Towards a New Generation of Anti-HIV Drugs

Interaction Kinetic Analysis of Enzyme Inhibitors Using SPR-biosensors

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Abstract
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As of today, there are 25 drugs approved for the treatment of HIV and AIDS. Nevertheless,
HIV continues to infect and kill millions of people every year. Despite intensive research efforts,
both a vaccine and a cure remain elusive and the long term efficacy of existing drugs is limited by
the development of resistant HIV strains. New drugs and preventive strategies that are effective
against resistant virus are therefore still needed. In this thesis an enzymological approach,
primarily using SPR-based interaction kinetic analysis, has been used for identification and
characterization of compounds of potential use in next generation anti-HIV drugs.

By screening of a targeted non-nucleoside reverse transcriptase inhibitor (NNRTI) library,
one novel and highly potent NNRTI was identified. The inhibitor was selected with respect
to resilience to drug resistance and for high affinity and slow dissociation – a kinetic profile
assumed to be suitable for inhibitors used in topical microbicides. In order to confirm the
hypothesis that such a kinetic profile would result in an effective preventive agent with long-
lasting effect, the correlation between antiviral effect and kinetic profile was investigated
for a panel of NNRTIs. The kinetic profiles revealed that NNRTI efficacy is dependent on
slow dissociation from the target, although the induced fit interaction mechanism prevented
quantification of the rate constants.

To avoid cross-resistance, the next generation anti-HIV drugs should be based on chemical
to entities that do not resemble drugs in clinical use, either in structure or mode-of-action.
Fragment-based drug discovery was used for identification of structurally new inhibitors of
HIV-enzymes. One fragment that was effective also on variants of HIV RT with resistance
mutations was identified. The study revealed the possibility of identifying structurally novel
NNRTIs as well as fragments interacting with other sites of the protein.

The two compounds identified in this thesis represent potential starting points for a new
generation of NNRTIs. The applied methodologies also show how interaction kinetic analysis
can be used as an effective and versatile tool throughout the lead discovery process, especially
when integrated with functional enzymological assays.

Keywords: drug discovery, fragment, screening, reverse transcriptase, microbicides,
interaction analysis, NNRTIs

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This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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Abbreviations

ADME Absorption, distribution, metabolism and excretion
AIDS Acquired immunodeficiency syndrome
CA Carbonic anhydrase
CRF Circulating recombinant form
CRI Co-receptor inhibitor
dNTP Deoxynucleotide triphosphate
DMSO Dimethyl sulfoxide
EDC 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
FBDD Fragment based drug discovery
FI Fusion inhibitor
HIV-1 Human immunodeficiency virus type 1
HIV-2 Human immunodeficiency virus type 2
HSA Human serum albumin
HTS High throughput screening
IN Integrase
INI Integrase inhibitor
NMR Nuclear magnetic resonance
NNRTI Non-nucleoside reverse transcriptase inhibitor
NNIBP Non-nucleoside inhibitor binding pocket
NRTI Nucleoside reverse transcriptase inhibitor
PD Pharmacodynamics
PI Protease inhibitor
PK Pharmacokinetics
PR Protease
RT Reverse transcriptase
SIV Simian immunodeficiency virus
STD Sexually transmitted disease
SPR Surface plasmon resonance
TDF Tenofovir disoproxil fumarate
To Micke, Alfred and Klara

The more you drink from the goblet of knowledge, the thirstier you get.
A grand scientific challenge

The work presented in this thesis shows how analysis of interaction kinetics can be used as an effective tool for drug discovery. The methodologies are applied specifically to the development of drugs and preventive methods against HIV. New experimental strategies for how interaction kinetic information from SPR-based biosensor assays can be used for identification, characterization and evaluation of potential drug molecules are presented. Effective inhibitors of HIV-reverse transcriptase with resilience to resistance mutations have been identified.

In 1983, the human immunodeficiency virus (HIV) was identified as the causative agent of acquired immunodeficiency syndrome (AIDS) [1]. Thanks to the development of anti-HIV drugs, HIV infections have changed from being a fatal condition, into a chronic infection that – if correctly treated – rarely is lethal. However, the existing treatments remain a privilege for the few, and development of resistance towards anti-HIV drugs in clinical use is a growing problem. Moreover, despite more than 25 years of intensive research, no vaccine or cure is in sight [2]. HIV therefore continues to spread, and the latest statistics reported by UNAIDS offers somber reading [3]; 33 million people are estimated to be living with HIV, and every year another 2 more million people get infected and 1.8 million people die from AIDS. The price paid for this in terms of medical and social costs and human suffering is enormous.

To change the situation, new effective preventive measures are needed, along with a new generation of more potent anti-HIV drugs. However, it is a difficult task to develop new drugs. It takes on average about 12–15 years and costs around US$ 1 billion to develop one new drug [4, 5]. One reason for these high costs is that many drug candidates fail late in the drug development process. By improving the methods at the early stages of the process, and thereby reducing attrition rates in later stages, the process could become more effective and the overall cost could be reduced. The development of drugs or preventive agents against HIV, however, faces also other challenges.

From a medical and pharmaceutical perspective, HIV is an unusually difficult opponent. It belongs to the family of retroviruses, and as such incorporates its own genetic material into the genome of an infected host cell. In this
way, the virus can hide from the immune system and engage in a relentless war of attrition with the immune cells. In addition to its capacity to hide, HIV also has a high proliferation rate and an extraordinarily high inherent mutation rate. Thus the virus is capable of quickly adapting to new conditions, for example by becoming resistant to drugs. Resistant HIV strains against all of the currently available anti-HIV drugs already exist. A new generation of anti-HIV drugs must therefore be effective not only against the wild-type virus, but also against resistant virus strains and preferably have high resilience to new mutations. This adds to the difficulties of drug development against HIV.

Treatment and prevention of HIV is also complicated by social factors. Expensive multi-drug regimens that must be kept cold and that include several pills a day, at specific hours, may represent an effective treatment for wealthy patients with a structured life. However, for a drug-addict living on the street and for a poor woman living in sub-Saharan Africa without a clock and a fridge, this kind of treatment is not an option. These kinds of social limitations must be considered when new drugs and preventive methods are developed. Moreover, the issue of prevention and accessibility to treatment is marred by prejudice and stigma associated to HIV [3, 6]. For this reason, a medical or technical answer will only ever make up part of the solution.

Drug research may take somewhat different directions when social aspects are considered early in the development of a drug or preventive measure. [Topical] microbicides for women represents one example where a social need has been recognized, and a medical solution has been identified and now is under development [7]. Microbicides are self-administered prophylactic agents that are intended for use by women as “chemical condoms” to prevent HIV infection via sexual intercourse. Women now account for a majority of all new infections globally [3]. In sub-Saharan Africa where the HIV epidemic is most serious, young women aged 15–24 years are eight times more likely than men to be HIV positive. This is a result of their higher biological vulnerability to infection during sexual intercourse, in combination with economic dependence, gender based violence and other socioeconomic and cultural disadvantages [3]. Approval from the partner is not required for using microbicides, in contrast to when condoms are used.

The fight against HIV is thus a grand scientific challenge where knowledge and experiences stemming from many disciplines are needed. Novel methods implemented in early drug discovery could lead to a new and more potent generation of microbicides and anti-HIV drugs. This thesis focuses on these areas with the added benefit that the methodologies developed also may help reduce the cost for drug development by making it more targeted and reduce the attrition rates in later stages of the drug development process.
The human immunodeficiency virus

“Know your enemy and know yourself.”

This is a piece of advice from the famous Chinese military strategist Sun Tzu, living in the 6th century BC. It was certainly not referring to wars against pathogens, nevertheless, it hits the core of medicine and drug discovery; the better you know the pathogen causing a disease, and the better you know the system it is interfering with, the higher are your chances of finding a way to defeat it. A brief introduction to HIV along with current strategies for prevention of HIV transmission and anti-HIV drugs are therefore presented in the following background section.

HIV-1 and HIV-2

Today two types of HIV, type 1 (HIV-1) and type 2 (HIV-2) are known. Both originate from non-human primates in Central and West Africa, and were transmitted to humans on several occasions [8]. HIV-1 was discovered in 1983 by Françoise Barré-Sinoussi and Luc Montagnier [1], who received the Nobel prize for their work in 2008. This was only a few years after the first AIDS cases had been recognized and reported in the USA [9]. HIV-1 is responsible for the global epidemic, and is the form normally referred to (including in this thesis), unless otherwise specified. HIV-2 also causes AIDS, but has a longer latency phase and lower morbidity [10]. Due to its lower infectivity it is not as widely spread.

HIV is a spherical, enveloped and complex retrovirus with a diameter of about 100 nm (Figure 1A). It has a single stranded positive RNA genome (~9 kb) (Figure 1B), present in 2 copies inside the viral capsid. The genome encodes three polyproteins common to all retroviruses (Gag, Gag-Pol, Env), and six additional proteins. The polyproteins are cleaved into nine proteins distinct proteins, Figure 1C, why the genome in total encodes 15 proteins. Three of these are essential enzymes: an integrase, a polymerase (reverse transcriptase, RT) and a protease [10].
Figure 1. A) The spherical and enveloped HIV virion with the glycoproteins protruding through the membrane and the RNA genome and viral enzymes inside the capsid. B) The HIV genome with the three large genes (Gag, Pol, Env) and the six genes encoding for vif, tat, vpr, rev, nef and vpu. LTR stands for long terminal repeat and is the control center for gene expression. C) The three polyproteins (Gag, Gag-Pol and Env) are translated into nine distinct proteins.

Genetic variability and its implications

The genetic variation of HIV is very large. Presently HIV is divided into four groups (M, N, O and P). This classification is based on phylogenetic analyses, where group M (for major) is responsible for the global epidemic. Group M contains at least nine subtypes, or clades, and over 20 circulating recombinant forms (CRFs) [11]. Subtype A, B and C account for more than 70% of all infections. Clade C is alone responsible for almost half of all infections and is mainly present in Africa and Asia. Clade A is also present in Africa, whereas subtype B is the dominating subtype in North America and Europe [12].

The great variability among the HIV isolates, both within and between infected individuals, has several causes. It is a result of the exceptionally error-prone reverse transcription performed by the viral RT [13], a high replication rate [14] and an inherent ability to recombine [15]. The natural variability and enormous evolutionary potential in response to e.g. medication has large implications for both drug design and regimen management. The most obvious problem is the fast development of drug resistance, making existing drugs useless unless applied in combination therapies. But the design of new drugs is further complicated by the many target variants. Although most current anti-HIV drugs have been designed against subtype B, they seem to be effective also against other subtypes [16]. The larger genetic differences between HIV type 1 and type 2 make some of the drugs used to treat HIV-1 infection ineffective against HIV-2.
Life cycle and tropism

A good understanding of the HIV life cycle is key to identifying weaknesses that can be exploited by new drugs. HIV infects human immune cells, predominantly CD4+ T-lymphocytes, macrophages and dendritic cells [17]. The type of cells the virus infects (tissue tropism) is determined by the presence of specific surface host cell receptors. Interactions between complexes of glycoproteins (gp120 and gp41) attached to the lipid capsule of the virion, and host cell receptors (CD4 and a co-receptor such as CCR5 or CXCR4) is critical to viral infectivity (Figure 2, step A).

![Figure 2. Schematic illustration over the HIV life cycle: A) attachment, B) fusion, C) reverse transcription, D) nuclear import, E) integration, F) export and translation G) virion assembly and budding, H) virion maturation.](image)

After attaching to the host cell, the viral membrane fuses with the cell membrane, allowing entry of the viral capsid into the cytoplasm (step B). The capsid is subsequently uncoated. Exactly how, where, and when this occurs remains uncertain, but it is known to be a critical process for infectivity and is hence a possible mechanism to target [18]. The viral RNA is reverse transcribed by RT into duplex DNA, either in the cytosol or inside the capsid (step C) [18]. A pre-integration complex, containing the viral DNA, the viral integrase (IN), and some other viral and cellular proteins [19], is formed once reverse transcription is terminated. This large nucleoprotein complex is actively imported to the nucleus via nuclear pore complexes (step D) [20], giving HIV-1 its unique ability to replicate efficiently also in non-dividing cells, such as macrophages [18].
Once inside the nucleus, the viral cDNA is integrated into a host cell chromosome by IN (step E) [21]. At this stage it is called a provirus, and represents the form in which HIV can stay latent and effectively evade immune surveillance for extended periods (up to a decade or longer) [22]. This slow course of infection is the reason why HIV belongs to a group of retroviruses called Lentivirus, *lenti*- meaning slow in Latin.

Like all viruses, HIV hijacks parts of the host cell machinery to propagate. Both cellular and viral proteins are indispensible for this complex, yet not fully understood, infection process. After integration of the provirus into the host genome, transcription of viral mRNAs by cellular DNA polymerases is initiated, and the mRNAs are exported out of the nucleus (step F). Some of the mRNAs are translated into polyproteins (Gag, Gag-Pol and Env) whereas other remain as intact copies of the complete viral RNA genome. The products are packaged into nascent virions that assemble close to the cell membrane (step G). The virions that bud off are immature and noninfectious. A maturation process, in which the viral protease (PR) cleaves the polyproteins and a structural rearrangement of the particle takes place, is needed for the virion to become infectious (step H).

**AIDS – when the immune system fails**

If left untreated, an HIV infection eventually results in AIDS; a condition where a severely compromised immune defense cannot hold back opportunistic diseases, often caused by bacteria, fungi, parasites or other viruses. The first symptoms of an HIV infection usually resemble an ordinary flu, but during this phase the number of viral particles (viral load) in the blood is very high. This period is followed by a long asymptomatic period known as clinical latency. During this latency phase of persistent infection (lasting from months up to many years), the viral loads are kept relatively low. However, during this time, HIV silently exhausts the immune system by killing its cells, mainly CD4+ T lymphocytes. When the number of CD4 T cells becomes very low (~200 cells/µL blood), most of the serious opportunistic infections and cancers associated with AIDS can develop [23]. Treatment with anti-HIV drugs is recommended by WHO to be initiated once a CD4 T cell count falls below 350 cells/µL [24].
HIV transmission routes

HIV is transmitted through three major routes.

Sexual transmission

Sexual transmission accounts for more than 80% of all new HIV infections worldwide. According to UNAIDS, it remains the most difficult mode of transmission to address [3]. Both in homo- and heterosexual intercourse, the semen recipient partner is the one at highest risk of becoming infected. Occurrence of other sexually transmitted diseases (STDs) significantly increases the infection risk [25]. In the case of genital ulcers this is due to a loss of integrity of the genital mucosa and because target cells for infection (immune cells) are present at higher concentrations in the epithelial cells during inflammation [26].

Transmission via blood

Needle sharing among intravenous drug users is today the most common way in which blood contact results in transmission of HIV-1, accounting for an estimated 250 000 cases per year [27]. However, medical settings around the world still account for a significant number of new HIV infections either via blood transfusions, medical injections, medical waste and occupational exposures [28].

Mother-to-child transmission

Transmission of HIV-1 from a mother to her child (vertical transmission) occurs either in the uterus via the placenta or, which is more frequent, at the time of delivery, as a result of contact with a contaminated genital tract. Breastfeeding is a third source of mother-to-child transmission, since the milk can contain infected cells [29].

Preventing HIV transmission

There are protective strategies for each route of HIV transmission. The currently available methods range from physical HIV barriers to the use of anti-HIV drugs and social preventive efforts, such as education, safer-sex practices and needle-sharing programs. While these approaches have been relatively effective [3], more powerful and complementary methods are needed to further reduce transmission rates. A vaccine that has the ability to induce immunity against HIV is expected to be a very important tool, since vaccines have previously proven to be highly effective in the prevention of a variety
of serious diseases [2]. An HIV vaccine, however, remains elusive. Some HIV vaccines have been tested in large scale clinical trials [30-32], but most have been failures. In late 2009, the first report of a vaccine that protected from HIV acquisition was presented [33], but the trial reported a modest 31% efficacy and the protective effect was not long-lasting.

Topical microbicides
A preventive method that has recently showed proof-of-concept in a clinical trial [34], and hence is much hoped for, is the use of topical microbicides. A [topical] microbicide against HIV is a pharmaceutical agent that is applied to the vagina or rectum in order to prevent HIV from being passed on during intercourse [35]. It can be formulated in many ways such as in a gel, foam, suppository, or in a vaginal ring that slowly releases the active ingredient [36].

First and second generation microbicides
Research on HIV microbicides started almost 20 years ago. The first generations of microbicides were based on one of three non-specific mechanisms of actions [37]:

1) Inactivation of the virus by disrupting the viral envelope or lipid membrane with surfactants or detergents.
2) Creation of a physical barrier by polyanions that prevents the virus from binding to the target cells in the genital mucosa.
3) Strengthening of the body’s normal defense. The pH in the vagina is normally acidic (~ pH 4), making it a rather hostile environment for pathogens, such as fungi and viruses. However, semen is basic and causes the pH in the vagina to increase during sexual intercourse, making the vaginal environment less protective. By applying a microbicide that functions as a buffer, the lower and more protective natural pH of the vagina can be maintained.

Several microbicides with these non-specific modes of action have completed phase III clinical trials, but all have failed due to adverse effects [38], no statistically significant effects or trial-related problems [38, 39].

Third generation microbicides
New approaches, using specific anti-HIV agents (also referred to as antiretroviral agents) as the active ingredient in microbicides, are now tested. Microbicides based on antiretroviral agents have the advantage of being highly specific and effective. Moreover, they are not contraceptive (which is important to encourage regular use in many parts of the world) and can be delivered through vaginal rings or other sustained release methods. Potential
limitations with this type of microbicide are reduced protection against other STDs and uncertainties related to toxicity and resistance development. In 2010, a phase II clinical trial in South Africa for a microbicide gel containing the antiretroviral drug tenofovir, showed a reduced HIV acquisition by an estimated 39% overall, and for women with high gel adherence 54% [34]. This is the first proof-of-concept showing that microbicides can work.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are a type of anti-HIV drugs with properties well suited for use in microbicides. They are effective, highly specific and don’t need to be metabolically activated before achieving antiviral activity. They can exert their activity directly against both cell-free and cell-associated HIV-1 and tight binding NNRTIs [25]. Paper I and paper II in this thesis concerns the identification and kinetic characterization of NNRTIs for use in microbicides.

As described above, the great diversity of HIV strains is an obstacle for all preventive methods, including microbicides. Microbicides must confer protection against a broad panel of HIV variants circulating in the population. Thus, similar to other therapies that involve multiple drugs, formulations of microbicides containing 2–3 anti-HIV agents acting through different mechanisms and with different resistance profiles have been presented as a potential solution [35].

Anti-HIV drugs

Since the approval of the first anti-HIV drugs in 1987 [40], biomedical research has resulted in a diverse battery of drugs against HIV.

Classes of currently approved drugs

There are presently 25 drugs approved for use against HIV infection and AIDS. They can be divided into six different classes based on their mode of action [41].

1. Nucleoside reverse transcriptase inhibitors (NRTIs)

NRTIs was the first class of drugs that came into clinical use against HIV in 1987 [40]. They are substrate analogues that act as chain terminators and thereby block reverse transcription performed by HIV RT. NRTIs are administered as pro-drugs that need to be phosphorylated by cellular kinases into their triphosphate form before becoming active. They are unspecific and therefore cause severe side effects. Resistance develops rapidly against all available NRTIs if they are used as monotherapy. Currently, there are seven approved NRTIs: zidovudine (AZT), didanosine, zalcitabine, stavudine,
lamivudine, abacavir, emcitrabine. The structure of the first approved NRTI, zidovudine (AZT), is shown in Figure 3. There is also a related drug, tenofovir, that belongs to the class nucleotide reverse transcriptase inhibitors (NtRTIs) [41]. Tenofovir is an acyclic analogue of adenosine monophosphate. It only requires two intracellular phosphorylations to become active since it already has a phosphonate group attached to it, Figure 3. However, due to poor bioavailability, tenofovir now exists as the ester prodrug tenofovir disoproxil fumarate (TDF).

![Zidovudine (NRTI) and Tenofovir (NtRTI)](image)

**Figure 3.** The structures of the first approved NRTI, zidovudine (AZT), and the first approved NtRTI, tenofovir.

### 2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTIs also target the polymerase activity of HIV RT, but through allosteric inhibition. They are highly specific and are therefore better tolerated than NRTIs, causing fewer adverse side effects. Rapid resistance development is a problem also with NRTIs and they are not functional towards HIV-2. There are currently four licensed NNRTIs: nevirapine, delavirdine, efavirenz and etravirine (Figure 4). One more (rilpivirine) is expected to be approved soon [41].

![Nevirapine, Efavirenz, Delavirdine, Etravirine](image)

**Figure 4.** The structures of the four clinically approved NNRTIs.

### 3. Protease inhibitors (PIs)

PIs inhibit the function of HIV protease, preventing the virus from replicating and making new infective virions. The drugs mimic a peptide substrate in its transition state and were discovered through rational, structure-based drug design [42]. Once bound to the active site, the inhibitor cannot be cleaved and thereby blocks further catalytic activity. Some PIs inhibit both
HIV-1 and HIV-2. This is the largest group of inhibitors with ten licensed drugs: saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, atazanavir, lopinavir, fosamprenavir, tipranavir, and darunavir [41]. The structure of indinavir is shown as an example in figure 5.

![Indinavir](image)

**Figure 5.** The structure of the early protease inhibitor indinavir.

### 4. Integrase inhibitor (INI)

The first, and so far only, approved anti-HIV drug that targets IN is raltegravir, approved for clinical use in 2007 [43], Figure 6. Viral IN has two catalytic functions used in the process of integrating the transcribed viral DNA into the host genome: processing of the 3’ ends and a strand transfer reaction, i.e. joining of the viral and cellular DNA. Raltegravir is a specific inhibitor of the rate-limiting strand transfer reaction [44]. IN has no counterpart in humans, reducing the risk for side effects.

![Raltegravir](image)

**Figure 6.** The structure of the integrase inhibitor raltegravir

### 5. Co-receptor inhibitor (CRI)

Maraviroc is a CCR5 antagonist that prevents binding of the HIV virion to the host cell. It was approved in 2007 and is the first drug of its kind, see Figure 7. Maraviroc selectively interferes with the binding of the viral membrane glycoprotein gp120 to the co-receptor CCR5 [45]. It is hence only effective against CCR5-tropic HIV strains.
Figure 7. The structure of the co-receptor inhibitor maraviroc.

6. Fusion inhibitor (FI)
Enfuvirtide is a synthetic 36-amino acid peptide that binds to the viral envelope glycoprotein 41 [46]. It selectively hinders the anchoring and subsequent fusion of HIV with the host cell membrane. Due to its large size and chemical properties, it has very poor oral bioavailability and must be injected subcutaneously twice daily. It is therefore primarily used in salvation therapy, i.e. when all other therapies have failed.

Concerns related to anti-HIV drug treatments

Life-long and difficult treatments
None of the existing anti-HIV drugs can cure an HIV infection. Treatments are life-long and aim at prolonging the asymptomatic latency phase, during which viral loads are kept at a low and steady level. The pressure on the immune system is thereby kept low and the onset of AIDS can be postponed or prevented. This kind of long-term treatments put very high demands on drug safety. The unspecific action of many existing anti-HIV drugs requires high dosages, which give rise to severe side effects and can after sustained use can result in organ failure.

Drug-drug interactions, simultaneous treatment of other AIDS related diseases, the need for adherence to the treatment protocols, and development of drug resistance are other complicating aspects of anti-HIV treatments.

Resistance development
When only one antiretroviral drug (monotherapy) is used in HIV treatment, drug resistance develops rapidly against all classes of drugs [47]. The resistance mutations that occur differ with respect to the specific drug and class, but unfortunately cross-resistance mutations (when one mutation renders resistance to several drugs) are frequent. To stop resistance development viral replication must be completely blocked. In order to achieve this, a combination of drugs are needed [47]. Combination therapy has therefore been established as the common practice for HIV treatment. A combination
of three or more drugs from at least two different classes is used. This approach, known as highly active antiretroviral therapy (HAART), has proven very effective against drug resistance development and has the additional advantage of lower toxicity and synergistic effects [43].

A new generation of anti-HIV drugs

Problems with toxicity and resistance development make the search for new anti-HIV drugs a continuous process. New targets and completely new mechanisms for inhibition of viral propagation are being explored. The drug classes INI, Fl, and CRI are successful examples of drugs against new targets. Inhibition of the RNase H activity of HIV RT and blocking of the interaction between IN and the host cell factor lens epithelium-derived growth factor (LEDGF) important for DNA integration are alternative strategies [48, 49]. The maturation process, budding inhibitors, and inhibition of the uncoating process have also been suggested therapeutic strategies [18, 49]. Research investigating how the dimerization process of the viral enzymes can be interrupted by small molecules is ongoing [50-52]. As new details regarding the infection process, target cell specificity, and the immune response become available, new weak spots in the viral life cycle will certainly appear and present opportunities for new therapeutic interventions.

However, existing targets are still important in anti-HIV drug discovery. They have the advantage of being thoroughly validated and well characterized. A new generation of drugs that are more effective, has potency against existing resistance mutations, and has higher resilience to new mutations is therefore required. The articles in this thesis concern the identification and characterization of a new generation of HIV enzyme inhibitors.
Drug Discovery

On a molecular level, the human body is a tremendously complex system of biochemical reactions that interact in tightly regulated networks. Drug discovery is fundamentally the identification of a molecule that can alleviate a physical problem or a disease by interfering with one, or sometimes several, biochemical processes within these interconnected networks. Historically, drugs have been identified from natural substances such as herbs, roots, and berries, either through serendipitous findings or by trial and error [4]. In the wake of the great advances in biochemistry, genetics and medicine during the last century, a "rational approach" to drug discovery has emerged. This approach is a more or less standardized process, where chemical, structural, mechanistic, and kinetic information about the drug target and its interaction with potential drug molecules form the basis for drug design. In Figure 8, the drug discovery process has been depicted as a flowchart with four consecutive steps. The articles in this thesis concern the two middle steps, pre-clinical drug discovery.

The drug discovery process

Figure 8. A simplified outline of the drug discovery process.

Target identification and validation

The first step in the drug discovery process is to identify a suitable molecular target that is instrumental in a given disease. This is typically a receptor, an enzyme, or an ion channel [53]. Once identified, the target needs to be validated. This means that the role of the target in the disease has to be clearly understood. Experimental evidence must also show that interference with the
target results in the anticipated therapeutic response [5]. Failure to correctly validate a target can be extremely costly since it commonly takes several years before a drug can be tested in animals and humans.

Early pre-clinical studies
After target identification and validation, the next step is hit/lead identification and optimization. In this phase, molecules that interact with the target in the desired way (inhibition, activation etc) are identified. There are two main strategies:

Rational drug discovery is an approach for design of drug molecules that can be used for discovery of enzyme inhibitors. It makes use of available knowledge about the structure of the enzyme, its mechanism of action and its natural substrates. HIV-protease inhibitors are good examples of drugs that were rationally designed [42]. They are peptidomimetic transition state analogues that were designed on the basis of the catalytic mechanism, for which the transition state structure is known. The initial hit/leads were optimized using crystallography, NMR, computational studies, and advanced chemical synthesis.

Screening of large collections of chemical compounds (libraries) is another strategy that is common among pharmaceutical companies. It is used to identify initial molecules that interact with the drug target. This method is suitable for various types of targets and no structural data is needed initially. The optimization using structure-based methods is the same as for the “rational” approach. The compounds included in the libraries are usually a historic collection of synthetic drug-like compounds, but may also be retrieved from nature. More recently, smaller libraries consisting of fragments (MW<300 Da) have become popular. These are designed with respect to large coverage of chemical space, i.e. maximal diversity. (See below.)

In this early key step of the drug discovery process, the focus is on the interaction between the molecules with their target. Compounds are usually selected on the basis of chemical properties, such as affinity and selectivity. Often, a number of tractable substances (hits or leads), with attractive characteristics are identified. These “immature” drug molecules usually possess also undesirable properties such as low solubility, absorption difficulties, or metabolism related problems.

Late pre-clinical studies
The identified hit/lead molecules are subsequently subjected to a lengthy, iterative process of chemical optimizations in order to amplify the desired
activity and to eliminate undesired properties. The goal is to develop drug-molecules with high affinity and selectivity, along with good bioavailability and low toxicity. When a lead compound has been developed into a compound worthy of extensive biological and pharmacological studies, it can be referred to as a candidate drug (CD) [4]. The safety profile of the candidate drug is normally determined using pharmacodynamic (PD), pharmacokinetic (PK) and ADMET (absorption, distribution, metabolism, excretion and toxicity) testing in animals. The information gleaned from these studies are evaluated and used for safe clinical testing on humans.

Clinical studies
Clinical studies refer to the thorough evaluations of toxicity, adverse side effects, efficiency, and dosage that have to be conducted on large numbers of humans, before a drug can be approved. Clinical trials are divided into three major phases [49]:

**Phase I**, in which a small group of healthy volunteers are exposed to the drug to assess safety, PD, PK and tolerability (dosage levels and side effects).

**Phase II**, in which larger groups of patients are enrolled to verify efficacy, dosage and toxicity.

**Phase III**, in which randomized and controlled studies are performed on thousands of patients at several places simultaneously, to assess drug efficacy and adverse effects from long-term use. After successful phase III trials the drug may be approved for marketing by one of the regulatory agencies (e.g. U.S. Food and Drug Administration (FDA) or the European Medicines Agency (EMEA)).

The whole process from target validation to approved drug, takes on average 12–15 years, and costs in the region of US$ 0.8–1 billion [4, 5]. High attrition rates in late stages of the process contribute substantially to the high costs and relative inefficiency of the process. There is hence a lot to gain from reducing the failure rate in later stages of the process. Over the last decade, the number of new drugs emerging per year has decreased, despite continuously high investments [54]. Improvements that make the drug discovery process more targeted and resource efficient are needed [54, 55]. Detailed interaction kinetic analysis, as implemented in this thesis, may be useful for making better informed decisions at an early stage of the drug discovery process and increase the productivity by reducing attrition rates in later stages.
Enzymes as drug targets

Due to their pivotal roles in most biological systems and their dependence on a specific structure and chemistry for catalysis, enzymes often serve as excellent drug targets [56]. Almost half of all marketed drugs in 2002 targeted enzymes [53].

Targeting viral enzymes

Also viruses are critically dependent on the function of enzymes. Since they make use of host enzymes for many metabolic processes, viral-specific enzymes are typically few. This limits the number of possible enzyme targets, but on the other hand, makes the identification of viral drug targets relatively straightforward.

HIV is critically dependent on the function of three viral enzymes: a polymerase, a protease and an integrase. All but two of the 25 existing anti-HIV drugs target these enzymes. Three of the studies included in this thesis focus on NNRTI inhibitors of the polymerase HIV RT. The enzyme and the mechanisms of RT-inhibition are described in further biochemical detail below.

HIV reverse transcriptase

HIV RT (EC.2.7.7.49) is the primary target in the studies of this thesis. It is a flexible, multifunctional enzyme that catalyses a series of complex reactions necessary to transcribe single stranded viral RNA into double stranded DNA. The enzyme has two active sites, but three distinct enzymatic activities: RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity and RNase H activity.

Structure

The biologically relevant form of the enzyme is a stable heterodimer, composed of two related subunits (p66 and p51) [57], Figure 9. These are identical, except that the smaller p51 lacks the RNase H domain. Subunit p51 is generated through proteolytic cleavage of the C-terminal end of p66 by PR. The enzyme forms a classical polymerase structure [56] with four distinct subdomains referred to as the fingers, palm, thumb and connection, according to the nomenclature of Kohlstaedt et al. [58].
The large subunit hosts the polymerase as well as the RNase H active sites. The fingers, palm, and thumb subdomains form a nucleic-acid binding cleft in which the polymerase active site is located, Figure 9. Subunit p51 lacks catalytic activity and its role is to provide structural support and to enable for loading of the tRNA primer onto p66 [50, 59]. Despite having the same primary sequence, the polymerase domains in p51 and p66 differ markedly in spatial arrangement [58]. In the monomeric form, both subunits are catalytically inert and viruses that are defective in RNase H activity are non-infectious [57].

**Mechanism**

The process of transcribing a single stranded RNA genome into double stranded cDNA is a complex mechanism. New DNA strands are formed using both RNA and DNA templates, and the RNA strand of the RNA:DNA duplex is specifically degraded. Short segments of the RNA strand are however left intact by RNase H, to be used as primers. The enzyme must therefore coordinate its two different catalytic activities and discriminate between very similar substrates and templates [60].

HIV RT uses a mechanism known as an ordered bisubstrate reaction, in which the template-primer (T/P) complex forms the first substrate [56], Figure 10, step 1.
Figure 10. Schematic illustration of the DNA polymerization mechanism of HIV RT. Binding of the template-primer (T/P) complex to the enzyme (E) and a concomitant conformational change (step 1). Binding of a dNTP and a concomitant conformational change (step 2). Phosphoryl transfer (step 3). A conformational change (step 4). Pyrophosphate release (step 5). Enzyme dissociation (step 6) or continued synthesis (step 7). Modified from [47, 61].

The reaction begins when the T/P-complex bind to the enzyme active site, which results in a conformational change of the p66 thumb position (step 1) [47]. This is followed by a separate binding of the second substrate, the deoxynucleotide triphosphate (dNTP) (step 2). Upon dNTP-binding, the enzyme goes through another conformational change that positions the dNTP properly relative to the primer (step 2), which is necessary for phosphoryl transfer (step 3) [61]. This is followed by a new conformational change (step 4) that allows for formation of a phosphodiester bond, with concomitant release of a pyrophosphate (step 5) [47, 62, 63]. However, one round of catalysis is typically not ended by product release (step 6), since the extended T/P-complex (i.e. the product) remains bound in the active site, ready to react with a new incoming dNTP (step 7). The ability to continue strand elongation without dissociation from the T/P-complex defines the processivity of the polymerase [61].

Mechanisms of RT inhibition

The approved classes of HIV RT inhibitors exploit two different inhibition mechanisms.

Chain termination by NRTIs
NRTIs are structurally diverse analogues of naturally occurring dNTPs (Figure 11), lacking the 3’-hydroxyl group of the ribose sugar, compare Zidovudine in Figure 3. They are incorporated into the newly synthesized DNA
chain and inhibit the enzyme activity by blocking further chain elongation. The NRTI forms a “dead-end complex” with the enzyme, the T/P-complex and the next complementary dNTP [64].

![Figure 11. The dNTP adenosine triphosphate, a natural substrate.](image)

NRTIs have poor selectivity because they can be used also by cellular polymerases, although less readily than by HIV RT. This results in unwanted side effects and toxicity. Another problem is their susceptibility to resistance mutations. HIV becomes resistant to NRTIs by improving their capacity to discriminate between natural dNTP substrates and the NRTIs. Two basic mechanisms are known: NRTI exclusion and NRTI excision (pyrophosphorolysis) [47]. These mechanisms are in focus when new drugs are designed. The NtRTI TDF is for example less sensitive to excision by 3’→5’ exonucleases, due to incorporation via the more stable phosphonate group, Figure 3 [43].

**Allosteric inhibition by NNRTIs**

NNRTIs are chemically diverse, non-competitive inhibitors. The first generation NNRTIs (nevirapine and delavirdine) were discovered by random compound library screening, whereas subsequent generations of NNRTIs (efavirenz and etravirine) were developed as result of structure-based drug-design [65]. They all inhibit HIV-1 RT by binding to a deep hydrophobic pocket termed the non-nucleoside reverse transcriptase binding pocket (NNIBP). The NNIBP is unique to HIV-1 RT, conferring high selectivity with few toxicity problems. Unfortunately, NNRTIs are ineffective against HIV-2.

The NNIBP is located approximately 10 Å away from the active site in the palm of the p66 subunit. It is not visible in the crystal structure of the free enzyme because it is blocked by the side chains of Y181 and Y188 [65, 66]. A local conformational change that opens up the NNIBP is therefore required for binding of NNRTIs [65, 67]. The binding pocket has different conformations depending on the NNRTI that is bound [68]. This conformational elasticity has important consequences for drug design. Subsequent to NNRTI binding, a second conformational change (induced fit) is suggested to occur, closing the pocket [67].
Despite their chemical heterogeneity, NNRTIs are generally hydrophobic in character. This enables favorable interactions with the hydrophobic residues lining the binding pocket [68]. NNRTIs bind either to the free enzyme, the enzyme-T/P complex or to the ternary enzyme-T/P-dNTP complex with similar or different affinities, depending on the NNRTI [56].

Several mechanisms for the inhibition of HIV RT by NNRTIs have been suggested. These include a distortion of the geometry of the polymerase active site, restriction of the mobility of the thumb which prevents translocation of the T/P, and a change in the p66/p51 interface interactions [65]. Some NNRTIs have been shown to inhibit activity by stabilizing the heterodimer, whereas others have prevented proper dimerization and thereby inhibited the enzyme [51]. Proper dimerization is obviously very important for enzyme activity.

Like for the NRTIs, mutations conferring resistance to NNRTIs quickly develop. The binding site is not conserved and resistance mutations to all approved NNRTIs exist. Structural and biochemical studies along with computational modeling indicate three major classes of resistance mechanisms (Figure 12): 1) Loss or change of key hydrophobic interactions (e.g. Y181C), 2) steric hindrance (e.g. L100I) and, 3) mutations at the NNIBP entrance (e.g. K103N) [47].

![Figure 12. Three amino acids of HIV RT located in the p66 palm subdomain that when mutated represent three major classes of resistance mechanisms towards NNRTIs: K103 (mutations at the NNIBP entrance), L100 (mutations resulting in steric hindrance) and Y181 (mutations resulting in loss or change of hydrophobic interactions).](image)

*Alternative inhibition mechanisms of RT*

NRTIs and NNRTIs only represent two mechanisms for RT inhibition. There are at least two other possible inhibition strategies: 1) Disruption of key protein-protein interactions in the dimerization interface, making an inactive enzyme [57], 2) Inhibition of RNase H, or in other ways interfering with the secondary structure of this domain [48].
Compound library screening

Three of the articles in this thesis concern to the use of compound library screening in the pre-clinical phases of drug discovery. Compound library screening often serves as an entry point for identification of molecules suitable for further optimization. In this initial stage, the criteria for selection of molecules is often restricted to a verified interaction or inhibitory effect and selectivity towards the target [69].

There are three main types of compound libraries; 1) libraries for high throughput screening (HTS), 2) targeted libraries and 3) fragment libraries. Behind each type of library there is a different rationale. The number and type of compounds in the library influences what screening method can be used, and hence what information can be obtained. The chemical composition of the library (i.e. chemical properties such as MW and chemical diversity) determines what molecules can be identified and how sensitive the screening method must be. The choice and design of the compound library is therefore critical for the outcome of the screening.

High throughput screening libraries

The most common type of library is the corporate HTS library; i.e. very large collections ($10^5$–$10^6$) of chemically diverse and drug-like substances (MWs of around 400–700 Da). These historical collections of molecules are screened for a particular biological activity (or behavior) with the drug target of interest, using automated assays with very high throughput and relatively low information output. The rationale behind the “random screening” of huge libraries is that the probability of identifying good starting points (hits) for subsequent lead optimization, increases by sheer force of numbers. The more molecules that are tested, the higher will the number of hits be. HTS screening has rendered some successes since they began in the 1990s (e.g. the discovery of NNRTIs [65]), but identifying good starting points using these large “raw” libraries has turned out to be more difficult and less effective than was anticipated [70, 71].

In attempts to address the problems with low hit rates and few successful leads, new approaches and concepts to library filtering and design have emerged. In 1997, Christopher Lipinski published a seminal paper where he formulated five simple rules for prediction of good oral bioavailability of drugs [72]. These rules became referred to as “Lipinski’s rule of five”.

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They have, together with other predictive models and concepts [73-75], had a significant influence on the design of compound libraries and hit identification strategies. As a result, also HTS libraries are nowadays often subjected to some basic filtering and crude selection [76].

Targeted compound libraries

Targeted libraries evolved in response to the HTS libraries, and are also widely used. They are substantially smaller \((10^3-10^4)\) than HTS libraries and contain compounds that represent variants of chemical scaffolds known to inhibit a given target [77]. By screening a smaller, targeted set of molecules, the probability of finding a potent inhibitor is higher than when screening a large, random selection of molecules [77]. The lower number of compounds in this type of library reduces the demand on throughput, enabling the use of screening techniques with medium throughput and higher information output, such as SPR-based biosensors.

Fragment libraries

The third and most recent type of library that has been developed is the fragment library. The name refers to the very small molecules they contain, typically with MWs below 300 Da [78]. Also, these libraries hold relatively few molecules \((10^3-10^4)\). The principle behind fragment libraries and fragment-based drug discovery (FBDD) is that there is a lot to gain from identifying very small molecules as starting points for drug discovery [79]. Michael Hann et al. showed in 2001 that the probability of finding a compound with the right binding properties decreases considerably when the complexity of the molecule increases [80]. The hit-rate for a fragment library is therefore higher than for a library with larger and more complex molecules. In addition, the optimization efforts that follow hit identification frequently result in an increased MW and hydrophobicity of the molecules. These properties are often associated with poor bioavailability [81] and are hence undesired for drug molecules. Starting with a small and less complex molecule is more advantageous also for this reason. Because the total “chemical structure space” is smaller for fragments than for larger molecules, a small library

<table>
<thead>
<tr>
<th>Lipinski's rule of five</th>
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<tr>
<td>An oral drug should obey at least three of these criteria:</td>
</tr>
<tr>
<td>• MW (\leq) 500 Da</td>
</tr>
<tr>
<td>• (\leq) 5 hydrogen bond donors</td>
</tr>
<tr>
<td>• (\leq) 10 hydrogen bond acceptors</td>
</tr>
<tr>
<td>• (\log P \leq 5)</td>
</tr>
</tbody>
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allows the exploration of a relatively larger portion of chemical space. This increases the chance of finding novel scaffolds [78]. However, since fragments typically bind with weak affinities to their targets (K_D values typically >100 μM [82]), fragment screening puts high demands on the sensitivity of the techniques employed [83].

As with HTS and targeted libraries, there is a continuous search for methods that would improve the design of fragment libraries and the associated screening techniques [76]. Lipinski’s rule of five has for example been modified into “a rule of three” to better suit fragments [84]. And new concepts such as ligand efficiency (LE) [85], aimed at improving the prioritizing of compounds in early drug discovery, continuously emerge.

Once identified, a fragment must undergo chemical improvements to increase binding affinity and to acquire drug-like properties [86]. There are two main ways to achieve this. The most common method is “fragment-growing”. This is a stepwise and systematic addition of chemical functionalities to the fragment, together with a continuous feedback for pharmacological and physicochemical properties. The second strategy is known as “fragment linking” and can be used if two or more fragment hits are found to bind at proximal sites in e.g. the active site of an enzyme. By using three-dimensional structural information the different fragments can be joined through appropriate spacers to produce one high affinity ligand [82]. There are several examples of successful fragment-linking [87].

Biomolecular interactions

Drug discovery fundamentally concerns the understanding of biomolecular interactions. The formation of a binary complex between a drug and its target is pivotal for in vivo drug action [69]. Methods to evaluate, describe, and predict these interactions are hence at the core of drug development.

Methods for interaction analysis

A wide range of techniques can be used to study interactions. For compound library screening in early-phase drug discovery, in vitro-based, and highly reductionist methods, are most often used. Depending on the information needed, different methods are suitable. But a complete picture of an interaction can only be obtained by employing several orthogonal techniques. Some methods, such as x-ray crystallography and nuclear magnetic resonance (NMR), can give detailed, structural information about interactions. Calorimetric methods, like isothermal titration calorimetry (ITC), report thermodynamic parameters for interactions. However, in early drug-discovery there
are two parameters, affinity and selectivity, that usually are in focus [69]. These parameters can be assessed through different techniques that either provide direct binding assays (in which for example, the binding of an inhibitor to the target is monitored directly) or indirect and often functional assays (in which, for example, the binding of an inhibitor to an enzyme is detected as a decrease or increase in substrate or product formation). One highly sensitive and label-free method for measuring direct binding is surface plasmon resonance (SPR-) based biosensor analysis.

**SPR-based biosensor technology**

SPR-based biosensors make use of an optical phenomenon associated with total internal reflection for detection of interactions [88]. The phenomenon, called surface plasmon-polaritons [89], is electromagnetic waves that can arise at the interface between two media with different refractive index. These surface plasmon waves are very sensitive to changes in refractive index at the interface, and the technology is based on optical detection of such changes [89]. By immobilizing one molecule (the ligand) onto the sensor surface, and letting the other interactant (the analyte) flow over the surface in solution, interactions can be monitored in real time (Figure 13). Interactions result in changes of mass at the sensor surface interface, giving a change in refractive index, which can be optically detected as a dip in light intensity [90]. The intensity changes are translated into a sensorgram, Figure 14.

![Figure 13. SPR technology as implemented in the Biacore™ instrument series. A sensor surface made of a glass support is covered by a thin gold layer to which a carboxylated dextran matrix is covalently bound. This surface forms one wall in a microfluidic system in which buffer continuously flows. Plane-polarized light is reflected through a prism from the back of the gold film, not letting any light pass through the sample. A ligand (e.g. an enzyme) is immobilized onto the sensor surface and buffer containing analytes is injected automatically.](image-url)
Albeit measured indirectly through detection of a refractive index change, it is considered a direct binding assay. The results are presented as sensorgrams, with the binding signal plotted against time, Figure 14.

![Diagram](image.png)

**Figure 14.** A typical sensorgram depicting an interaction as an association and a dissociation phase. The start and stop of the analyte injection are indicated by arrows.

**Enzyme kinetics**

Kinetics is the study of reaction rates and is highly relevant for describing drug-target interactions. A drug based on enzyme inhibition is – with few exceptions – only efficacious as long as the drug molecule stays bound to its target [69]. The formation and disintegration of an enzyme-inhibitor complex can be described and quantified in terms of enzyme kinetics.

When kinetics of enzyme-inhibitor interactions are studied in direct binding assays, the situation is simplified as compared to in indirect assays where a substrate also is present. This makes direct binding assays highly suitable for interaction mechanism studies and for determinations of kinetic parameters. Indirect assays are necessary for determining inhibition of enzyme activity and the mode of inhibition (e.g., competitive or non-competitive).

**Affinity**

Target affinity is a popular kinetic parameter for characterization and ranking of interactions in early-phase drug discovery. Affinity defines the amount of complex formed at a certain drug concentration, i.e., the effectiveness of an interaction. It is commonly quantified in terms of the dissociation equilibrium constant (K_D), or as the half-maximal inhibitory concentration (IC_{50}). A relatively high affinity, i.e., a K_D-value in the low µM-nM range, is a basic requirement for most drug interaction as it affects the effective dose concentration [69].
The simplest interaction between an enzyme (E) and an inhibitor (I) can be described as

\[
\begin{align*}
\text{E} + \text{I} & \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} \text{EI} \\
\end{align*}
\]

(Eq 1.)

where \(k_{\text{on}}\) represents the association rate constant and \(k_{\text{off}}\) the dissociation rate constant, and \(K_D\) is defined as

\[
K_D = \frac{k_{\text{off}}}{k_{\text{on}}} = \frac{[\text{E}][\text{I}]}{[\text{EI}]} \\
\]

(Eq. 2)

**Rate constants**

While having relevance and being frequently used, affinity is still a crude descriptor of an interaction. From Equation 2 it can be deduced that interactions with equal \(K_D\)-values can have different association and dissociation rate constants. Interactions with the same \(K_D\) can be distinguished from each other by their kinetic rate constants, providing means for ranking of compounds on a kinetic basis.

Moreover, Tummino and Copeland *et al.* [69, 91] argues that rate constants derived from measurements in open systems better reflect the *in vivo* situation for drug-target interactions than equilibrium-based metrics such as \(K_D\) and \(IC_{50}\). These are often determined in closed systems at steady-state conditions, where the concentration of the compound stays the same throughout the measurements. The duration of the binary complex (i.e. drug efficacy) is dependent on the rate constants, predominantly the dissociation rate constant, and not on equilibrium conditions. The common use of equilibrium based methodologies in drug discovery is questioned also by David Swinney [92], who concludes that ”[…] methodologies that measure kinetics, non-equilibrium binding events and conformational diversity might have greater value than previously realized.”

**Interaction mechanisms**

Inhibitor binding to an enzyme is seldom as simple as depicted by Equation 1. Often, the initial binary complex (EI) goes through a conformational change (isomerization), resulting in a second, high affinity (low energy) enzyme-inhibitor complex (EI*) [56]. This is called an *induced fit* mechanism.
Another mechanism, in which the free enzyme exists in equilibrium between two different energy-conformations (E and E*), and the inhibitor preferably binds to the less stable high-energy conformation (E*), is referred to as selected fit [93]. Both these mechanisms can be described by a four-state model, such as presented in Figure 15.

![Figure 15. A four-state binding model combines the induced fit and selected fit mechanisms (modified from [94]). It assumes two isoforms of the free enzyme (E for the low energy form and E* for the high energy form) and two binary complex conformations (EI for low energy and EI* for high energy). The inhibitor binds to the low energy isoform of the free enzyme (E) in the induced fit path, whereas it binds to the high energy isoform (E*) in the selected fit path. There are eight rate constants involved.](image)

In some systems, such as for the binding of NNRTIs to HIV RT, the interaction mechanism may be even more complex. After having studied the kinetics of NNRTI binding to the K103N mutant of HIV RT, Geitmann et al. [67] propose that the interaction can be described by a three-step model. The free enzyme is assumed to exist in an open (E) and closed (E*) conformation and selected binding of an NNRTI to the open conformation results in a binary complex formation (EI). This high energy complex subsequently goes through an induced conformational change in which the NNIBP closes and forms a more stable complex (EI*). This model is described by figure 15, when the equilibrium between EI* to EI* is disregarded. In addition, the authors suggest the existence of a natural heterogeneity of RT, implying that there exist two different ground isoforms (E1 and E2) of the enzyme. Both these isoforms interact with NNRTIs according to the proposed three-state model, but with different reaction rates, resulting in a very complex model. Abbondanzieri et al. reports in Nature 2008, that HIV RT exists in one polymerase-competent and one RNase H competent state. This may support the hypothesis that HIV RT naturally exist in two heterogeneous forms.
Present Investigation

Interaction kinetics in drug discovery

Interaction kinetic data can be used as a tool for selection and characterization of drug molecules. At different stages in the drug development process, different levels of kinetic resolution are valuable [95]. In the initial stages of a drug discovery program, it may very well suffice to determine if a molecule binds to the target or not, and to make a simple ranking based on some parameter that, preferably, correlates with affinity. Later in the process, when fewer and more developed molecules are to be screened and characterized, quantification of the interaction using mechanistically defined parameters becomes relevant.

In the four papers included in this thesis, different approaches to interaction kinetic analyses of small molecules in early-phase drug discovery are presented. The studies have been performed using SPR-based biosensor technology, which offers a unique possibility to study interactions between small molecules and drug targets due to its very high sensitivity and capacity to detect interactions in real-time. Different types of compound libraries, varying experimental strategies and complementary orthogonal assays have been used in order to identify new HIV enzyme inhibitors. The first two papers present strategies for how interaction kinetic data can be used for targeted selection and characterization of NNRTIs (i.e. lead compounds), intended for use in topical microbicides. Paper III and paper IV present approaches for how SPR-based interaction kinetic analyses can be used for effective identification and ranking of fragments, for use as chemical starting points in (antiretroviral) drug development.

In the following sections, different aspects of the respective studies are discussed, along with their scientific contributions.

Kinetic criteria for lead selection (Paper I)

Compound selection criteria

There are a number of properties (e.g. affinity, specificity, and hydrophobicity), which are normally taken into consideration early in the process of de-
signing a new drug or pharmaceutical agent [69]. The intended clinical use of an inhibitor can affect the additional properties that are likely to be of importance. In paper I, a targeted library with molecules designed to be analogues of known NNRTIs is screened for inhibitors suitable for use in a topical microbicide. Because topical microbicides are intended to be employed by women in areas where resistant HIV strains are common, an important characteristic of the inhibitors is to show potency against resistant viral strains. In addition, microbicides have to be highly effective in suppressing HIV replication to reduce resistance development [47] and the need to be practical for regular use. A property that affects the user-friendliness, and consequently the adherence, is the intervals at which the microbicide has to be administered for retained efficacy. Long intervals between administrations are typically preferred by the user, and will encourage compliance. These properties – high and sustained efficacy along with potency against resistant HIV strains – formed the basis for the selection criteria used in the NNRTI screening in paper I.

High affinity and slow dissociation for sustained efficacy

It was hypothesized that NNRTIs showing high affinity and slow dissociation would provide high and long-lasting anti-HIV effect. This defined interaction profile was used as the primary selection criterion in the screening.

In order to efficiently inhibit all HIV RT molecules, and thereby prevent primary infection and resistance development [47], the enzymes have to be constantly occupied by the inhibitor. This means there has to be a sufficient concentration of inhibitor present in the mucosal lining of the vagina (or rectum) at the time for viral attack. Smart delivery systems, such as vaginal rings that continuously release a small amount of inhibitor, are useful to achieve this end. But affinity, mechanism and residence time, may be even more critical [69, 92]. High affinity compounds (with K_D-values around 10^{-9}–10^{-12} M) have generally the advantage that the drug concentrations in blood can be kept lower than for drugs with higher K_D, assuming the same inhibition mechanism [92]. This is desirable as high doses increase the risk for toxicity and adverse side effects, especially for non-specific inhibitors. Even though microbicides are not systemically, but topically, administered, a low concentration of inhibitor is likely to be beneficial from a toxicological perspective, since the microbicides are intended to be used regularly over many years.

The molecule exhibiting the highest affinity is not necessarily the most suitable drug compound. Affinity is a composite parameter dependent on both the association and dissociation rate constants (K_D= k_{off}/k_{on}) and dissociation is inversely related to residence time (1/k_{off}) of the inhibitor at its target.
Long residence time equals in most physiological situations a long inhibitory effect [69]. Dissociation rate can therefore be a suitable optimization parameter. If the turn-over rate of the enzyme exceeds the longevity of the binary complex, the advantage of slow dissociation is limited to the lifetime of the enzyme. This is an important aspect for many biological systems, where new enzyme molecules are continuously produced. However, when the microbiocide inhibitors are intended to act, i.e. before primary infection has been established, the number of HIV RT molecules is finite, and new enzymes are not produced. In summary, an inhibitor exhibiting high affinity and slow dissociation, in combination with a system for constant delivery of the inhibitor, would be likely to ensure that the HIV RT molecules are blocked at all times.

Resilience to resistance mutations for long-term clinical efficacy

The second selection criterion used in the NNRTI screening was potency against multiple resistant HIV-1 strains. To enable selection based on resistance resilience, the experiment was designed so that four different HIV-1 RT variants were screened in parallel. The targets were wild type HIV-1 RT and three clinically resistant single “mutants”. The amino acid substitutions in the mutant enzymes (K103N, L100I and Y181C) are all located in the NNIBP and are representative for three different types of resistance mechanisms. They frequently appear in response to treatment with the approved NNRTIs nevirapine, delavirdine and efavirenz. Because it is important that a microbicide is potent also against resistant HIV strains, only inhibitors that fulfilled the first selection criterion (i.e. showed high affinity and slow dissociation) for all four enzyme variants were considered an interesting hit.

A reductionist approach to data analysis

The use of a targeted lead compound library enabled sufficient kinetic resolution for selection based on a kinetic interaction profile. To efficiently evaluate the large amount of interaction data generated in the screening, a reductionist approach was used. By expressing the kinetic profile of each individual interaction in only two variables, one representing affinity and the other dissociation rate, a robust and simple method for data analysis was developed.

One interesting compound

Out of the 800 compounds that were screened, two structurally unrelated NNRTIs showed the desired interaction profile: high affinity and slow dissociation with all four enzyme variants. One of the compounds turned out to be a false positive when validated in a competition-assay and an enzyme
inhibition assay. This result underlines the importance of employing orthogonal assays for hit validation, especially when the selection criteria have an inherent bias towards unspecific binding – as is the case when the selection criteria include binding to several targets. The remaining hit compound, the thiourea derivative MV026340 (Figure 16), was confirmed to bind to the NNIBP both by the competition assay, the enzyme inhibition assay and by co-crystallization with HIV-RT (T. Unge, personal communication 2008).

![Figure 16. The structure of MV026340.](image)

The full potential of using interaction kinetic data in drug design, and for predictions of therapeutic responses, can be reached only when large, varied, high-quality data sets have been analysed and correlated to physiological data. Presently, there is a paucity of this kind of studies, which makes it too early to draw any general conclusions about optimal kinetics for high drug efficacy. In paper II, we attempt to investigate how well the hypothesized kinetic profile in paper I correlates with antiviral efficacy.

A relationship between kinetics and antiviral effect (Paper II)

Many researchers have over the last years reported on the value of obtaining detailed interaction kinetic data for a more purposive drug design [69, 91, 92]. The hypothesis presented in paper I, and discussed above, states that high affinity and slow dissociation is likely to be positively correlated with sustained antiviral efficacy. In order to investigate how well this assumption holds, the kinetics of a novel high affinity NNRTI (MIV-170, Figure 17), as well as three clinical and one pre-clinical NNRTI, were studied and correlated to antiviral effect in cell culture. Four different variants of HIV-1 RT, wild type and the three mutants in paper I, were used as targets in the SPR-based biosensor assays.

![Figure 17. Structure of MIV-170.](image)
A qualitative analysis revealed an induced fit mechanism

SPR-based biosensor technology is acclaimed for its sensitivity and unusually information rich output. Kinetic parameters such as affinity and rate constants can typically be quantitatively determined for molecules with high affinity, a property normally associated with high MW. However, other experimental limitations than low affinity, may result in a decreased information output. In this study we show how a limited dataset can allow for a qualitative analysis of the kinetic characteristics.

In order to determine the kinetic mechanism of the NNRTI-HIV RT interactions, sensorgrams were simulated with a set of mechanistic models representing steps of the complex mechanism previously found to describe the interactions of NNRTIs with the K103N variant of HIV RT [67]. An induced fit mechanism turned out to be the simplest model capturing the basic features of the experimental data (Figure 18).

![Figure 18](image)

**Figure 18.** The enzyme and the NNRTI form a high energy binary complex (EI) that goes through a conformational change to the more stable low energy complex (EI*).

In this model, the NNRTI first binds to the free enzyme and forms an initial, energetically relatively unstable complex. Subsequently, a more stable, low energy complex is formed through conformational changes. The low energy and high energy complex formations are governed by two different sets of rate constants. By simulating sensorgrams (Figure 19) with fixed values for the two association rate constants (k₁ and k₃), but varying the values for the dissociation rate constants (k₂ and k₄), sensorgrams similar to the experimental dataset were obtained.
Figure 19. Simulated sensorgrams representing interactions with an induced fit mechanism. All sensorgrams were simulated with the same enzyme concentration (5.12 μM) and fixed $k_1$ and $k_3$ values. The dissociation rate constant $k_4$ varies between panel A and B and dissociation rate $k_2$ varies within the same panels.

It could be concluded that the first dissociation rate constant ($k_2$), describing the rate by which the initial enzyme-inhibitor complex dissociates into free enzyme and inhibitor, had a large impact on the maximal amount of complex formed. The second dissociation rate constant ($k_4$), representing the rate by which the induced complex relaxes into the initial complex, influenced the slope of the dissociation curves.

There is both structural and kinetic data supporting a complex interaction model for NNRTI-binding to HIV RT. The enzyme is known to be highly flexible [58, 66] and the NNIBP is most likely only temporarily available for inhibitor binding in the free form of the enzyme [65, 67]. The conformational change that occurs in the enzyme upon binding of NNRTIs, support an induced fit mechanism.

Rate constants affect the antiviral efficacy

The kinetic characteristics of the interactions were used for a qualitative correlation analysis with antiviral efficacy data. It was clear that the best antiviral efficacy was achieved when both the relaxation rate from the induced complex ($k_4$) and the dissociation rate from the initial complex into free enzyme and inhibitor ($k_2$) was slow, as exemplified by MIV-170. For the other NNRTIs that were tested, their varying antiviral efficacies also could be satisfactorily explained by different rates of $k_2$ and $k_4$. This qualitative kinetic analysis illustrates how elucidation of a kinetic mechanism of a drug-target interaction can allow identification of critical kinetic features that correlate with antiviral efficacy. The results support the hypothesis that the efficacy of NNRTIs is dependent on slow dissociation from the target.
Conclusions from paper I and paper II

Paper I and paper II show the value and potential of kinetic characterization of high affinity drug-target interactions in lead discovery. They provide an example of how understanding of the kinetic mechanism of a drug-target interaction can be useful for prediction of antiviral effect. Paper II also shows that simulation of interaction data for qualitative analysis of tight-binding drugs can be informative.

The lead molecules in the targeted screening library in article I were, however, based on known NNRTI structures, eliminating the chance of identifying completely new structures. A drug that is similar to a known NNRTI may interact with the same amino acid residues in the NNIBP, and thereby be susceptible to cross-resistance development. When searching for new inhibitors of HIV enzymes, it is therefore logical to look also outside the known structure space. One way to increase the chance of identifying completely new chemical entities is by screening fragment libraries. In paper III and paper IV this relatively young approach to drug discovery is explored for identification of new inhibitors of HIV enzymes using interaction kinetic analysis.

Fragment library design (Paper III)

In recent years, fragment-based drug discovery (FBDD) has become an important approach to drug discovery [96]. It is a methodology that is based on fragment library screening, for which the composition of the library is critical. Ideally, a fragment library should contain structurally diverse molecules that efficiently explore the chemical space relevant for drug development [97]. The composition of a library may need adjustment with respect to the screening method and the condition used for screening. Many strategies for library design have emerged [84, 98-100], but there is as yet little knowledge about how suitable existing libraries are to current applications.

Screening against three enzymes and a plasma protein

In paper III, a unique study, designed to experimentally validate a new fragment library for its quality and suitability for use in SPR-based biosensor screening, is presented. A set of three prototypical drug targets (HIV-protease, thrombin and carbonic anhydrase, CA) and one non-target protein (human serum albumin, HSA) were used in an explorative pre-screening. The aim was to investigate properties of the new fragment library and to identify fragments that exhibited unsuitable characteristics for screening
using SPR-based biosensors, prior to its use for an actual screening against a target.

The 930 fragments in the library had been selected from a database with 4.6 million commercially available molecules, by use of generally accepted physicochemical and chemical filters. The fragments were screened in duplicates at a single concentration (200 µM) in a buffer containing 5% DMSO to ensure solubility.

**Selectivity**

Initially, a profile of all the fragment-target interactions was made based on detectable signal relative baseline signal, showing that 29% of the fragments interacted with all four target proteins. Only six fragments did not interact significantly with any of the targets. Selectivity is not expected to be a critical factor for fragments, since the small molecules will inevitably undergo an optimization process in which their specificity can be augmented [101]. Nevertheless, a fragment library is expected to show a unique interaction profile to each individual target, otherwise it could imply that the fragments simply stick to the sensor surface or any protein covered surface. The interaction profile of the validated library confirmed target discrimination. However, a different experimental setup would be required to obtain detailed specificity data.

**Super-stoichiometry**

The library was further evaluated for presence of super-stoichiometric fragments. Fragments that interact with more than a few defined binding sites on the target are unspecific (promiscuous [102]) and hence undesirable in a library. When fragments interacted with a stoichiometry $\geq 5$ they were defined as super-stoichiometric. The analysis showed that 26 of the fragments behaved super-stoichiometrically with at least one target protein, but not a single fragment showed this behavior for all four proteins. This proved the value of the concept with several parallel targets. If fewer target proteins had been used, a number of compounds would have been disqualified from the library on the grounds of promiscuity [103]. A compound that shows promiscuous binding to one target, may very well interact in a specific way with another target. It is therefore important to be cautious when permanently excluding a fragment from a library based on the result from interactions with only one target.
Interaction kinetic profile

Slowly dissociating fragments are also undesirable, since they can block the sensor surfaces in a screen and thereby give rise to false negative in succeeding injections. All interactions were therefore analysed with respect to their kinetic profile. A majority of the interactions showed “square-pulse sensorgrams”, representing both fast association and dissociation rates. This was true for all target proteins. In order to detect fragments that exhibited slow dissociation rates, a reductionist report-point based method was used (similar to the one used in paper I). This analysis identified 35 proteins with very slow to no dissociation from at least one target protein. Two fragments showed essentially irreversible interactions with all four proteins, suggesting that these compounds should be excluded or run at the end of a screen.

The value of multiple targets

To the best of our knowledge, this study is the first to compare how fragments in a library interact with several targets. When identifying super-stoichiometric fragments and slowly dissociating fragments (both considered undesirable properties for fragments in a FBDD library) for exclusion from a library, the use of multiple targets with different physiochemical properties turned out to give important information. 61 fragments would have been excluded from the present library on the basis of super-stoichiometry or slow dissociation, if results regarding only one target would have been used. However, taking the behaviour with all four target proteins into consideration, only 2 fragments were indicated for exclusion. These two fragments have later been found to dissociate relatively rapidly from another target, supporting the idea that fragment interaction behaviour is target dependent.

Fragments for a new generation of NNRTIs (Paper IV)

Low kinetic resolution

In paper I and II it was shown how detailed interaction kinetic data from SPR-based biosensors can be integrated in the lead discovery and characterization process. When fragments are studied, much lower kinetic resolution is normally obtained, due to their low MWs and typically weak affinities. This is, however, rarely a problem given that fragment data is only relevant in the initial hit identification steps of a drug development program. In these stages, the number of compounds is high and quantitative and mechanistic aspects are of little importance, Figure 20.
Figure 20. Early in the fragment-based drug discovery process there are typically numerous compounds with relatively low complexity and low possible kinetic resolution. At later stages, fewer and more complex molecules remain, and the kinetic information that can be obtained is more detailed.

At this early point in the FBDD process, kinetic interaction data can serve as an effective method for reliable identification and ranking of compounds that interact with the target. In paper IV, interaction kinetic data and inhibition data was used for identification of novel scaffolds for allosteric inhibitors of HIV-1 RT.

Similarity to existing NNRTI structures
For the purpose of identifying novel scaffolds, a fragment library is likely to be more suitable than a library with larger and more structurally limited molecules. However, to evaluate how well the particular library used in paper IV would serve as a source for novel NNRTIs, the fragments were compared to 826 existing NNRTIs and checked for similarity. This initial analysis showed that less than 3% of the fragments were substructures of known NNRTI structures and hence that the library would be appropriate for its purpose.

A pragmatic approach to ranking
The fragment library screening was performed in three consecutive steps, each serving as a filter. In the first step, all 1040 compounds were tested at
four different concentrations against wild type HIV-1 RT. This enabled the identification of fragments that appeared to interact with the target with a simple 1:1 mechanism, by calculating apparent affinities ($K_D^{\text{app}}$). Estimations of more well defined $K_D$-values would have required significantly higher screening concentrations. The use of apparent affinities is a pragmatic method useful for confirming binding to the target, and for an initial ranking.

Few binding fragments

The second step consisted of two parallel screening steps, also targeting the wild type enzyme. One of the screens was an SPR-based competition analysis, in which the 96 fragments remaining from step 1 were tested for competition with nevirapine. This was performed to determine if the fragment bound to the NNIBP or not. The parallel screening step was an enzyme inhibition assay, in which it could be verified that the fragments inhibited HIV-1 RT. Since the NNIBP is a quite hydrophobic binding site that lacks specific functional groups for interaction, it was somewhat surprising that only 2% of the fragments were identified as binders.

One potent novel scaffold

At the outset of the last step, 10 of the original 1040 fragments remained. All these fragments were structurally novel compared to the NNRTIs used in the initial comparison. More detailed interaction and inhibition analyses were performed with these compounds. Three resistant variants of HIV-1 RT were added as targets, to select for resilience to resistance. The determinations of both apparent affinity ($K_D^{\text{app}}$) and inhibition ($EC_{50}$) were more accurate in the final experiments due to experimental replicates, but a low kinetic resolution remained due to weak affinities. The absence of an efficient ligand binding “hot spot” in the NNIBP [104] makes high affinity fragments rare. One interesting fragment was identified, a bromoindanone (Figure 21) that repeatedly showed more than 50% inhibition against all four enzyme variants and displayed $IC_{50}$ values lower than 25μM. The fragment had a ligand efficiency (LE) of 0.44 kcal mol$^{-1}$, providing an excellent starting point for new NNRTIs, if the LE can be kept constant throughout lead optimization.

![Figure 21](image)

**Figure 21.** The bromoindanone fragment identified as hit in the fragment screening.
Conclusions from paper III and IV

A pre-screening of a fragment library, of the type presented in paper III, can give valuable information about how suitable the library is for the intended screening technique, as well as if any changes in library composition, experimental setup, and sample order should be made before using the library for screening. The most important finding may be that fragments can behave like a promiscuous binder with one target but interact in a good way with another. This has implications for library optimization, since permanent exclusion of fragments from a library should be made with caution if based on results obtained from interactions with only one target.

The stepwise hit selection and validation strategy used in paper IV provides an example of how interaction kinetic analysis, in combination with inhibition assays, can be used for efficient identification and ranking of low-affinity fragments. One novel fragment that showed inhibition of HIV RT and resilience to resistance mutations was identified, and may constitute a promising starting point for the development of a new generation NNRTIs.
Concluding remarks and future perspectives

The search for new drugs against HIV is likely to be a continuous process due to the limited long-term efficiency of drugs in clinical use. Even when used in combination therapy, multi-drug resistant HIV strains evolve, though at a much lower frequency. However, the more mechanisms that are exploited by the anti-HIV armamentarium, the better are the chances of finding efficient combination therapies that completely block viral resistance development. HIV RT is a well validated drug target, but as yet no drug in clinical use is targeting RNase H activity. In paper IV, a number of compounds that bind to HIV RT, but not to the NNIBP, are identified. These compounds represent an interesting collection of molecules that potentially could inhibit RT activity through new mechanisms, such as inhibition of RNase H. This would be an interesting path to pursue.

Modern drug discovery is based on a reductionist approach. Vast amounts of information is gathered from simplified *in vitro* and *in vivo* assays and used for ranking and prioritizing of compounds. But it is exceedingly difficult to understand a complex system such as the human physiology by only looking at its pieces. Unfortunately, there are few alternatives since the possibility to perform studies in humans and other primates for good reasons are limited.

I see it as a key challenge for present and future drug discovery to figure out how to best apply the reductionist approach to mirror and predict physiological responses. This is needed to reduce the high attrition rates in drug discovery. New methods, such as those presented in this thesis, where the use of high resolution interaction kinetic approaches help guide drug discovery at an early stage, will probably be important for better interpretations of the dynamics of molecular interactions. Studies of detailed thermodynamics of drug interactions, and how it relates to interaction kinetics, is also a highly interesting and relevant topic at the very core of understanding interactions. An improved understanding of interactions, and implementation of this knowledge in the drug discovery process, is likely to improve the predictions of drug action in humans. More correlation studies between in vitro parameters, drug action and therapeutic response are also fundamental.
Med sikte mot en ny generation läkemedel mot HIV

*Denna avhandling redogör för forskning som visar hur en avancerad kartläggning av interaktioner mellan små molekyler (läkemedel och deras målenzymer) kan användas som ett mångsidigt och effektivt verktyg vid utveckling av nya läkemedel. Metodologin är applicerad på läkemedel och preventionsmetoder mot HIV. Nya experimentella strategier för hur detaljerad kinetisk information från SPR-baserade biosensorförsök kan användas för både identifiering, karakterisering och utvärdering av potentiella läkemedelsmolekyler presenteras.*

En bred vetenskaplig utmaning


Utvecklingen av läkemedel mot HIV möter även andra stora utmaningar möter. Ur ett medicinskt perspektiv är HIV en ovanligt svår motståndare, främst på grund av sin benägenhet att mutera och därigenom utveckla resistens mot läkemedel. Prevention och behandling av HIV försvåras dessutom av sociala faktorer såsom fattigdom, bristande jämställdhet mellan män och kvinnor, religiösa föreställningar etc. För att nya läkemedel ska bli så effek-
tiva som möjligt bör sådana faktorer tas i beaktning. Ett exempel på hur ett socialt behov har påverkat utformningen av en (potentiell) medicinsk lösning är den pågående utvecklingen av topiska mikrobicider. Det är ett slags ”kemisk kondom” som kvinnor själva kan applicera före samlag för att skydda sig mot HIV. Till skillnad från när kondom används är inte kvinnan i samma utsträckning beroende av sin partner för att kunna använda en mikrobicid.

Dessa ekonomiska, medicinska och sociala svårigheter gör kampen mot HIV till en bred vetenskaplig utmaning där kunskaper och insikter från en rad olika vetenskapsområden är viktiga. Nya metoder för att studera molekylinteraktioner i ett tidigt skede av läkemedelsutvecklingen förväntas underlätta utvecklingen av en ny generation effektivare läkemedel mot HIV. Den här avhandlingen fokuserar på detta, men metodologin som utvecklats kan också appliceras på läkemedelsutveckling mer generellt.

Interaktionskinetik som verktyg vid läkemedelsutveckling

De flesta läkemedel baserar sig på interaktioner mellan den aktiva substansen i läkemedlet (läkemedelsmolekylen) och dess målmolekyl, vanligen ett enzym eller en receptor. Läkemedelsmolekylen ska interagera med målmolekylen och genom att antingen stimulera eller inhibera (blockera) dess aktivitet ska en önskvärd fysiologisk respons erhållas. Studiet av interaktioner mellan läkemedel och deras målmolekyler bidrar därför till viktig kunskap för läkemedelsutveckling.

För att identifiera molekyler som interagerar med varandra utförs vanligen studier (screeningar) i vilka stora samlingar av molekyler (kemiska bibliotek) testas för interaktion (eller aktivitet) mot en eller flera målmolekyler. I denna avhandling har olika typer av kemiska bibliotek screenats mot HIV-enzyme i syfte att hitta nya inhibitorer som har lämpliga egenskaper för användning i mikrobicider eller läkemedel. Den experimentella metodologin och analysen är ny och förväntas kunna bidra till ökad och mer ändamålsenlig tidig läkemedelsutveckling.

Metoden som huvudsakligen har används för studierna i denna avhandling heter yt-plasmon resonans (SPR)-baserad biosenorteknologi. Det är en mycket känslig teknik som kan användas för att studera interaktioner mellan små molekyler. Informationen man får fram är tidsupplöst, vilket innebär att den redovisar sekund för sekund hur molekyler binder till varandra (associerar) och sedan släpper igen (dissocierar). Vanligen studeras bindningar mellan läkemedelsmolekyler med metoder som kan säga med vilken affinitet (ung. effektivitet) som molekylerna binder till varandra. Detta är ett användbart sätt att kvantifiera interaktioner med, men ger mindre detaljerad information än SPR-tekniken. Denna kan även visa hur associationen re-
Spektive dissociationer ser ut, och ofta kvantifiera detta genom att ge hastighetskonstanter för interaktionens association- respektive dissociationshastighet. Studiet av interaktioner utifrån deras hastighetskonstanter kallas interaktionskinetik.

Screening av kemiska bibliotek

I den första artikeln testas ett kemiskt bibliotek bestående av analoger till kända inhibitorer av HIV RT (omvänt transkriptas), mot fyra olika varianter av RT-enzymet. Utgångspunkten är ett antagande om att en interaktion mellan en inhibitor och RT som kännetecknas av hög affinitet och långsam dissociation passar bra i en mikrobicid. Detta förmodas ge en långvarig skyddande effekt, vilket är lämpligt för en mikrobicid. Analysen identifierar en molekyl som uppvisar den efterfrågade interaktionsprofilen både mot vildtypsenzymet och mot de tre resistentena enzymvarianterna.

I artikel två undersöks om den interaktionsprofil som antogs vara lämplig i artikel ett korrelerar med antiviral effekt i cellkultur. En ny inhibitor (MIV-170) med mycket hög affinitet och långsam dissociation undersöktges tillsammans med fyra kliniska inhibitorer. Det visade sig att det gick att se en korrelation mellan antiviral effekt och vissa hastighetskonstanter i den komplexa bindningsmodell som presenterades (induced fit).

Artikel tre och fyra är fokuserade på screening av kemiska bibliotek med mycket små molekyler (s.k. fragment), för identifikation av nya inhibitorer av HIV enzymer. Sammansättningen av fragmentbiblioteken är viktig för resultaten från screeningarna. I artikel tre redovisas en metod för hur ett fragmentbibliotek har validerats med avseende på lämplighet för användning vid SPR-baserade screeningar. Fyra olika målproteiner, däribland HIV-proteas, användes vid valideringen. Det visade sig att fragment som uppvisar olämpliga interaktioner med vissa målproteiner beter sig bra i interaktionen med andra. En viktig slutsats är alltså att inte bedöma ett fragments generella kvalitet utifrån dess beteende med endast ett (eller ett par) målproteiner.

I artikel fyra screenades ett fragmentbibliotek mot fyra varianter av HIV RT. Syftet var att identifiera nya kemiska strukturer av specifika RT-inhibitorer, s.k. NNRTIs. Fragment binder normalt med mycket låg affinitet till mål molekylerna, vilket kan göra dem svåra att studera, särskilt med avseende på kinetik. En experimentell strategi som baserade sig på stegvis selektion av fragment utifrån resultat från SPR-baserade biosensorförsök och enzymatiska inhibitionsmätningar möjliggjorde en effektiv screening och analys av ett bibliotek med 1040 fragment. Ett fragment uppvisade bindning och minst 50% inhibition mot samtliga RT-varianter.
Sammanfattningsvis har två föreningar med potential som nya läkemedelsmolekyler mot HIV identifierats. Därtill har metoder utvecklats som visar hur detaljerad interaktionskinetik effektivt kan användas för identifiering och karakterisering av läkemedelsmolekyler i tidig läkemedelsutveckling. Förhoppningsvis kan metodologin vidareutvecklas och bidra till att minska kostnaderna för framtida läkemedelsutveckling.
During my time as an undergraduate student at Uppsala Graduate School in Biomedical Research, I came to realize that when choosing a PhD position, it is far more critical to find the right supervisor than the right research project. This insight made me make the decision that I would only ever engage in PhD-studies if I found both a perfect supervisor and an interesting project. As luck would have it – I did.

*Helena,* as my supervisor you have inspired, encouraged, instructed, challenged and trusted me. I find your forthrightness relieving, and your enthusiasm and fairness admirable. I have truly enjoyed our many and vivid discussions about writing, books, and about different aspects of enzymology and biochemistry. Thank you for giving me the opportunity to do research in your group!

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References

40. FDA, Antiretroviral drugs used in the treatment of HIV infection. 2010.


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