedral metallacarboranes, 34th FEBS Congress - Life's molecular Interactions, Prague, Czech Republic.

3. Pokorná, J., Cígler, P., Kožíšek, M., Řezáčová, P., Brynda, Lepšík, M., B. Grüner, B., V. Šícha, V., H.-G. Kraeusslich, H.-G.and J. Konvalinka, J. (2010) Inorganic Polyhedral Metallacarborane Inhibitors of HIV Protease and its Resistant Mutants, 12th International Symposium on Proteases, Inhibitors and Biological Control, Portoroz, Slovenia.

4. Pokorná, J., Heyda, J. and Konvalinka, J. (2012) Ion specific effects of alkali cations on the catalytic activity of HIV protease, Ion Specific Hofmeister Effects: Faraday Discussions No. 160, Oxford, the United Kingdom.

2.4 Paper I: **Current and Novel Inhibitors of HIV Protease.**

Pokorná, J., Machala, L., Řezáčová, P. and Konvalinka, J.

Viruses (2009) **1**, 1209-1239

Brief overview

2.4.1 Introduction

Protease inhibitors impede an essential step in the life-cycle of human immunodeficiency virus, maturation. It is morphological rearrangement of the immature, non-infectious virus particle to the mature infectious virion. This transition is triggered by the viral enzyme protease, which cleaves the viral Gag and Gag-Pol polyprotein precursors into mature structural proteins and functional enzymes. Since immature particles are non-infectious, the protease has been a prime target for pharmaceutical research. Introduction of protease antiviral inhibitors to the combination therapy for HIV in 1995 resulted in a significantly reduced HIV-related morbidity and mortality. Currently, nine PIs are used in clinical practice. However, the usefulness of PIs is compromised by extensive cross resistance, caused by very high replication rate of HIV and by the lack of HIV-1 RT proof-reading activity. Further limitations of this class of virostatics are their tolerability and toxicity, adherence, and high price.

This paper reviews the PIs currently in clinical use, a few compounds that entered clinical testing and examples of experimental inhibitors targeting protease binding cleft. Finally, PIs targeting other important enzyme regions, namely the protease dimerization and flap domains, are mentioned.

2.4.2 Inhibitors currently used in clinical practice

All PIs currently used in HAART, with the exception of tipranavir, are peptidomimetic competitive inhibitors, which bind to the active site of the protease. The first generation HIV protease inhibitors includes in order of their introduction: saquinavir (1995), ritonavir (1996), indinavir (1996), nelfinavir (1997), and amprenavir (1999)/ fosamprenavir (2003). The second generation HIV protease inhibitors were designed with the aim to inhibit HIV PR strains resistant to the first generation PIs and reduce adverse effects. This group consists of lopinavir (2000), atazanavir (2003), tipranavir (2005), and darunavir (2006). To

increase their plasma level, they are mostly co-administered with low doses of a booster, a potent inhibitor of cytochrome P450, such as ritonavir.

2.4.3 Inhibitors of HIV protease "in the pipeline" and in the development

Promising PIs in the pipeline involve Ambrilia Biopharma compound PPL-100, Sequoia Pharmaceuticals inhibitor SPI-256, and molecules based on darunavir scaffold (or its derivative TMC 126), such as GRL series by Ghosh or GS-8374 inhibitor developed by Gilead Sciences.

Very attractive scaffold for PIs design represents the 7-membered ring of cyclic urea (compound DMP323 and DMP450). Unusual chemistry was used in the case of C_{60} fullerenes, icosahedral carboranes (binding to the flap-proximal region of the HIV PR active site), and Nb-containing polyoxometalates (targeting the cationic pocket on the outer surface of the flaps).

Because HIV PR is only active as a dimer and dimeric interface is conserved among most HIV-1 isolates and drug-resistant strains, this domain represents further attractive target for the development of ligands preventing dimerization. The most potent inhibitor, containing the minimal peptide core, attained a sub-nanomolar inhibition constant for the *in vitro* inhibition. An antibody recognizing the N-terminus of HIV PR with inhibition constant in low nano-molar range has also been reported.

2.4.4 Conclusions

Development and clinical application of PIs represents the successful example of rational drug design in the history of medicinal chemistry. The inclusion of PIs to the therapeutic armamentarium has dramatically improved AIDS therapy. However, there is continuing need for novel safer and cheaper antiviral inhibitors targeting wild type as well as resistant strains of HIV, imposing low pill burden and little side-effect to the patient.

Author´s contribution to Paper I:

I performed the research of the literature, provided the first draft of the manuscript, prepared several figures and edited the manuscript under the guidance of my supervisor.

2.5 Paper II: **Design of HIV protease inhibitors based on inorganic polyhedral metallacarboranes.**

Rezácová, P., Pokorná, J., Brynda, J., Kozísek, M., Cígler, P., Lepsík, M., Fanfrlík, J., Rezác, J., Grantz Sasková, K., Sieglová, I., Plesek, J., Sícha, V., Grüner, B., Oberwinkler, H., Sedlácek, J., Kräusslich, HG, Hobza, P., Král, V. and Konvalinka, J.

J Med Chem (2009) **52**(22), 7132-41

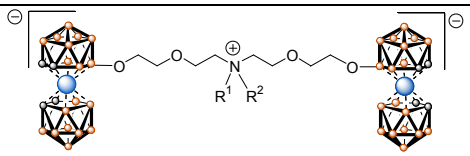
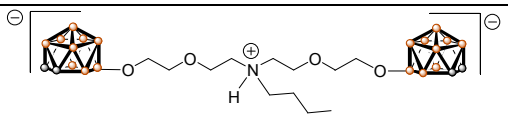
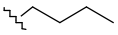
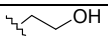
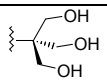
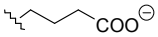
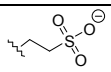
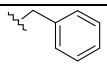
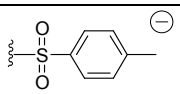
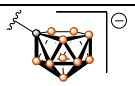
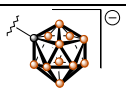
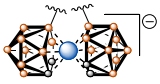
Brief overview

In search for novel unconventional inhibitor structures, our group has recently identified icosahedral carbon/boron (carborane) cluster complexes as promising scaffold for a new class of non-peptidic protease inhibitors (*194*). We previously reported that substituted cobalt bis(dicarbollides) are potent and specific inhibitors of the wild-type HIV-1 protease as well as its resistant variants (*197*). The crystal structure analysis of the parental compound in complex with wild-type HIV-1 protease revealed two molecules of ligand bound in the flap proximal region, uniquely in the semi-open form of the enzyme. In a structure guided drug design effort, we connected the two metallacarborane cages with an amino group containing linker and obtained a series of compounds. We determined inhibition potency and mechanism of this set of substituted compounds, analyzed the HIV protease:inhibitor X-ray structure, and computationally explored the conformational space of the flexible linker.

2.5.1 Metallacarboranes as the inhibitors of HIV protease

The series of metallacarboranes was tested *in vitro* by spectrophotometric assay using a chromogenic substrate and recombinant wild-type HIV-1 PR. Inhibitor structures, the IC_{50} values, inhibition mechanisms, and inhibition constants (K_i values) for six compounds with competitive mode of inhibition are shown in Table 1.

Table 1. Structures and inhibitory constants of metallacarborane inhibitors of HIV-1 PR.

General formula[a]			**14** [a]		
					
Compound description			***in vitro* enzyme assay**		
	R^1	R^2	IC_{50}	Mechanism	K_i (nM)
1	H	H	140 nM	competitive	4.9 ± 2.1
2			160 nM	concentration dependent[b]	N.D.
3		H	100 nM	competitive	2.2 ± 1.2
4		H	140 nM	competitive	4.7 ± 1.2
5		H	130 nM	competitive	2.7 ± 1.1
6		H	190 nM	noncompetitive	N.D.
7		H	110 nM	competitive	4.2 ± 1.5
8		H	110 nM	noncompetitive	N.D.
9		H	140 nM	concentration dependent [b]	N.D.
10		none	70 nM	concentration dependent [b]	N.D.
11[c]		H	50 nM	noncompetitive	N.D.
12 [c]		H	58 nM	concentration dependent [b]	N.D.
13 [c]			250 nM	competitive	0.27 ± 0.33
14	see Fig. above		8.5 µM	N.D.	N.D.

(a) Color coding: orange, BH groups or B (if substituted); black, CH groups or C (if substituted); blue, Co atom. (b) With the increasing concentration, the inhibition mechanism changes from noncompetitive through mixed toward competitive. (c) The metallacarborane cluster substituents are covalently bound to the central nitrogen atom of the linker (*200*).

2.5.2 *In vitro* resistance profile of metallacarboranes

The ability to inhibit a panel of seven HIV PR variants representing enzyme mutations for various FDA-approved protease inhibitors was tested for five metallacarboranes from this series. Proteases PR1 - PR4 were prepared by site-directed mutagenesis and harbor primary mutations conferring resistance to nelfinavir, ritonavir, indinavir, and lopinavir. Three other highly resistant HIV PR species PR5 - PR7 were derived from HIV-positive patients failing antiretroviral therapy with PIs. The resistance profile is expressed as a vitality value representing the relative capacity of the mutated enzyme to cleave its substrate in the presence of an inhibitor. Seven inhibitors used in clinical practice were included in our inhibition assay for comparison. While metallacarboranes demonstrated low relative loss of inhibition potency, the presence of mutations caused dramatic increase in vitality for saquinavir, indinavir, nelfinavir, lopinavir, amprenavir, atazanavir, and darunavir (Fig. 17).

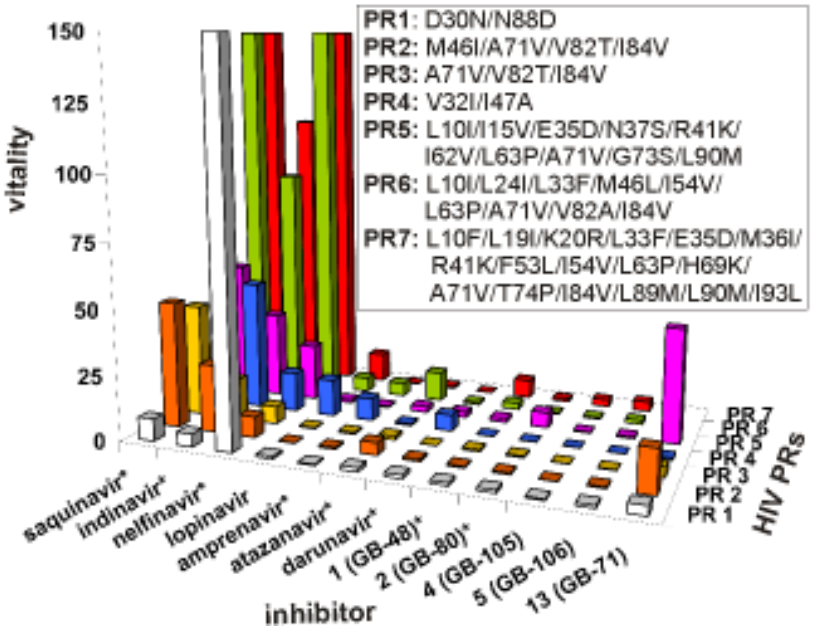


Figure 17. Vitality values of seven clinical inhibitors and five cobaltacarborane compounds analyzed with the panel of HIV-1 PR resistant species. Mutations in HIV-1 PR variants are shown in the figure inset. The vitality (*240*) is defined as $(K_i k_{cat}/K_m)_{MUT}/(K_i k_{cat}/ K_M)_{WT}$, where MUT and WT are the mutated and wild-type enzyme variant, respectively. The vitality values could not be determined for compound 13 and proteases PR6 and PR7 because the inhibitor concentration needed to inhibit these enzyme variants was above the compound solubility limit. The asterisks mark data published in our previous work (*197, 200*).

2.5.3 Crystal structure of HIV protease in complex with compound 1

The crystal structure of compound **1** bound to HIV protease was determined at 1.7 Å resolution (Fig. 18). One molecule of cobalt bis(1,2-dicarbollide) clusters occupies the binding site of the HIV PR symmetrically in the hydrophobic pockets corresponding to S3 and S3´ substrate binding subsites. The binding of compound **1** blocks the flap closure, thus the overall enzyme dimer structure is in unique semi-open conformation, as was observed in the structure with parental metallacarborane (*194*). Although both cages of the ligand could be modeled into well-defined electron density, continuous map of the connecting inherently flexible linker was missing. To determine energetically feasible conformers of the linker, which amino group is in interactions with catalytic aspartates, we employed molecular modeling and calculations. Interestingly, five identified possible conformers shared a spherical electron density in place of the predicted nitrogen atom position.

a) b)

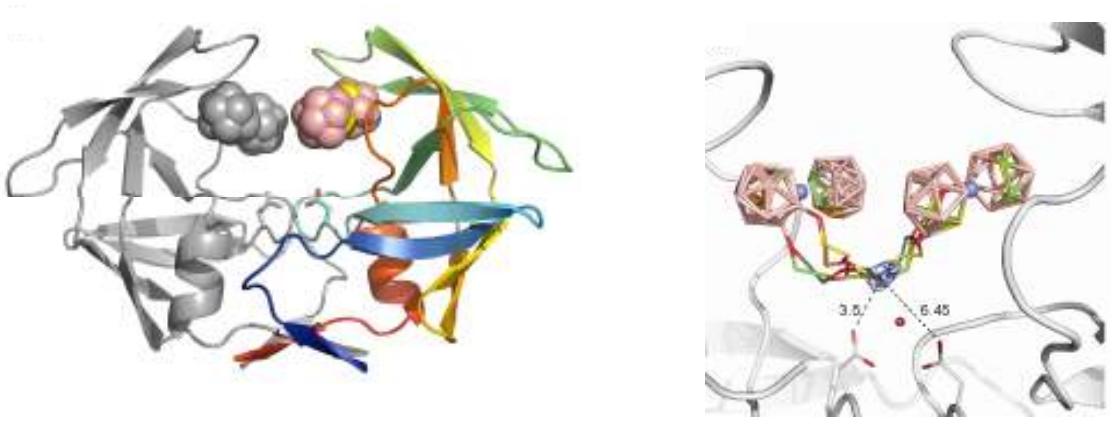


Figure 18. (a) Overall view of the crystal structure of the HIV PR:1 complex. (b) The four lowest energy 1 conformers obtained from molecular modeling and calculations. Catalytic water is depicted as a red sphere. Catalytic aspartates are shown in sticks in both figures (*200*).

2.5.4 Different modes of inhibition for various metallacarboranes

Interestingly, we observed different mode of inhibition for various compounds of our series: competitive, noncompetitive, and for some inhibitors the mechanism of inhibition was dependent on the compound concentration. The competitive mode of inhibition means that the inhibitor competes with the substrate for binding to the enzyme binding cleft. This mode of action corresponds to the published crystal structure of the parent bis(1,2-dicarbollide) ion bound to the active site of HIV PR (*194*) as well as to the crystal structure presented in this work. Non-competitive type of inhibition could suggest specific binding of compounds outside the enzyme active site. Concentration dependent type might be related to aggregation of cobalt bis(dicarbollides) in solution that was described previously (*241*). It was shown that the formation of aggregates in aqueous solutions is fairly complex and depends on numerous factors, including compound concentration (*241*). Detailed explanation of these different types of inhibition mechanisms are subject of our further studies.

Author´s contribution to Paper II:

Together with my colleague Milan Kožíšek we identified metallacarboranes as the inhibitors of HIV-1 protease through random testing of various organic and inorganic compounds. I have determined IC_{50} values and the type of mechanism of inhibition for the series of compounds *in vitro* by spectrophotometric assay. For competitive inhibitors I determined the K_i values.

2.6 Paper III: **HIV-1 protease inhibitor mutations affect the development of HIV-1 resistance to the maturation inhibitor bevirimat.**

Fun, A., van Maarseveen, NM., Pokorná, J., Maas, EM, Schipper, PJ., Konvalinka, J. and Nijhuis, M.

Retrovirology (2011) **8**:70

Brief overview

Maturation inhibitors represent an experimental class of antivirals that hamper the maturation of HIV. The processing of the precursor Gag and Gag-Pol polyproteins by the viral protease is required to form infectious viral particles, as was mentioned above. The most advanced maturation inhibitor, often used as a model system, is bevirimat. In contrast to protease inhibitors, bevirimat prevents viral maturation by targeting the CA/p2 substrate cleavage site within the Gag instead of the viral enzyme itself. It was reported that the presence of bevirimat resistance mutations is associated with the number of protease mutations and that they are co-selected during the failure of prolonged protease inhibitor therapy (*221*). Since maturation inhibitors are likely to be included as part of salvage therapy, it is necessary to understand the consequences of prior treatment with PIs. In all of resistance *in vitro* selection studies performed by our collaborators in Utrecht, one of four mutations was selected in Gag: V362I, A364V, S368N or V370A. The data showed that resistance patterns were significantly different for viruses with PI resistant proteases compared to wild-type proteases. Mutations V362I and S368N resulted in much higher levels of resistance when introduced into the viruses with PI resistance mutations in the viral protease compared to the viruses with wild-type or less mutated protease. Viral replication was not found to be a major factor during emergence of bevirimat resistance. Furthermore, accelerated processing of the CA/p2 cleavage site in the presence of the bevirimat resistance mutation A364V was observed. These results emphasize that for the prediction of treatment responses for maturation inhibitors, complex interactions between the viral protease and its substrate should be taken into account.

2.6.1 Effect of bevirimat resistance mutations on the CA/p2 processing efficiencies

To describe the differences in resistance levels caused by bevirimat resistance mutations in different genetic backgrounds, we carried out a biochemical analysis of the specific cleavage efficiencies. Three different substrates derived from Gag (wt), Gag (V362I), and Gag (A364V), represented by the nonapeptides: KARVL↓AEANle-NH_2, KARIL↓AEANle- NH_2, and KARVL↓VEANle-NH_2, were processed with either the wild-type (HXB2) or the highly mutated (PR-2) protease (Tab. 2). Despite the lower absolute cleavage efficiency of PR-2 compared to HXB2, the relative increase in substrate conversion in the presence of bevirimat resistance mutations is greater in the context of the PR-2 protease compared to the HXB2 protease. In addition, both proteases processed substrate with A364V mutation ten times faster than substrate with V362I mutation. This might explain the high levels of bevirimat resistance conferred by A364V in all backgrounds and enhanced resistance levels observed for the PR-2 viruses.

Table 2. CA/p2 processing efficiencies of HXB2 and PR-2 proteases

substrate	Relative substrate conversion		
	HXB2	**PR-2**	**Ratio (PR-2/HXB2)**
WT	1	1	-
V362I	0.87	1.3	1.49
A364V	7.6	11	1.45

The cleavage efficiency of the WT substrate was set to 1, conversion of substrates with V362I or A364V was measured relative to the conversion of the WT substrate (*222*).

Author´s contribution to Paper III:

I prepared two recombinant HIV-1 proteases used in this study and tested their enzymatic activities with three different substrates using HPLC. I also drafted the methodological part of the manuscript.

2.7 Paper IV: **Ion specific effects of sodium and potassium on the catalytic activity of HIV-1 protease.**

Heyda, J.*, Pokorná, J.*, Vrbka, L., Vácha, R., Jagoda-Cwiklik, B., Konvalinka, J., Jungwirth, P. and Vondrášek, J.

* These authors contributed equally to this work.

Phys Chem Chem Phys (2009) **11**, 7599-604.

Brief overview

The specific regulation of enzymatic activity by small alkali cations, especially sodium and potassium, has an important role in many biological processes (*118, 132, 242-244*). Moreover, non-specific, "salting-out" effect of inorganic ions has been described already in 19th century as "Hofmeister series". Its molecular mechanism, however, remains elusive. A higher affinity of Na^+ over K^+ to the protein surface was recently quantified by molecular dynamic simulations and conductivity measurements (*135*). Several groups have reported the observation that higher salt concentration increases the activity of HIV-1 protease (*130, 245*), however, the majority of previous analyses have studied the effect of different anions. We performed experimental and computational studies to demonstrate and rationalize the cation-specific effect of Na^+ *vs*. K^+ on the enzymatic activity of HIV-1 PR in aqueous solution. Our experiments showed the specific cation-dependence of the enzymatic activity of HIV-1 protease that grows with increasing salt concentration. This effect is stronger in KCl-containing buffers than in buffers containing NaCl. Furthermore, experimentally determined catalytic efficiency (k_{cat}/K_M) of HIV-1 PR is significantly higher in the presence of K^+ compared to Na^+ at comparable salt concentrations. Molecular dynamic simulations showed sodium affinity to the surface of HIV-1 PR to be twice as high as potassium affinity, with the carboxylic side chain groups of Glu and Asp and the backbone carbonyl oxygens being responsible for this cation-specific attraction. The most relevant could be the interaction with the Asp pair at the active site, where the increased presence of sodium over potassium can lead to a decreased efficiency of substrate binding in NaCl-containing buffers compared to KCl-containing buffers.

2.7.1 Experimental studies

To characterize the dependence of HIV-1 PR activity on Na^+/K^+ ion concentrations, we measured the initial velocities and kinetic constants (*i.e.* K_M and k_{cat}) in three different buffers: phosphate and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 6.0, and acetate buffer, pH 4.7 (data not shown). We used spectrophotometric assay with a chromogenic peptide substrate derived from the processing site spanning the CA/p2 cleavage site with a *para*-nitrophenyl residue in the P1‘ position of the substrate (Peptide 1: KARVNle*F(NO_2)EANle-NH_2). In order to dissect the possible influence of negatively charged Glu residue of Peptide 1 on this phenomenon, we also analysed the kinetics with a second peptide substrate (data not shown). Peptide 2: 2,2-aminobenzoyl-TINle*F(NO_2)QR-NH_2, is based on the p2-NC cleavage site of the HIV Gag polyprotein. We observed a monotonous growth in initial velocities with increasing NaCl or KCl concentration over the whole concentration. In all cases, a higher initial velocity was determined in the presence of KCl than in the presence of NaCl (Fig. 19).

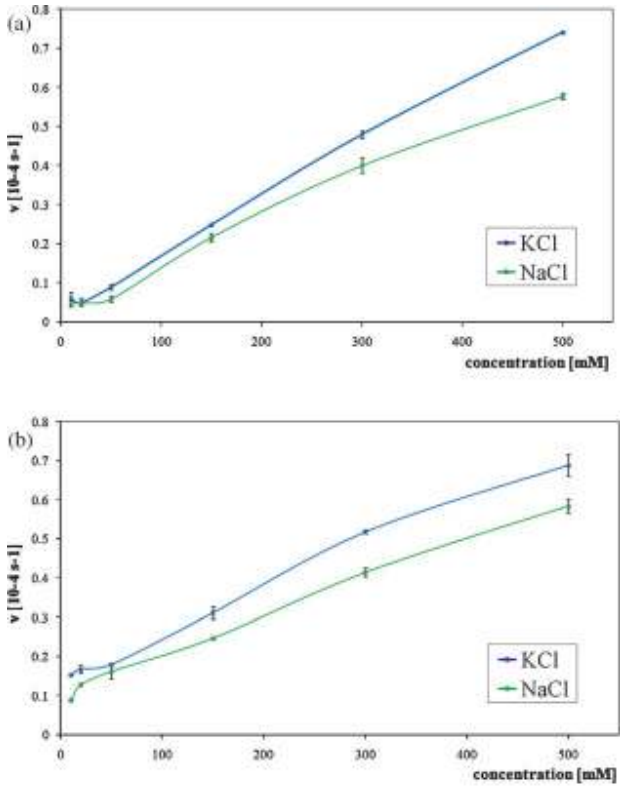


Figure 19. Dependence of the initial velocities of the hydrolysis of Peptide 1 by HIV-1 PR on increasing concentrations of NaCl or KCl. The kinetic measurements were performed in 50 mM HEPES (a) and phosphate (b), pH 6.0 (*136*).

The kinetic constants K_M and k_{cat} at various salt concentrations were determined at pH 6.0 to quantify the dependence of Peptide 1 (Tab. 3) and 2 (data not shown) hydrolysis on salt concentration. The K_M values decrease with the ionic strength for both ions, suggesting the improved substrate binding, whereas the differences in the turnover numbers (k_{cat} values) are less pronounced. The higher overall catalytic efficiencies (k_{cat}/K_M) in the presence of potassium are in agreement with the higher activity of HIV-1 PR in the presence of K^+ ions.

Table 3. Kinetic analysis of HIV PR activity dependency on the salt concentration (hydrolysis of Peptide 1 in the phosphate buffer, pH 6.0 (*136*).

Salt	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
NaCl 50 mM	120 ± 20	10 ± 1	89 ± 14
NaCl 300 mM	86 ± 10	11 ± 1	130 ± 20
KCl 50 mM	110 ± 20	16 ± 1	150 ± 30
KCl 300 mM	41 ± 2	17 ± 1	400 ± 30

Author´s contribution to Paper IV:

I prepared recombinant wild-type HIV-1 protease used in this study and performed all the kinetic analyses described in the paper. I also drafted parts of the manuscript.

2.8 Paper V: **Ion specific effects of alkali cations on the catalytic activity of HIV protease.**

Pokorná, J.*, Heyda, J.* and Konvalinka, J.

* These authors contributed equally to this work.

Faraday Disc (2013) **160** (1), 359 - 370

Brief overview

We have previously reported cation specific effects of sodium and potassium on the enzymatic activity of the HIV protease in aqueous solutions (*136*). Now we have extended our studies for other alkali cations using enzyme kinetics and molecular dynamic simulations. We have shown that the dependence of the initial velocity of peptide substrate hydrolysis on the nature of the cation follows the Hofmeister series with the exception of caesium. Substantially higher effect of K^+ on catalytic efficiency both in terms of substrate binding (K_M) and turnover number (k_{cat}) was found compared to Na^+ or Li^+ at corresponding salt concentrations. Molecular dynamic simulations showed higher preference of protein surface for lithium and sodium over potassium and caesium, mostly due to stronger affinity to negatively charged side chain groups of aspartates and glutamates. Surprisingly, unexpected decrease of the K_M value for a specific substrate was observed for the very low salt concentration.

2.8.1 Molecular dynamics of the alkali cations interaction with HIV-1 PR surface

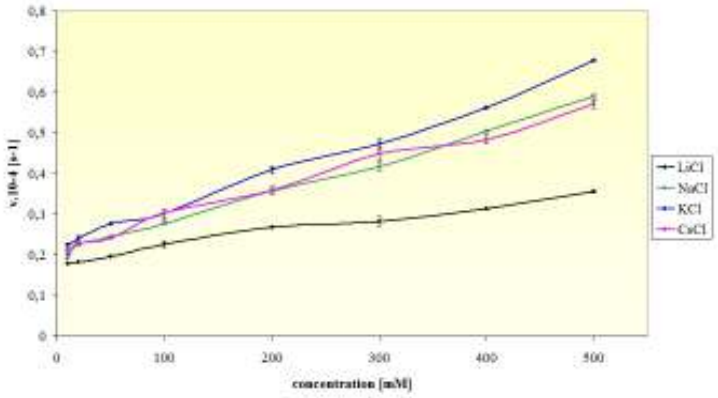
MD simulations were employed to investigate the interaction of HIV-1 protease with lithium, sodium, potassium, and caesium cations in aqueous salt solutions. The most important sites occupied by cations are located near the aspartic dyad (i.e. below the flaps) and near the entrance to the HIV-1 protease binding cavity, where the negatively charged amino acids are most abundant. The results suggest that the affinity of Li^+ and Na^+ is significantly stronger and more complex than that of K^+ and Cs^+. In contrast, the Cl^- does not demonstrate so strong interactions with the surface of HIV-1 protease, even though the surface is overall positively charged.

2.8.2 Experimental analysis of activation of HIV protease by alkali ion concentration

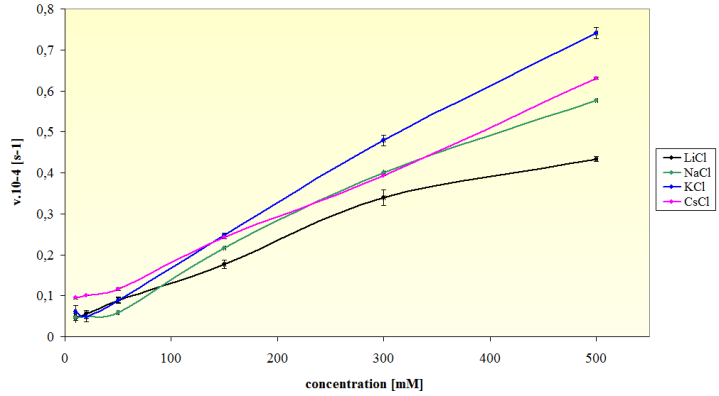
The initial velocities of the hydrolysis of the model chromogenic peptide substrate of HIV PR (Peptide 1: KARVNle*F(NO_2)EANle-NH_2) were determined in varying concentrations of sodium, potassium, lithium, and caesium ions (Fig. 20). Spectrophotometric studies show a monotonous growth in the initial velocities with increasing ionic strength for all the tested salts in the phosphate buffer and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 6.0. KCl enhances the enzymatic activity most effectively, followed by NaCl and CsCl, with comparable influence. The concentration of lithium influenced the enzymatic activity less significantly. Similar results were also obtained in the 2-[Bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol (BIS-TRIS) buffer pH 6.0 and in the acetate buffer, pH 4.7, as well as with another peptide substrate (Peptide 2: 2-aminobenzoyl-TINle*F(NO_2)QR-NH_2) in the 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0 (data not shown).

Surprisingly, significant increase in the enzymatic activity of HIV PR in the very low salt concentration range (10-20 mM) has been observed for the cleavage of the Peptide 1 substrate in the MES buffer, pH 6.0, while the dependence in the higher concentrations follows similar pattern as in all the other buffers mentioned above.

(a)



(b)



(c)

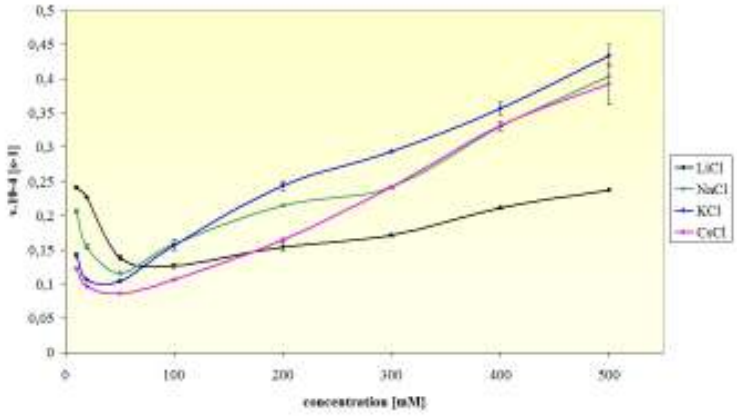


Figure 20. Dependence of the initial velocities of the hydrolysis of the Peptide 1 by HIV-1 PR on increasing concentrations of alkali ions. The kinetic measurements were performed in 50 mM phosphate (a), HEPES (b), or MES (c), pH 6.0 (*137*).

The values of the determined kinetic constants K_M and k_{cat} at various salt concentrations in the phosphate (Tab. 4) and MES buffer (Tab. 5) reflect different activity course in different buffers. A higher activity of HIV-1 PR in the presence of K^+ compared to other alkali ions corresponds to the higher catalytic efficiencies (k_{cat}/K_M) for both salt concentrations measured at phosphate buffer pH 6.0. Increasing salt concentration leads to the drop in the K_M value for all analyzed ions with the exception of lithium, where, somewhat surprisingly, the increased concentration leads actually to small increase, rather than decrease of the K_M value. The dramatic decrease in the K_M values (i. e. more effective substrate binding) for 10 mM salt concentration for all tested ions in comparison to 300 mM salt concentration in the MES buffer suggests that the unexpected activation of HIV PR in this buffer in the range of very low salt concentrations is caused by improved substrate binding rather than catalytic efficiency. At present we have no specific explanation for this surprising observation. We speculate that in the low salt concentration, a semi-stable complex between the substrate and MES anion is formed that binds more effectively to the HIV PR binding site than the substrate alone. The attempt to analyze this hypothesis on structural level will continue.

Table 4. Kinetic parameters of the cleavage of peptide substrate KARVNle*F(NO_2)EANle-NH_2 by HIV-1 PR in the phosphate buffer pH 6.0 under various concentrations of alkali ions (*137*).

Salt	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
LiCl 50 mM	170 ± 20	13 ± 1	79 ± 13
NaCl 50 mM	120 ± 20	10 ± 1	89 ± 14
KCl 50 mM	110 ± 20	16 ± 1	150 ± 30
CsCl 50 mM	160 ± 20	12 ± 1	73 ± 10
LiCl 300 mM	190 ± 20	17 ± 1	88 ± 14
NaCl 300 mM	86 ± 10	11 ± 1	130 ± 20
KCl 300 mM	41 ± 2	17 ± 1	400 ± 30
CsCl 300 mM	60 ± 3	12 ± 0.4	200 ± 10

Table 5. Kinetic parameters of the cleavage of peptide substrate KARVNle*F(NO_2)EANle-NH_2 by HIV-1 PR in MES buffer pH 6.0 under various concentrations of alkali ions (*137*).

Salt	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
LiCl 10 mM	2.5 ± 0.2	3.9 ± 0.1	1600 ± 200
NaCl 10 mM	10 ± 1	2.4 ± 0.1	240 ± 20
KCl 10 mM	19 ± 3	4.9 ± 0.7	260 ± 50
CsCl 10 mM	47 ± 6	6.1 ± 0.3	130 ± 20
LiCl 300 mM	180 ± 20	20 ± 2	110 ± 20
NaCl 300 mM	150 ± 10	16 ± 2	100 ± 20
KCl 300 mM	57 ± 5	14 ± 1	240 ± 30
CsCl 300 mM	67 ± 6	16 ± 1	240 ± 30

Author´s contribution to Paper V:

I prepared recombinant wild-type HIV-1 protease used in this study, performed all the kinetic analyses discussed in the paper and drafted parts of the manuscript.

3. Discussion and Conclusions

In this PhD thesis, HIV protease inhibitors that are among most effective anti-HIV drugs, are reviewed. The HIV protease plays indispensable role in the late phase of viral cycle. Its inactivation by genetic mutation or chemical inhibition leads to the formation of noninfectious virions, thus slowing or even stopping the spread of HIV. Many different classes of HIV-1 protease inhibitors have been developed, some of them showing excellent antiviral profiles, since the first protease antagonist was identified in 1987. At present, there are nine HIV PIs at clinical practice approved by the FDA as antiviral drugs. They are usually taken in drug combination regimens to achieve the highest possible benefit, tolerability, and adherence and to diminish the risk of the development of drug-resistant HIV strains. Individual classes of PIs, their development, pharmacokinetic properties, most common side-effects, off-target activities, and resistance profiles are covered in Paper I.

While screening a number of unconventional structural motifs for their ability to inhibit HIV protease, we identified icosahedral boranes and carboranes as potent and selective inhibitors of this enzyme. The compounds were characterized enzymologically by spectrophotometric assay. The crystal structure of parental compound complexed to HIV PR revealed two cobaltacarborane cages bound into the HIV-1 PR active-site cleft. It was the first crystal structure of a carborane-protein complex ever solved. To increase binding affinity to the viral protease we connected the two parental cages with a hydrophilic linker enabling substitution of the central part of the molecule. Indeed, resulting series of double cluster compounds showed IC_{50} values in the submicromolar range and for most potent competitive inhibitors low nanomolar inhibition constants were determined. The X-ray structure of wild-type HIV-1 PR in complex with inhibitor containing two metallacarborane clusters was analyzed and refined to 1.7 Å resolution. Because there was no continuous experimental electron density for the disordered linker, its possible positions were modeled with help of computational procedures. Several tested compounds proved their ability to effectively inhibit a panel of multi-resistant HIV PR variants.

It remains to be seen to what extent these findings open the way to novel treatment

options for AIDS and pharmacologic use of carborane inhibitors in human medicine. Although the compounds are specific and potent inhibitors of viral enzyme, there are still numerous obstacles. First one is the activity of tested carborane compounds in tissue cultures. The self-assembly of cobaltacarboranes in aqueous solutions and formation of the nanoparticles with the radius changing with concentration, ionic strength, and aging of solution were observed by light scattering and microscopy techniques (*198*). Quantum mechanic calculations suggest that the attraction of metallacarborane anions in the implicit solvent model is relatively weak, only slightly stronger than contribution of electrostatic repulsion (*246*). This aggregation behavior in aqueous solutions could be explained by amphiphilic character of cobaltacarboranes. Since the overall negative charge of cobalt bis(dicarbollides) is delocalized, these compounds exhibit superacidity and their salts are fully dissociated in water. It has been shown that the amphiphilicity affects the surface activity and it is well-established fact that some of surface-active compounds can form multimolecular aggregates and micelles in aqueous solutions (*247-251*). These interesting phenomena in aquaeous solutions, however, cause irreproducible and time-dependent behaviour of cosanes in biological tests.

Second obstacles on the way "from bench to bed" is pharmacokinetics and bioavailability of tested compounds. There are almost no data in the literature on the bioavailability of metallacarboranes, however encouraging unpublished data of Prof. Hajdúch from the Palacký University of Olomouc suggest oral availability of several of tested cosanes and their slow clearance from the plasma of tested mice (Hajdúch, M., Grüner, B. et al., unpublished observation).

The third and probably the most critical obstacle is economic consideration. There are 26 different anti-HIV agents on the market already. Another antiviral compound would have not only to meet very stringent FDA criteria of drug safety and efficacy, but also perform better than most of the compounds already on the market. Furthermore, the financial crisis and ongoing health care reforms generate pressure to reduce cost of drug development skyrocketing to 1 bilion per compound. It results in decreasing number of new drugs introduced each year on the market.

It is difficult to speculate whether or not cosanes will become new anti HIV drugs. What is, however, already clear is that this class of compounds enlarged the "chemical space" and represents new type of pharmacophore that should be taken into account in rational drug design as well as in combinatorial approaches for novel therapeutic targets, even

outside HIV field. Recently, sulfamide derivatives of carborane and metallacarborane clusters were shown to inhibit enzymatic activity of several human carbonic anhydrase (CA) isoenzymes (Brynda, J., Cigler, P., Grüner, B., unpublished results). These metalloenzymes catalyse conversion between carbon dioxide and the bicarbonate ion and play key roles in intracellular and extracellular pH homeostasis, in the transport of CO_2 and bicarbonate in respiration in both various physiological and pathological processes. The transmembrane CA isoenzyme CAIX is associated with cancer and tumor progression (*252*). Boron cluster derivatives were successfully used to replace "ring" moiety in classical CAs inhibitors and to inhibit isoforms CAII and CAIX with IC_{50} values in low micromolar and submicromolar range, some of the inhibitors being more than 50-times more selective toward the tumor specific CAIX than for CAII present in normal tissue. Furthermore, it was demonstrated that the specificity of compounds toward certain CA isoenzyme can be modulated by the nature of the cluster and/or the *exo*-skeletal cluster substitution.

In further part of the thesis, we analyzed the viral resistance development towards new type of antiviral drug, bevirimat. It is the most advanced compound of the novel experimental class of maturation inhibitors. It hampers the ultimate rate-limiting cleavage step during processing of the Gag polyprotein by targeting the structural protein itself. Furthermore, it was reported that bevirimat has a stabilizing effect on the immature Gag lattice. Our resistance selection studies show that the protease background has impact on the resistance profile for the bevirimat, which becomes more diverse for viruses with a mutated protease compared to viruses with a wild-type protease. The major mutations were identified and analyzed: Gag V362I, A364V, S368N, and V370A. Our results indicate that change of the processing of CA/p2 cleavage site by mutated HIV proteases as compared to the wild type HIV PR can explain the protease-dependent bevirimat resistance and replication level.

It was previously reported that bevirimat is effective only in 50 percent of patients not having a specific group of mutations in the glutamine-valine-threonine (QVT) motif found in the C-terminal Gag region. Novel mechanism of resistance development, suggested by our study, represents another obstacle on the way to clinical use of this compound. Nevertheless, the knowledge gained during the work on this inhibitor could provide help in the development of new maturation inhibitors, ideally exhibiting synergy with protease inhibitors.

Finally, ion specific effects of alkali cations on the HIV-1 protease have been studied by means of molecular dynamic simulations and enzyme kinetics. Computational analysis showed higher affinity of sodium and lithium over potassium and caesium to the HIV-1 protease surface, mainly due to stronger interactions with carboxylate side chain groups of aspartates and glutamates. Experimental studies demonstrated that the proteolytic activity of the HIV-1 protease in principle follows the Hofmeister series, with the exception of caesium. More effective substrate binding (K_M) and turnover number (k_{cat}) are observed in the presence of K^+ compared to Na^+ or Li^+ at corresponding salt concentrations. Furthermore, unexpected decrease in the K_M value was observed for a specific substrate and buffer at very low salt concentration.

This surprising observation shows how little we still understand about the regulation of proteolysis in complex biological systems and how much there is still left to analyze and study. The crucial problem of the activation of PR in the context of Gag-Pol polyprotein might be addressed by biological chemistry approaches, such as photoinactivable inhibitor of HIV PR. This photolabile inhibitor would serve as a possible "switch-on" for the protease and would enable to study the hypothesized autocatalytic cleavage of PR from the Gag-Pol polyprotein as well as its irreplaceable role in maturation process of the virus. The inhibitor, cleaved by a quantum of light, would release a fully functioning unaltered protease at the desired moment, for example at different stages of virus budding. The infectivity as well as structure of the virus itself would indirectly show whether a host protease is needed for the cleavage of the polyprotein and also whether the viral particle is capable of full maturation after PR activation even after the budding of immature viral particle (Schimer, J., unpublished data).

To summarize, in the first part of this PhD thesis, the current literature on HIV protease inhibitors was reviewed. Next, metallacarboranes were identified as potent and specific HIV-1 protease inhibitors. Further, our results highlighted the complex interaction between the HIV-1 protease and its substrate Gag that affects the development of HIV-1 resistance to the maturation inhibitor bevirimat. These findings can help to develop the next generation of protease and maturation inhibitors. Finally, specific effects of alkali cations on the catalytic activity of HIV-1 protease were studied.

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Appendix: Reprints of the publications included in the thesis

My contribution to the publications included in the thesis

Paper I: Pokorná et al., *Viruses* 2009, 1, 1209-1239

I performed the research of the literature, provided the first draft of the manuscript, prepared several figures and edited the manuscript under the guidance of my supervisor.

Paper II: Řezáčová et al., *J Med Chem* 2009, 52 (22), 7132-41

Together with my colleague Milan Kožíšek we identified metallacarboranes as the inhibitors of wild-type HIV-1 protease through random testing of various organic and inorganic compounds. I have determined IC_{50} values and the type of mechanism of inhibition for the series of compounds *in vitro* by spectrophotometric assay. For competitive inhibitors I determined the K_i values.

Paper III: Fun et al., *Retrovirology* 2011, 8:70

I prepared two recombinant HIV-1 proteases used in this study and tested their enzymatic activities with three different substrates using HPLC. I also drafted the methodological part of the manuscript.

Paper IV: Heyda, Pokorná et al., *Phys Chem Chem Phys* 2009, 11(35), 7599-604

I prepared recombinant wild-type HIV-1 protease used in this study and performed all the kinetic analyses described in the paper. I also drafted parts of the manuscript.

Paper V: Pokorná, Heyda et al., *Faraday Disc* 2013, 160 (1), 359 – 370

I prepared recombinant wild-type HIV-1 protease used in this study, performed all the kinetic analyses discussed in the paper and drafted parts of the manuscript.

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Jana Pokorná

I hereby confirm

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Jan Konvalinka, Supervisor

Declaration

Prohlašuji, že jsem tuto práci, ani její podstatnou část, nepředložila k získání stejného ani jiného akademického titulu.

Jana Pokorná

V Praze dne 25.1.2013