Characterization of HCV Protease Inhibitors

Inhibition and Interaction Studies with Applications for Drug Discovery

SOFIA SVAHN GUSTAFSSON
In this thesis, different approaches based on inhibition and interactions studies, have been used to characterize inhibitors of the non-structural protein 3 (NS3) from the hepatitis C virus (HCV). This involves identification of enzyme inhibitory effects and characterization of interaction mechanisms and kinetics, as well as effects on replication in a cell based system and serum protein binding. All this information contributes to HCV drug discovery.

By using an inhibition assay it was possible to evaluate the effects of NS3 protease inhibitors, tested or used in the clinic, on NS3 variants, representing different model systems often used for drug discovery. This study illustrates the importance of accounting for differences in catalytic properties in comparative analyses, for making relevant interpretations of inhibition data. An SPR biosensor-based assay expanded the first study, and provided kinetic and mechanistic information, by direct interaction analyses of the inhibitors. It revealed significant differences between the different genotypes and model systems, and provided data that can be used to better understand the efficacy of inhibitors.

Additionally, novel NS3 protease inhibitors were evaluated with respect to their potential to interfere with protease activity, their sensitivity to resistant mutants and effect on HCV replication. The most potent compounds were also characterized by their bioavailability, solubility and metabolic stability. This provides information for design of improved NS3 protease inhibitors, suggesting potential peptidomimetic structures for the backbone as well as for peptide substituents. These modification strategies allowed inhibitors to be truncated and less peptide-like, still with retained inhibitory effect.

A new strategy for analysis of serum protein binding, of importance for drug distribution was also developed. By defining and using the concept of binding efficiency, serum protein interactions of moderate affinity, as described by rapid kinetics, were characterized. This strategy is also applicable for analysis of low affinity interactions.

Taken together, all these studies provide knowledge and strategies for HCV drug discovery, and by using this information we might take a step closer to the final goal, which is to eradicate HCV.

Sofia Svahn Gustafsson, Uppsala University, Department of Chemistry - BMC, Box 576, SE-751 23 Uppsala, Sweden.

© Sofia Svahn Gustafsson 2013

ISSN 1651-6214
ISBN 978-91-554-8591-7
urn:nbn:se:uu:diva-193256 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-193256)
Lyckan är inte att nå sitt mål, lyckan är att vara på väg

Ingvar Kamprad
List of Papers

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


Reprints were made with permission from the respective publishers.
Abstract

Drug discovery

Hepatitis C

Prevalence and transmission
Progression and symptoms of infection
Treatment

Hepatitis C virus

The viral life cycle
The viral proteins
HCV genotypes, subtypes and quasi-species

HCV drug discovery

Drug targets
Viral enzymes as drug targets
The NS3 protein
NS3 protease
Substrate specificity
Structure
Mechanism of action

Discovery of NS3 protease inhibitors
Peptidomimetic NS3 protease inhibitors
NS3 model systems
Assays for evaluation of NS3 inhibitors
Enzyme inhibition analysis
Enzyme-inhibitor interaction analysis
Cell-based analysis
Pharmacokinetic profiling

Present investigation

Aim

Results

Evaluation of NS3 protease inhibitors
Characterization of NS3 inhibition (Paper I)
Kinetic analysis of inhibitor interactions (Paper II)
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADME</td>
<td>adsorption, distribution, metabolism, excretion</td>
</tr>
<tr>
<td>AGP</td>
<td>$\alpha_1$-acid glycoprotein</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ARF</td>
<td>alternative reading frame</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine tri-phosphate</td>
</tr>
<tr>
<td>DAAs</td>
<td>direct acting antivirals</td>
</tr>
<tr>
<td>E1 and E2</td>
<td>envelope proteins 1 and 2</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydro chloride</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmatic reticulum</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>HBV</td>
<td>hepatitis B virus</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HSA</td>
<td>human serum albumin</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>NCR</td>
<td>non coding region</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NS</td>
<td>non-structural protein</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>RU</td>
<td>resonance unit</td>
</tr>
<tr>
<td>SR-B1</td>
<td>scavenger receptor class B type 1</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>SVR</td>
<td>sustained viral response</td>
</tr>
</tbody>
</table>
Drug discovery

The main goal for the pharmaceutical industry is to discover, develop and market drugs for treatment of different diseases. This process can be very long, and a period of 10-20 years from target identification to drug approval is not rare (Figure 1).

Figure 1. Illustration of the discovery process (pre-clinical studies and clinical trials) for a hypothetical drug, where target validation is followed by identification and characterization of hits and leads. The figure also illustrates drug attrition (black area), starting with 10,000 compounds, a number that is highly reduced to 250 lead compounds and further 5 candidate drugs (CDs) in different developmental processes, and finally results in 1 approved drug.

The starting point for drug discovery is often the identification and validation of a target that is somehow connected to a diseased state, often an enzyme or a receptor, involved in important processes in the human body or in a pathogenic virus or bacteria. For identification of compounds that interact with the target, the so-called hit/lead selection, different approaches can be used, for example screening of big compound libraries or a more rational approach, where target structure and mechanism is the basis for design of leads. This is followed by characterization of structure/activity and struc-
ture/kinetic relationships, SAR and SKR, and pharmacokinetics, involving studies of ADME (absorption, distribution, metabolism, and excretion) characteristics, and pharmacodynamics, evaluating biochemical and physiological drug effects. *In vitro* analyses, cell-based assays and animal models in the pre-clinical phase are later, for the most potent candidate drugs (CDs), combined with human testing and drug evaluation in clinical trials. The number of compounds is highly reduced in the drug discovery process, which, at the best, ends up with a drug approval.

In addition to long time, the drug discovery process is also very costly, ending at billions of US dollar for each new approved drug, which is to a great extent the result of the high attrition rate [1]. The most common causes for excluding drugs today are problems with efficacy and toxicity, however poor pharmacokinetics and low drug availability also contribute [2]. In order for the pharmaceutical industry to reduce time and cost, efficient optimization procedures and suitable developmental strategies, preferably applied in pre-clinical studies, are required. The purpose of this thesis is to identify biochemical strategies, based on analysis of inhibition and interactions, for drug discovery against hepatitis C.
Hepatitis C

In 1975, a new virus was found in patients with a liver disease and who had received a blood transfusion. It was first described as non-A, non-B hepatitis virus, until 1989, when it was identified and classified as hepatitis C virus (HCV) [3]. HCV is the causative agent of hepatitis C and also the most common cause of chronic liver disease (cirrhosis and liver cancer) in the Western world [4].

Prevalence and transmission

About 3% of the world population is estimated to be infected with HCV, and as many as 130-200 million are chronically infected [5]. The global prevalence and distribution varies, with the number of cases being highest in Africa and Eastern Asia.

The virus is transmitted through blood-to-blood contact. Common sources for getting infected used to be hemodialysis, blood transfusions, and organ transplantation. Thanks to the highly controlled screening that started in the 1990s, the risk is much lower today, especially in developed countries. However, among intravenous drug users and in other situations where sharing of needles is common, the risk of transmission is still very high.

Progression and symptoms of infection

HCV infections mostly afflict liver cells, but there have been reports of HCV replication in other tissues, such as the central nervous system and lymph nodes [6, 7]. The course of infection is a dynamic process. During the acute phase (1-2 weeks post-infection) viral RNA appears in the blood and the levels of alanine aminotransferase (ALT) are elevated, which is an indication of liver damage [8, 9]. The initial infection is often asymptomatic but symptoms, such as fatigue, loss of appetite, skin rashes, and jaundice, can occur [9].

About 20% of infected individuals recover from hepatitis C, meaning that viral RNA is completely cleared from the body. However, in the remaining cases, the infection proceeds into a chronic phase. Although the chronic infection can be asymptomatic, approximately 15% develop liver cirrhosis
(10-20 years post-infection) [10], which is fibrosis or scarring of the liver caused by the death of hepatocytes. This prevents blood from passing through the liver and leads ultimately to liver failure. It is clearly seen as an accumulation of toxins in the blood. 1-5% of cirrhotic patients develop liver cancer with death as common outcome [11]. Transplantation is often the only solution when dealing with severe liver cirrhosis, but this is not a permanent solution since there are often problems with re-infection of the new organ [5].

Disease progression and severity is not completely understood. It is very individual. A healthy lifestyle can have a great impact on the disease once infected. Excessive alcohol intake and co-infection with other viruses, such as the hepatitis B virus (HBV) and the human immunodeficiency virus (HIV) are major risks for development of liver cancer [10].

Treatment

The ultimate goal for treatment of HCV is to totally eradicate the virus. The standard treatment for chronic hepatitis C today is an indirect combination therapy, using pegylated interferon (IFN) and ribavirin. IFN is a chemical messenger that induces an immunological response upon viral infection [12]. By adding extra interferon, the body’s immune system is improved and has a better ability to inhibit the viral infection of other cells. HCV has however mechanisms to evade the cytokine response of the body. By combining IFN with ribavirin, the chances to get rid of the virus are at least doubled. Ribavirin is an antiviral drug affecting a combination of mechanisms such as the immunological response, the viral replication, and/or viral mutagenesis by reducing the fitness of the virus [13, 14]. However, the drawbacks with this combination therapy are high cost, long treatment times, severe side-effects, and the fact that it is not effective for all HCV infections. Vaccines or drugs directed against host- and/or HCV proteins involved in the viral life cycle (direct acting antivirals, DAAs) are alternatives to the indirect viral inhibition using IFN and ribavirin.

In patients suffering a secondary HCV infection, after viral clearance of a primary infection, the immunological response is changed [15, 16]. This supports the existence of an induced memory function and is encouraging for the possibility to develop HCV vaccines. However, vaccines have unfortunately been shown to affect the evolvement of virus that can escape the immune system [17-19]. To date, there are no vaccines with full potential to clear HCV.
Hepatitis C virus

HCV is a RNA virus belonging to the *Flaviviridae* family of viruses [20]. Its genome consists of a 9.6 kb single stranded (+) RNA which is composed of a 5’-non coding region (NCR), an open reading frame (ORF) that encodes a 3000 amino acid polyprotein, and a 3’-NCR. The 5’-NCR is highly conserved and contains an internal ribosome entry site (IRES) that enables cap-independent translation [21, 22]. The genome is surrounded by a nucleocapsid and a highly glycosylated envelope [23]

The viral life cycle

Viral particles in blood serum form aggregates with lipoproteins of low- or very low density. It is thought that cell entry is guided through interactions between the lipid protein aggregates and host cell receptors, followed by recognition through other cellular receptors. For example the tight junction proteins occludin and claudin-1, the scavenger receptor class B type 1 (SR-B1), and tetraspanin (CD81) are known to be involved [24, 25].

![Image of the HCV life cycle](image)

**Figure 2.** The HCV life cycle within the hepatocyte (A) endocytosis, (B) RNA release, (C) translation and genetic processing into viral proteins, (D) RNA synthesis, and finally (E) maturation and assembly and (F) release of new particles.

The HCV life cycle is illustrated in **Figure 2**. Following receptor-mediated endocytosis (A) the virus is uncoated and the viral RNA is released (B) [26]. The genome is translated by the ribosomes and generates a viral polyprotein (**Figure 3**), associated with the ER membrane. The polyprotein is processed, co- and post-translationally, by host and viral proteases and the result is ten
mature viral proteins (C) [27-29]. Some of these proteins build up the repli-
cation machinery in the membranous web, which is responsible for the syn-
thesis of new viral RNA (D) [30]. This RNA is then enclosed and assembled
into viral particles (E), which are matured and later leave the cell through
exocytosis (F). To date, the regulation of translation, replication and packa-
ging is unknown.

The viral proteins
Processing of the polyprotein (Figure 3) by ER peptidases and viral protease-
es generates three structural proteins (core protein C, and the envelope pro-
teins E1 and E2) and seven non-structural (NS) proteins (p7, NS2, NS3,
NS4A, NS4B, NS5A, and NS5B).

Figure 3. The HCV polyprotein. Arrows indicate the cleavage sites of host proteases
(thin arrows), the viral NS2-3 autoprotease (medium arrow), and the viral NS3 pro-
tease (fat arrows).

The structural proteins are involved in the formation of the new viral parti-
cle. The core protein (C) forms the viral nucleocapsid, and the envelope pro-
teins (E1 and E2), both highly glycosylated, form together the viral outer
surface [31-33]. The non-structural proteins are all involved in the replica-
tion of the genetic material.

The p7-protein has ion channel activity and is essential for viral infection
[34-36]. NS2-3 is a unique type of cysteine protease, with the catalytic activ-
ity situated in the C-terminal half of NS2 and in the N-terminal one-third of
NS3. The result of NS2-3 action is the autocleavage of the NS2-NS3 junc-
tion. [37-39]. NS3 has several functions. It serves both as a serine protease,
cleaving the downstream polyprotein junctions, and as a RNA heli-
case/NTPase, involved in the unwinding of double stranded RNA at the cost
of NTP [40]. The NS4A polypeptide functions as a cofactor for the NS3
protease, enhancing its activity [41, 42]. NS4A is also responsible for the
anchoring of NS3 to the ER membrane. NS4B is a transmembrane protein
that induces membrane alteration necessary for the viral replication [30, 43].
NS5A is a phosphoprotein hypothesized to be involved in the modulation of
viral replication and virus assembly [44, 45]. NS5B is an RNA-dependent RNA-polymerase that is responsible for the synthesis of new viral RNA [46].

In addition to the above mentioned viral proteins, there is another protein which is processed from an alternative reading frame (ARF) overlapping the core region [47]. This protein, usually called the F- or ARF-protein, is known to be expressed during infection, but its function is yet unknown. It is however speculated to be involved in the development of severe liver diseases [48, 49]. Additionally, the position of the F-protein in the beginning of the genome makes it ideal for regulation events.

HCV genotypes, subtypes and quasi-species

Hepatitis C is classified as a single disease but the infection can actually be caused by different variants of HCV. There are today eleven known genotypes of HCV, of which six (1 to 6) are more common than the others [50]. There are big differences in the genetic material of the different genotypes, with only about 65% sequence identity. The different genotypes are further divided into subtypes (1a, 1b, etc.), which share approximately 75% sequence identity [50].

Different genotypes have different global distribution. According to the World Health Organization (WHO), the most common genotype 1 predominates in Europe, North America, and Japan. Genotype 2 is found in similar regions as genotype 1, however much less frequent. Genotype 3 predominates in south-east Asia, genotype 4 in the Middle East, Egypt, and central Africa, whereas genotype 5 is almost exclusively represented in South Africa. Genotype 6 and other minor genotype groups are principally found in Asia.

HCV is a rapidly replicating virus, with approximately $10^{12}$ viral particles produced each day during a chronic infection [51]. In addition, HCV is very error prone since the viral polymerase lacks a proof-reading mechanism [51]. These two features contribute to a rapid viral evolution, creating a heterogeneous population of the virus with single and multiple mutations in the genetic material. These minor genomic alterations generate even more HCV variants, known as quasi-species [50].

Normally, a patient is infected by a single viral genotype, which does not change during the infection. However, since the virus has a high rate of mutation, it is not rare that the infected carries several quasi-species.

The importance of the genotype for pathogenesis is still controversial since environmental, genetic and immunological factors may contribute to the diseased state. However, there are studies that suggest a correlation between genotype 3 and more rapid progression of liver fibrosis [52, 53]. Spontaneous viral clearance is also hypothesized to be genotype dependent [54]. The outcome of HCV treatment, using IFN and ribavirin, is however
highly dependent on the infection genotype. Only 50% of patients with genotype 1 get a sustained virologic response (SVR), which is a measure of the viral load (viral RNA) in the blood, whereas for people infected with genotype 2 and 3 the SVR is 80% [55-59].
HCV drug discovery

Drug targets
In principle all proteins involved in HCV replication are potential targets for direct acting antivirals. Host-related factors involved in viral entry (SR-B1 and CD81), HCV replication (cyclophilins), and regulation of gene expression (microRNA-122) are also targets for viral inhibition [60, 61]. Additionally, an alternative strategy for inhibition of viral entry, assembly and release is to interfere with lipoprotein components [60].

There are today several drugs in clinical trials, directed against a variety of HCV targets. However, direct-acting drugs have shown to affect the emergence of drug-resistant variants of HCV, influencing the heterogeneity of the viral population (reviewed in [62]). This is a major problem in the development of new antiviral therapies. In order to reduce the risk for a selection of drug resistant HCV populations, one therapeutic approach is to use a combination of drugs directed against different targets, both viral and host ones.

Another challenge for HCV drug discovery is the difference in treatment outcome among the structurally different viral genotypes. An important goal is therefore to design drugs with inhibitory structures which are less affected by viral mutations and genetic differences.

Viral enzymes as drug targets
Due to their important physiological roles in all life forms, and the possibility to specifically inhibit their catalytic functions by small molecules, enzymes are excellent drug targets [63].

Among the enzymes encoded by HCV, the NS3 protease and the NS5B polymerase have gained most interest as drug targets. A number of potential drug-like compounds targeting these enzymes have been identified and many have reached clinical trials. But only NS3 protease inhibitors have yet been approved for clinical use. NS3 is also the focus of this thesis.
Enzyme kinetics

In an enzyme catalyzed reaction, a substrate (S) interacts with an enzyme (E) generating a product (P) [64]. This interaction can in the simplest case be described as

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_{cat}} E + P \xleftarrow{k_{-1}}
\]

ES is the complex formed between the enzyme and the substrate, which is a prerequisite for catalysis. The reaction conversion of substrate into product can be described by the Michaelis-Menten equation according to

\[
V_0 = \frac{d[P]}{dt} = \frac{[E]_T \cdot k_{cat} \cdot [S]}{K_M + [S]}
\]

\([E]_T\) is the total enzyme concentration, \(k_{cat}\) is the turnover number (or rate constant of product formation at saturating substrate concentrations) and \(K_M\) approximates the affinity for the substrate. Enzymes are often characterized based on their catalytic efficiency (\(k_{cat}/K_M\)).

The interaction between an enzyme and an inhibitor (I) can in the simplest case be described as

\[
E + I \xrightarrow{k_{on}} EI \xleftarrow{k_{off}}
\]

This scheme illustrates a simple reversible 1:1 interaction model where the EI is the complex formed between the enzyme and the inhibitor. The effectiveness of the interaction can be described using the equilibrium dissociation constant, \(K_D\). These can be defined by the association and the dissociation rate constants, \(k_{on}\) and \(k_{off}\), as illustrated by

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

The inhibition constant, \(K_i\), is commonly used as an equivalent of \(K_D\), but determined indirectly by the effect of the inhibitor on the catalytic activity. The lower the value of \(K_i\) or \(K_D\) the lower the amount of inhibitor is needed for reducing enzyme activity and the higher is the strength of the complex
interaction. $K_i$ is very useful for evaluation of the inhibitory potency of a compound, while both $K_i$ and $K_D$ can be used for characterization of the affinity of enzyme-inhibitor interactions.

Many enzyme-inhibitor interactions are however more complex than what can be described by a 1:1 interaction model. The simple model can easily be expanded so that it accounts also for complexities, such as conformational changes and molecular heterogeneity (Figure 4).

![Figure 4](image)

**Figure 4.** Interaction scheme illustrating a standard 1:1 interaction between an enzyme (E) and an inhibitor (I). The scheme also illustrates the induced fit or conformational change between EI and E*I, and the selected fit where the inhibitor has the possibility to selectively interact with both E and E* to form EI or E*I.

**Vitality values**

For comparative analyses of inhibitory effects ($K_i$) of lead compounds in their interactions with different enzyme variants, vitality values ($V$) are commonly used [65]. This approach accounts for possible differences in catalytic efficiency for the enzymes involved in the analysis, and uses one form of the enzyme as reference for the other variants. Vitality values can be described by

$$V = \frac{K_i \left( \frac{k_{cat}}{K_M} \right)_{var}}{K_i \left( \frac{k_{cat}}{K_M} \right)_{ref}}$$

**The NS3 protein**

NS3 is considered to be a very good target for direct acting HCV drugs, since it has a central role in the viral life cycle. Its role in cleaving the junctions between all the downstream proteins of the HCV polyprotein, thus releasing the functional proteins involved in replication, is clearly critical. In addition, the protease is also responsible for down-regulating the innate immune response of the host cell [66, 67].
Due to the high drugability of proteases and the availability of effective heterologous expression systems and reliable enzyme assays, NS3 is also easily studied at a biochemical level. The NS3 protein consists of about 630 amino acids. It is a multifunctional protein harboring both helicase/ATPase and protease activities (Figure 5). The helicase/ATPase domain constitutes two thirds of the protein (400 residues) and its activity is involved in the separation of dsRNA, using ATP as energy source. However, the mechanism by which the helicase is working is not completely understood. The other domain, constituting one third of the protein, is a protease that is involved in the cleavage of the viral polyprotein. It has been shown that the two enzymatic domains are interdependent since they both enhance the activity of each other [68, 69]. The covalent attachment of the helicase to the protease has shown to have strong influence on protease activity, suggesting a structural cooperation [68]. In addition, the protease is assumed to act as a cofactor for the helicase allosterically, stimulating the binding and unwinding of RNA [69].

Figure 5. The NS3 protein (PDB: 1CU1) highlighting the helicase domain in red, the protease domain in green and the NS4A cofactor in blue. The structural zinc and the ATP-binding site of the helicase are illustrated as black spheres. The black frame indicates the position of the protease substrate binding site with the catalytic triad in magenta.
NS3 protease

Substrate specificity

The substrate binding pockets or subsites of proteases are named S₁, S₁’, S₂, S₂’ and so on according to the terminology by Schechter and Berger [70] (Figure 6). Similarly, the residues of the substrate/peptide are named P₁, P₁’, P₂, and P₂’. The prime notations indicate C-terminal residues in reference to the cleavable peptide bond (the scissile bond). This nomenclature will be used throughout this thesis.

The formation of the protease-substrate complex involves hydrogen bonding as well as electrostatic and hydrophobic interactions. Generally, proteases have the tendency to recognize substrates with extended β-strand conformations, where the specificity is defined by the amino acids lining the subsites of the substrate binding site [71, 72]. Upon proteolytic cleavage the structure of the peptide is changed, which enables product release. A common approach for structure-based drug design of protease inhibitors is to modify a substrate and incorporate residues that stabilize the structure and enhance the affinity for the enzyme. Such compounds are known as peptidomimetics.

The consensus sequence of the cleavage sites of NS3 is presented in Figure 7. This is represented by an acidic residue (aspartic or glutamic acid) in P₆, a cysteine or a threonine in P₁, a serine or an alanine in P₁’, and a hydrophobic residue (H) in P₄’. The most important residue for substrate recognition is P₁ [70, 73, 74].
Figure 7. The cleavage sites and consensus sequences of NS3 protease from genotype 1b [73]. Upon cleavage of the scissile bond (arrows), N- and C-terminal products are generated.

Structure

The 3D-structure of the protease domain of NS3 was solved in 1996 [75, 76]. It revealed that the protease is stabilized by a zinc (Zn$^{2+}$) ion (Figure 5) [76]. This zinc is also important for the structure of the NS2-3 autoprotease, but neither of the two proteases use it for catalysis; it simply serves a structural role, similar to the disulfide bond that is conserved in other chymotrypsin-like serine proteases [77, 78].

The NS3 structure is further stabilized by complex formation with the 54 amino acid long NS4A protein, which contributes a missing β-strand to the NS3 structure (Figure 5). This interaction enables perfect positioning of the catalytic amino acids within the active, which enhances the catalytic activity of the enzyme [42]. Additionally, due to a hydrophobic N-terminal tail of NS4A, the heterodimeric NS3/NS4A complex is anchored to the ER membrane. This is of importance for NS3 function since it brings the protease in close proximity to the substrates that are also anchored to the ER membrane, but it also protects the protease from cellular degradation [41, 79].

Mechanism of action

The catalytic triad of the NS3 protease consists of His-57, Asp-81 and Ser-139 (Figure 8). NS3 acts via a ping-pong mechanism, in which the peptide substrate binds to the enzyme first. The C-terminal part of the peptide
(Figure 7) is released before the second substrate (water) binds, releasing the second product, the N-terminal part of the peptide (Figure 7).

![Figure 7](image1)

Figure 8. The catalytic triad of NS3, consisting of His-57, Asp-81 and Ser-139.

**Discovery of NS3 protease inhibitors**

The design of NS3 protease inhibitors took off in 1998, with the finding that the enzyme was inhibited by its own product [80, 81]. Serine proteases are often inhibited by millimolar concentrations of their C-terminal cleavage product, but in the case of the NS3 protease, it was the N-terminal cleavage products acted as competitive inhibitors with affinities in the micromolar range [80]. Optimization of these N-terminal peptides finally resulted in a potent inhibitor, Ac-Asp-D-Gla-Leu-Ile-Cha-Cys-OH, with nanomolar affinity (Figure 9) [79]. However, considering the size and peptidic nature of this hexapeptide, further optimization was required.

![Figure 9](image2)

**Figure 9.** Ac-Asp-D-Gla-Leu-Ile-Cha-Cys-OH (N-1725) (Gla: carboxy-Glu, Cha: cyclohexyl-Ala)
In general, peptides are not suitable as drugs. They are the substrates of proteases and are thus easily hydrolyzed and degraded. In addition, they exhibit poor bioavailability and are easily excreted. Peptidomimetics with increased bioavailability, and retained or improved biological activity have proven to be useful for HCV drug discovery.

A benefit of using inhibitors that are substrate-like is that the risk of drug-resistance is reduced [82]. The virus may otherwise develop resistance to its own substrate, which would be self-limiting [83]. Another benefit of interfering with the function of the protease is to help the immune response, since NS3 is known to be involved in the inhibition of IFN signaling and immune system interference [67, 84]. Therefore, inhibition of the protease has a dual approach; blocking replication as well as helping out in spontaneous viral clearance.

Peptidomimetic NS3 protease inhibitors
When modifying a natural peptide into a more drug-like compound, it is important to determine characteristics and roles of the different parts of the peptide. The length of the peptide and the required features of the N- and C-terminal residues are typically evaluated before the amino acid side chains and the peptide backbone are modified.

When redesigning the N-terminal cleavage products of NS3 protease into drug-like peptidomimetic inhibitors there were several characteristics of the initially identified peptides that were changed. The length of the peptides was problematic since an acidic side chain in P6 was required in order to pick up a strong electrostatic interaction with the enzyme. However, as the other part of the inhibitor was modified so that it contributed more to the affinity, it was possible to use a shorter peptide backbone.

Non-covalent inhibitors
One of the strategies that enabled the reduction of the length of the peptide backbone was the modification of the C-terminus, such as replacing the P1 cysteine for larger and even reactive groups. By also introducing unnatural amino acids into the peptide structure, as well as macrocyclic structures, it was possible to truncate the peptide chain. This also contributed to increased stability, membrane permeability, and biological activity.

BILN-2061 (ciluprevir, Figure 10) was discovered in 2003. The design of this inhibitor involved the replacement of the P1 cysteine by the vinylaminocyclopropane carboxylic acid, which resulted in dramatic improvement in potency [85-87]. The tripeptidic structure of this compound was made possible by introducing a macrocycle, which combines the P1- and P3-substituents, and a large P2 substituent. BILN-2061 was the first inhibitor in its class to reach clinical trials, where it showed important proof-of-concept [87]. It was however withdrawn due to cardiac toxicity in animals [88].
Another approach for reducing the length of the peptide backbone, was the C-terminal extension of an acyl sulfonamide group, which made it possible to more easily vary the P₁-substituent for elongation into the prime side of the enzyme [89]. Many clinical lead compounds (Figure 11), such as ITMN-191 (danoprevir), MK-7009 (vaniprevir), and TMC-435 (simeprevir), have been designed by this approach.

**Mechanism-based inhibitors**

Mechanism-based inhibitors are characterized by an electrophilic C-terminal group, such as an aldehyde, α-ketoamide, or α-ketoester, which forms a co-
valent but reversible bond with the catalytic serine [90]. These inhibitors are therefore reactive, and thus different to those designed initially. Two mechanism-based inhibitors (Figure 12), VX-950 (telaprevir) and SCH 503034 (boceprevir) were recently approved for treatment of HCV genotype 1.

Figure 12. Mechanism-based NS3 protease inhibitors in clinical use.

NS3 model systems

Elucidation of the characteristics of NS3 and the mechanistic details of its interaction with inhibitors is fundamental for design and optimization of lead compounds. For this purpose it is critical to have suitable model systems and methods that provide reliable and relevant information.

Characterization of enzymatic properties is typically performed using a native or modified variant of the enzyme and experimental conditions, selected with respect to high enzymatic activity and stability. The enzyme variant is ideally the naturally occurring form, but practical aspects, concerning the possibility of producing large amounts of stable protein, often make it necessary to use a modified form of the enzyme. This typically involves tagging, truncation or substitution of individual residues. The experimental conditions are based on standard conditions for biochemical studies, thought to be physiologically relevant. However, these can easily be modified regarding the buffer composition and other components, such as enzyme cofactors and stabilizing agents. For the purpose of drug discovery, the divergence from the natural system, both regarding the protein and the experimental conditions, should however be minimized in order for the assay to have physiological relevance.

Assays for evaluation of NS3 inhibitors

The elucidation of the mechanisms of viral entry, the viral life cycle, the role of individual viral proteins and the molecular details of pathogenesis was for a very long time hampered by the lack of suitable model systems for study-
ing HCV. The use of cell culture systems were not very efficient due to low viral replication, and the only animal model that could be used was the chimpanzee, which was of both ethical and economic concern [91]. The development of heterologous expression systems, the replicon system and the possibility to produce recombinant infectious virions have revolutionized the research around HCV. Today, there are several assays and model systems that can be used to study HCV, both in vivo and in vitro.

In order to fully understand the molecular and biochemical features of NS3 and to evaluate NS3 protease inhibitors, a variety of assays, providing kinetic, mechanistic, and functional information, need to be used. Kinetic information can be accessed via indirect inhibition assays or by using direct enzyme-inhibitor interaction analyses, where the latter alternative also provides mechanistic information. For further evaluation of the inhibitory functionality, i.e. the effect on HCV replication, a cellular system as in the replicon assay can be used. For evaluation of the physiological potential of leads, pharmacokinetic profiling is also valuable.

Enzyme inhibition analysis

Inhibition of NS3 can be studied using enzyme activity/inhibition assays. Fluorescence resonance energy transfer (FRET) methodology is commonly used since it can be very sensitive. It utilizes the transfer of excitation energy between two chromophors to describe interaction events. Inhibition analysis based on FRET is conveniently used for measurements of protease activity and inhibition.

![Figure 13](image)

**Figure 13.** Illustration of a protease activity assay utilizing FRET. The fluorescent signal is quenched as long as the peptide substrate is intact but is detectable upon substrate cleavage.

A peptide, which resembles the natural enzyme substrate, is labeled with an excitable fluorescent group and a quenching group, at the distal ends of the peptide (**Figure 13**). In the non-cleaved substrate the signal is quenched since the fluorescent and the quenching groups are close together. However,
when the substrate is cleaved, the two groups are separated and a fluorescent signal can be detected (Figure 13). This allows time-resolved measurement of product formation.

Interference with the activity of the protease, by for example addition of an NS3 inhibitor, will thus affect the fluorescent signal. Activity changes can in this way be used to characterize the catalytic properties of the enzyme, its dependence on the experimental conditions as well as inhibition characteristics.

Enzyme-inhibitor interaction analysis

In order to avoid some of the complexities that occur in an activity-based assay, an alternative strategy for evaluation of NS3 protease inhibitors is the use of a surface plasmon resonance (SPR) biosensor-based assay.

The SPR-based biosensor is a suitable tool for HCV drug discovery since it is useful for both characterization of enzyme-inhibitor interactions as well as for the evaluation of inhibitor pharmacokinetics (see below). The provided kinetic and mechanistic information can reveal important aspects for structure-based drug design, which is commonly used for NS3 protease inhibitors.

SPR interaction analysis

SPR is an optical phenomenon occurring under total internal reflection of polarized light, at the interface between two media with different refractive index. At a certain angle of the incident light (the SPR-angle), the light is absorbed instead of being reflected. This is due to electromagnetic waves (surface plasmons) arising at the boundary (sensor surface) between the two media. Changes in the refractive index caused by changes in mass near the sensor surface will change the detected SPR-angle [92].

SPR-based interaction analysis has the advantage of being a time-resolved method that can provide kinetic, structural, thermodynamic, and chemodynamic information about the interaction between the ligand and the target without the need for a substrate. It allows the use of different protein constructs even with poor catalytic efficiencies. Proteins can even be immobilized directly from cell lysates, which can avoid problems in producing pure and stable protein at high concentrations. Another possibility is to study the protein in a more natural environment, such as embedded in a lipid membrane.

A commonly used sensor surface consists of a glass plate covered with a thin film of gold positioned in a microfluidic system (Figure 14). Attached to the gold film is a thin layer of dextran, which is functionalized with carboxyl groups. By using different immobilization techniques, molecules can be covalently or non-covalently attached to the dextran. The continuous flow system allows injections of another molecule (analyte) over the immobilized
ligand surface. The changes caused by ligand-analyte interactions, are directly monitored.

**Figure 14.** Illustration of an SPR-based biosensor.

The SPR signal, measured in resonance units (RU), can thus be used to study real-time interactions between molecules. The interaction is visualized in a sensorogram which describes the signal as a function of time (**Figure 15**).

**Figure 15.** Schematic for a typical interaction analyzed using an SPR-based biosensor. The analyte is injected over the surface of immobilized ligand during the association phase. The dissociation is recorded after when only buffer flows over the surface.
**SPR data analysis**

Multiple injections of different analyte concentrations are often presented in an overlay sensogram (Figure 16). From the curvature of the sensogram, during the phase of analyte injection (association) and also after the injection has been stopped (dissociation), kinetic constants ($k_{on}$, $k_{off}$ and $K_D$) can be determined. If the association and dissociation phases display sufficient curvature i.e. are slower than the rate of diffusion and fast enough for a dissociation to be quantified, data can be used for global non-linear regression analysis using a suitable interacting model. Such analysis can also provide the stoichiometry of the interaction, as well as information of more complex interaction mechanisms.

For very rapid interactions a steady-state approach for determination of affinity is usable, provided that analyte concentrations higher than the $K_D$-value can be used. A saturation plot illustrating the relationship between analyte concentrations and equilibrium signals is shown in **Figure 16**.

![Overlay sensorgram (left) and the corresponding saturation plot (right).](image)

**Figure 16.** Overlay sensorgram (left) and the corresponding saturation plot (right).

As compared to an enzyme activity-based assay where the catalytic properties are fundamental for inhibition analyses, an SPR biosensor-based assay only requires enzyme functionality in the sense that it has to be correctly folded. Catalytic activity as such is not required. Functionality can be optimized by using suitable immobilization methods, buffer conditions and by adding components such as salt, detergent, and reducing agents. The degree of functionality can be determined by use of reference compounds for which interaction characteristics are known. The protein and the soluble analyte can also be kept untagged.
Cell-based analysis

**The HCV replicon model**

For evaluation of inhibitory effects on HCV replication, the replicon model system is useful. It can provide information on RNA synthesis in a cellular environment. This model system was developed in 1999 on the basis of the HCV 1b genotype [93]. For HCV replication to be followed in human hepatoma cells, the viral genome had to be genetically modified, and the genes for the structural proteins were removed. Additionally, a marker for resistance (Neo), as well as an additional IRES (E-IRES, from the encephalomyocarditis virus), were added (Figure 17).

![Figure 17](image)

Figure 17. Illustration of the HCV replicon genome expressing NS3-5B. E-IRES directs the translation of the NS proteins whereas HCV-IRES directs the development of resistance to neomycin (Neo).

Genome translation, initiated by E-IRES, results in cell expression of nonstructural proteins, whereas the HCV-IRES directs the production of neomycin phosphotransferase, which allows the cells to develop resistance to neomycin. In this way, replicon transfected cells harboring the replication machinery can be selected. It is therefore possible to induce the synthesis of viral RNA (replicons) in cell culture. Adaptive mutations in the replicon were further identified to improve RNA replication [94, 95]. Many of these mutations have not been identified in viral isolates from infected patients and are thus hypothesized as in vitro cell culture specific.

Since the report of the first replicon assay, different sub-genomic RNA structures have been developed and used. There are also reports of replicons from genotypes 1a and 2a, and the use of replicons in different cell lines [96]. In addition, the replicon model system have also been used for identification of drug-resistant variants of HCV [97].

For evaluation of NS3 protease inhibitors, the replicon assay is very useful, since it resembles a more complex system, as compared to the previously mentioned systems. It therefore represents a physiologically more relevant assay. The inhibitory potential is often evaluated by measuring the inhibitor concentration required to reduce replication by 50% (EC50).
Pharmacokinetic profiling

The evaluation of lead compounds also involves pharmacokinetic profiling, which explains how the body affects the drug once it has been administered to the body (Figure 18).

**Figure 18.** Schematic view of the fate of a drug once administered to the body. As long as the drug stays bound it stays in the body, since only the free drug can be metabolized and excreted.

The steps involved include absorption of the drug into the blood and its distribution to the site of action, but also its metabolism and excretion. These characteristics are usually summarized as the ADME characteristics, where ADME stands for absorption, distribution, metabolism, and excretion.

Absorption defines the drug bioavailability, which for an orally administered drug accounts for solubility, chemical stability and membrane permeability. Drug distribution in blood is to great extent determined by interactions between drug and proteins in serum. Metabolic aspects concern drug stability accounted by for example the drug half-life and degradation, whereas excretion properties involve processing of metabolites and how the drug is eliminated from the body.

*In silico* methods as well as *in vitro* and *in vivo* analyses are very useful tools for prediction of these drug characteristics, which all have a tremendous impact on drug discovery, both regarding attrition and cost.

**Serum protein binding**

The distribution parameter within ADME involves binding of drug compounds to serum proteins, which serve to transport the drug to its target. The binding is positive in the sense that the drug cannot be metabolized as long as it stays bound to these proteins. Serum proteins therefore serve as a drug reservoir. However, serum protein interactions must not stop the drug from its interaction with its specific drug target. Preferable characteristics of serum protein interactions are therefore rapid kinetics with efficient loading and unloading of drugs.
Present investigation

Aim

The overall aim of the present investigation was to provide tools, strategies, and information of value for pre-clinical drug discovery against hepatitis C. It has focused on characterization of inhibitory effects and kinetic interaction properties of NS3 protease inhibitors.

The first paper describes activity-based studies of different variants and genotypes of NS3. In this study the importance of accounting for differences in catalytic properties and selecting suitable experimental conditions and reliable model systems for evaluation of NS3 protease inhibitors is illustrated. In paper two, the same protein variants and inhibitors were analyzed by an SPR biosensor-based assay. This complementary strategy for evaluation of NS3 inhibitors has lower system complexity and enables a direct monitoring of the interactions, providing information on interaction mechanisms and kinetics. The application of the inhibition analysis is shown in the third and the fourth papers. These deal with strategies for design and synthesis of novel NS3 protease inhibitors with reduced sensitivity to a set of drug resistant mutations currently identified. The final paper provides a new strategy for evaluation of interactions between drugs and serum proteins, of relevance for interpretation of drug distribution in the body.
Results

Evaluation of NS3 protease inhibitors

There are several naturally occurring variants of NS3, represented by different viral genotypes and quasi-species. In addition, researchers use several different NS3 variants, which are genetically modified in order to simplify expression and purification of the enzymes. All of these variants can be used for evaluation of inhibitors but can be expected to give slightly different results. However, the magnitude and nature of the differences has not been known since a detailed comparative study has not previously been performed.

This has been the goal for Papers I and II. A panel of enzyme variants, all relevant for HCV drug discovery, was therefore produced. It encompassed the full length (fl) variants for genotypes 1a, 1b and 3a, an NS3/4A co-construct for genotype 1a and 1b, and the truncated protease domains (pd) of genotypes 1a and 1b. For resistance profiling in Paper I the resistant variant R155K for genotype 1a was also included.

The set of compounds chosen for evaluation represented structurally and mechanistically different NS3 protease inhibitors. Ac-Asp-D-Gla-Leu-Ile-Cha-Cys-OH (N-1725, Figure 9) is a hexapeptide analogue of the N-terminal cleavage product. BILN-2061 (Figure 10) and ITMN-191 (Figure 11) are macrocyclic inhibitors with individually optimized backbone structures, as well as different side chains and C-terminal groups. The two approved mechanism-based linear inhibitors VX-950 and SCH 503034 (only evaluated in Paper II) were also studied (Figure 12).

Characterization of NS3 inhibition (Paper I)

The goal in Paper I was to define experimental conditions and procedures for evaluation and comparison of NS3 protease inhibitors against a panel of variants of NS3, differing in their catalytic properties.

The catalytic efficiency was defined for each of the enzyme variants (Table 1). The choice of detergent as well as the concentrations of detergent, NaCl and glycerol in the assay buffer had a strong effect on the catalytic efficiency and kinetic properties of the enzyme. This can be attributed to differences in the physico-chemical properties of the proteins as well as dif-

36
ferential effects in the structural dynamics of the proteins and their interactions with the substrate and the NS4A cofactor. When the catalytic properties of the enzyme variants were determined, under for each enzyme variant optimized conditions, it was revealed that their characteristics varied significantly.

For example, the full length enzyme from genotype 1b was the most catalytically efficient enzyme, primarily due to a relatively high rate of catalysis. The full length variants from genotype 1a and 3a were similar. For the 1b genotype the co-construct with NS4A had a dramatically reduced catalytic efficiency, while it had little effect for the 1a genotype. Similarly, truncation of the NS3 protein reduced the catalytic efficiency only of the genotype 1b enzyme. It was expected that it is important to account for these differences in the catalytic properties of the enzymes when they are used for comparative evaluation of inhibitors.

Inhibition of NS3 variants
Once optimized conditions for each enzyme variant had been identified, the inhibitory effects ($K_i$) were determined. The differences in the inhibitory constants showed that the inhibitory effect is highly dependent on the protein variant as well as the experimental conditions (Table 1). All inhibitors were generally more efficient for the full length enzymes of genotype 1a and 1b than for genotype 3a. This was most evident for ITMN-191, where $K_i$ was more than 700 (1a) and 500 (1b) times higher than for the 3a genotype. Truncation of NS3 had great effect on all inhibitors for the 1b genotype however not for the 1a genotype. The reduced activity for 1b may be explained by the reduced catalytic efficiency for this variant. Thus, the selection of model system and the experimental conditions will affect the evaluation of inhibitors.

Table 1. Catalytic efficiency ($k_{cat}/K_M$) and inhibition ($K_i$) data for different enzyme variants and genotypes (GT) of NS3, determined in reference (R) and optimized buffer (O).

<table>
<thead>
<tr>
<th>Enzyme variant</th>
<th>GT</th>
<th>$k_{cat}/K_M$ ($\mu M^{-1}s^{-1}$)</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N-1725</td>
<td>BILN-2061</td>
</tr>
<tr>
<td>N33pd</td>
<td></td>
<td>R</td>
<td>O</td>
</tr>
<tr>
<td>N33n</td>
<td>1a</td>
<td>0.71</td>
<td>10.1</td>
</tr>
<tr>
<td>N33fl R155K</td>
<td></td>
<td>1.59</td>
<td>6.02</td>
</tr>
<tr>
<td>N33fl/4A</td>
<td></td>
<td>11.7</td>
<td>11.0</td>
</tr>
<tr>
<td>N33fl</td>
<td></td>
<td>0.86</td>
<td>5.1</td>
</tr>
<tr>
<td>N33pd</td>
<td>1b</td>
<td>0.08</td>
<td>0.25</td>
</tr>
<tr>
<td>N33n</td>
<td></td>
<td>6.27</td>
<td>33.3</td>
</tr>
<tr>
<td>N33fl/4A</td>
<td></td>
<td>0.93</td>
<td>5.2</td>
</tr>
<tr>
<td>NB3fl</td>
<td>3a</td>
<td>6.96</td>
<td>10.6</td>
</tr>
</tbody>
</table>

*apparent $K_i$ due to mechanism-based inhibition
Inhibitory effects on different genotypes

In order to evaluate the difference in effect of the inhibitors on the different genotypes, vitality values (see p.20) were used (Table 2). In this approach, individually optimized catalytic properties of the different enzyme variants were accounted for in the comparative analysis. NS3\textsubscript{1a} was set as reference. An inhibitor is less efficient against a certain variant (relative the reference variant) if it has a vitality value $>> 1$. Higher vitality values for NS3\textsubscript{1b} and NS3\textsubscript{3a}, as compared to the reference, indicated that all inhibitors were most effective against the genotype 1a enzyme.

Table 2. Vitality values (V) used for comparison of sensitivity of resistance for genotype 1a, as well as inhibitor selectivity among the enzyme genotypes 1a, 1b and 3a.

<table>
<thead>
<tr>
<th>Enzyme variant</th>
<th>GT</th>
<th>Vitality values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N-1725</td>
</tr>
<tr>
<td>NS3\textsubscript{1a}</td>
<td>1a</td>
<td>1</td>
</tr>
<tr>
<td>NS3\textsubscript{1a,R155K}</td>
<td>1a</td>
<td>35</td>
</tr>
<tr>
<td>NS3\textsubscript{1b}</td>
<td>1b</td>
<td>2.1</td>
</tr>
<tr>
<td>NS3\textsubscript{3a}</td>
<td>3a</td>
<td>21</td>
</tr>
</tbody>
</table>

Resistance profiling

The approach of using vitality values was also applied for evaluation of the sensitivity of the different inhibitors to a commonly occurring resistant mutation (R155K). The result indicated high sensitivity for all inhibitors to the R155K mutant (Table 2), as indicated by the high vitality values for this variant. The result correlated well with existing replicon data [98].

Kinetic analysis of inhibitor interactions (Paper II)

SPR biosensor-based interaction analyses

In Paper II the evaluation of NS3 protease inhibitors was expanded in order to determine interaction kinetics and enzyme-inhibitor mechanisms in more detail. The interaction analyses were performed using a standard buffer supplemented only with detergent and reducing agent. Detergent preferences were taken from Paper I. All protein variants were treated equally in their immobilization to the sensor surface using standard amine coupling.

The interaction mechanisms were determined by non-linear regression analysis of the data using different interaction models describing a 1:1 interaction, an interaction with a heterogeneous ligand (selected fit) and a two-state interaction (induced fit) (Figure 4). The two-state model was however chosen since it described all inhibitor interactions. The enzyme-inhibitor interactions can thus be divided into two steps; the formation of the EI complex followed by the complex isomerization. This is illustrated by
Kinetic evaluation

The kinetics of the inhibitors was rather different, with N-1725 having the slowest association and fastest dissociation kinetics, resulting in moderate affinities. Also the other two linear inhibitors, VX-950 and SCH 503034, had relatively slow association and fast dissociation kinetics. The two macrocyclic inhibitors, BI LN-2061 and ITMN-191, had significantly faster association rates and slower dissociation rates, resulting in higher affinities. It can be speculated that this is a result of the reduced compound flexibility.

The kinetics for the clinical inhibitors, VX-950 and SCH 503034, were clearly suboptimal in comparison to the non-covalent inhibitors. Thus, a suggestion for future drug design might therefore be to combine the different inhibitor characteristics, in order to provide optimal kinetics.

Comparative analyses

A comparison of the kinetic profiles ($K_D = k_{-1}/k_1$) and enzyme inhibitory effects ($V$) revealed that the reduced effect against genotype 3 is most evident for BI LN-2061 and ITMN-191 (Table 3). This may have a structural explanation, since these two are similar in structure and have large macrocyclic backbones. The low flexibility of their rigid macrocycles may increase their sensitivity to genetic variations. N-1725 was shown to be equally effective against genotypes 1a and 1b but less effective against genotype 3. The affinities are however less affected. This indicates that, in contrast to the macrocyclic structure, the extended structure of N-1725 is less sensitive to genetic variations. For VX-950 the inhibitory effect on replication, as well as the interaction affinity, is higher for genotype 1b than for genotype 1a. Vitality values indicate the opposite. This may be explained by the alternative mechanism of action for VX-950.

Some of the inhibitor interactions were clearly affected by the isomerization step, as indicated by differences between $K_D$ and $K_D^*$, which was due to slow dissociation rates of the E*I complex. This was most evident for VX-950 and SCH 503034, indicating that their affinities are very much dependent on their mechanism-based mode-of-action.

\[
\begin{align*}
E + I & \underset{k_{-1}}{\rightleftharpoons} EI & \underset{k_2}{\rightleftharpoons} E^*I \\
\end{align*}
\]
Table 3. Comparison of kinetic profiles and inhibitory effects, as reflected by vitality values (V), first-step affinities ($K_D$), overall affinities ($K_D^*$) and inhibition of replication (EC$_{50}$).

<table>
<thead>
<tr>
<th></th>
<th>NS3$^{1a}$</th>
<th>NS3$^{1b}$</th>
<th>NS3$^{3a}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N-1725</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>1</td>
<td>2.1</td>
<td>21</td>
</tr>
<tr>
<td>$K_D$ (nM)</td>
<td>3900</td>
<td>7200</td>
<td>1400</td>
</tr>
<tr>
<td>$K_D^*$ (nM)</td>
<td>200</td>
<td>5600</td>
<td>870</td>
</tr>
<tr>
<td>EC$_{50}$ (nM)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

| **BILN-2061** |            |            |            |
| V      | 1          | 12         | 1000       |
| $K_D$ (nM) | 3.0       | 220        | 3300       |
| $K_D^*$ (nM) | 1.3       | 67         | 160        |
| EC$_{50}$ (nM) | 3$^{[87]}$ | 4$^{[87]}$ | nd         |

| **ITMN-191** |            |            |            |
| V      | 1          | 7.2        | 1200       |
| $K_D$ (nM) | 2.5       | 1.6        | 21         |
| $K_D^*$ (nM) | 0.13     | 1.6        | 0.52       |
| EC$_{50}$ (nM) | 0.24$^{[98]}$ | 1.8$^{[99]}$ | nd         |

| **VX-950** |            |            |            |
| V      | 1          | 7.7        | 8.9        |
| $K_D$ (nM) | 2000      | 8.6        | 66000      |
| $K_D^*$ (nM) | 340      | 8.5        | 1000       |
| EC$_{50}$ (nM) | 1030$^{[98]}$ | 350$^{[100]}$ | nd         |

| **SCH 503034** |            |            |            |
| V      | nd         | nd         | nd         |
| $K_D$ (nM) | nd      | 16000      | 1700       |
| $K_D^*$ (nM) | nd     | 90         | 3.9        |
| EC$_{50}$ (nM) | nd     | 200$^{[90]}$ | nd         |

*IC$_{50}$

Conclusions

Paper I and II clearly show the importance of choosing a suitable model system, experimental conditions and a reliable assay for a relevant evaluation of NS3 protease inhibitors. Different strategies will affect the interpretation of data used for HCV drug discovery. The importance is however not to find a single perfect system but rather to take advantage of the broad spectra of techniques and analytical methods for this kind of inhibitor evaluations. The importance of correlating data with more functional cell-based systems should also be highlighted.

Evaluation of novel NS3 protease inhibitor design

A common backbone structure for clinical compounds is a proline in the P$_2$ position. This has been shown to be of importance for inhibitory potency. However, it has been suggested that this P$_2$ residue is the major structural determinant for the loss of inhibitory effect in resistant NS3 variants [82].
Common resistant mutations in the NS3 protein affect the inhibitory effect without changing the interaction with the substrate, as judged by $K_M$. Therefore, identification of other groups in the P₂ position is hypothesized to be a good strategy to reduce the susceptibility of resistance. One possibility is to introduce a phenylglycine [81, 101, 102]. This promising strategy requires careful evaluation, which has been the topic for Paper III and IV.

The work presented herein was based on previous results from studies of phenylglycine, where combinations of various P₃ residues, different phenylglycine substituents, and P₁-P₁’ blocks were analyzed [101]. This study pointed out some interesting structures with good inhibitory potency in combination with the P₂ phenylglycine. These were different alkenylic extensions of the P₁ acyl sulfonamide, as well as different substituents on the phenylglycine, such as the quinolinoxyloxy- or pyrimidyloxy-groups as well as the vinyl group.

In Papers III and IV novel inhibitors were designed and synthesized based on two different peptide backbone structures (Figure 19). These allowed different strategies of modification in order to affect the inhibitory potency.

![Figure 19](image.png)

**Figure 19.** Inhibitor backbone structures evaluated in (a) Paper III and (b) Paper IV with different relevant sites for modification, A: P₁’ elongation of the acyl sulfonamide, B: P₁ modification, C: formation of regioisomers, D: P₂ modification, E: P₃ modification.
New phenylglycine-based inhibitors with alkenylic P₁’ groups  
(Paper III)

The goal of the third study was to evaluate the effect of modifications of the P₁’-residue of vinyl- and quinolinyloxy-substituted phenylglycine-based inhibitors, based on their inhibitory properties and their sensitivity to mutations of resistance in NS3. Vinyl groups are commonly used for macrocyclization of inhibitor structures, but were here hypothesized also to improve the inhibitory effect of acyclic inhibitors when in combination with different alkenylic P₁’ groups. Stereoisomers of these inhibitors, since phenylglycine can racemize, were also obtained and evaluated.

**Effect of inhibition**

The evaluation of the new inhibitors involved analysis of their inhibition of NS3 protease and the effect on viral replication in the replicon model system. The result showed that the inhibitory effect (K_i) varied with the size of the P₁’-group. However, the influence of the vinyl group was rather small, as determined by comparative analysis of previous inhibition data of inhibitors without the vinyl group [101]. Additionally, only small differences in inhibition were illustrated for the stereoisomers (Table 4). This was also supported by molecular modeling, since both epimers can be fitted into the enzyme binding pocket with only small adjustments of the peptide backbone. However, only inhibition by D-isomers was measurable using a replicon model system as indicated by the EC_{50}-values in Table 4.
Table 4. Comparison of P1 alkenylic substituted phenylglycine-based inhibitors including L- and D-isoforms. The inhibition constant (Ki) was determined using an enzyme inhibition assay, and the inhibition of replication (EC50-values) was determined using a subgenomic replicon assay (SD: standard deviation, na: not measurable)

<table>
<thead>
<tr>
<th>R</th>
<th>Compound</th>
<th>Ki ± SD (nM)</th>
<th>EC50 (µM)</th>
<th>R</th>
<th>Compound</th>
<th>Ki ± SD (nM)</th>
<th>EC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19a</td>
<td>63 ± 9</td>
<td>&gt;10</td>
<td>19b</td>
<td>48 ± 2</td>
<td>8.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19b</td>
<td>48 ± 2</td>
<td>8.9</td>
<td>20a</td>
<td>120 ± 26</td>
<td>&gt;10</td>
<td>20b</td>
<td>100 ± 14</td>
</tr>
<tr>
<td>21a</td>
<td>330 ± 60</td>
<td>nm</td>
<td>21b</td>
<td>190 ± 40</td>
<td>nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22a</td>
<td>290 ± 40</td>
<td>nm</td>
<td>22b</td>
<td>130 ± 20</td>
<td>nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23a</td>
<td>120 ± 20</td>
<td>&gt;10</td>
<td>23b</td>
<td>47 ± 6</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24a</td>
<td>140 ± 40</td>
<td>&gt;10</td>
<td>24b</td>
<td>35 ± 2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>500 ± 130</td>
<td>nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Resistance profiling

To evaluate the sensitivity of this new class of inhibitors to resistance, the most potent inhibitors (19a, 19b, 20a, 20b, 23a, and 23b) were also evaluated using mutant versions of NS3 (A156T and D168V). In order to compare the inhibitory effect and to compensate for effects of the mutations on the catalytic properties of the enzyme, both inhibition constants (\(K_i\)) and vitality values (V) were determined. The wild-type enzyme was set as reference. The inhibitory effect for this set of compounds was more or less unaffected by the studied mutations, as indicated by vitality values near 1 (Table 5). The substitution mutation on the 168 residue was to some extent beneficial for some of the inhibitors (1<V<6.2). This trend is not seen with some previously studied proline-based inhibitors [102]. As an example, vitality values for BILN-2061 against the two resistant enzyme variants are 1600 and 3200, respectively.

Table 5. Effect of resistance mutations (A156T and D168V) on inhibition (\(K_i\)), and vitality values (V) of phenylglycine-based inhibitors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>A156T</th>
<th>D168V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_i \pm SD \text{ (nM)})</td>
<td>V</td>
</tr>
<tr>
<td>19a</td>
<td>180 (\pm 20)</td>
<td>0.9</td>
</tr>
<tr>
<td>19b</td>
<td>86 (\pm 20)</td>
<td>0.9</td>
</tr>
<tr>
<td>20a</td>
<td>380 (\pm 80)</td>
<td>1.6</td>
</tr>
<tr>
<td>20b</td>
<td>270 (\pm 60)</td>
<td>1.4</td>
</tr>
<tr>
<td>23a</td>
<td>250 (\pm 70)</td>
<td>1.1</td>
</tr>
<tr>
<td>23b</td>
<td>180 (\pm 10)</td>
<td>0.9</td>
</tr>
</tbody>
</table>

NS3 protease inhibitors spanning the P\(_2\)-P\(_1\)' region (Paper IV)

The goal of the second study was to evaluate vinyl- and pyrimidyloxy-substituted phenylglycine-based inhibitors with an aromatic P\(_1\) moiety. Also the influence of different P\(_3\) capping groups was studied, in order to evaluate the possibility of developing smaller and less peptide-like inhibitors. The evaluation was based on inhibitory properties, the sensitivity of the inhibitors to resistance mutations in NS3, and pharmacokinetic characterizations. Regioisomers of these inhibitors, since the aromatic group enables the synthesis of the aryl acyl sulfonamide group in ortho-, meta or para position, in addition to the stereoisomers formed by racemization of the phenylglycine, were also obtained and evaluated.

Effect of inhibition

The evaluation of the new inhibitors involved analysis of their inhibition of NS3 protease activity. 4-(trifluoromethyl) phenyl and pent-4-enyl was cho-
sen as alkenyllic $P_1'$ substituents, based on previous result indicating promising inhibitory effects for these groups [101].

The result showed that the position of the substituted sulfonamide group had none or only small effect on the inhibitory potency for tripeptidic inhibitors (Table 6). The ones with 4-(trifluoromethyl) phenyl were slightly more effective than those with pent-4-enyl. Pent-4-enyl in ortho position was however more potent than the other regioisomeric forms. The difference between stereoisomers was negligible.

Table 6. Comparison of $P_2$ phenylglycine-based inhibitors with quinolinyl-oxy- and vinyl substituents spanning the $P_3$-$P_1'$ region. Included are the R-substituted sulfonamide group in ortho, meta and para position. The R-group is represented by a group of either 4-(trifluoromethyl) phenyl (compound 14, 15 and 16) or pent-4-enyl (compound B17, 17 and 18). The $P_3$ capping group is represented by a Boc-L-$\alpha$Leu.

<table>
<thead>
<tr>
<th>R</th>
<th>$K_i \pm SD$ (nM) (compound)</th>
<th>$K_i \pm SD$ (nM) (compound)</th>
<th>$K_i \pm SD$ (nM) (compound)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ortho</td>
<td>meta</td>
<td>para</td>
</tr>
<tr>
<td>L D</td>
<td>$K_i \pm SD$ (nM) (compound)</td>
<td>$K_i \pm SD$ (nM) (compound)</td>
<td>$K_i \pm SD$ (nM) (compound)</td>
</tr>
<tr>
<td></td>
<td>L D</td>
<td>L D</td>
<td>L D</td>
</tr>
<tr>
<td></td>
<td>45 ± 5.0 (14)</td>
<td>24 ± 3.1 (15)</td>
<td>30 ± 4.0 (16)</td>
</tr>
<tr>
<td></td>
<td>30 ± 4.0 (16)</td>
<td>30 ± 4.0 (16)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26 ±2.5 (B1*)</td>
<td>33 ± 3.3 (B1*)</td>
<td>88 ± 10 (17L)</td>
</tr>
<tr>
<td></td>
<td>100 ± 10 (17D)</td>
<td>96 ±20 (18L)</td>
<td>110 ±20 (18D)</td>
</tr>
</tbody>
</table>

* manuscript in production

Similar experiments were performed with truncated peptides spanning $P_2$-$P_1'$ instead of $P_3$-$P_1'$. Also these compounds showed small differences in their potential to inhibit protease activity (Table 7). Meta and para inhibitors with the 4-(trifluoromethyl) phenyl $P_1'$ substituents were slightly more effective
Table 7. Comparison of P₂ phenylglycine-based inhibitors with quinolinyl-oxy- and vinyl substituents spanning the P₂-P₁' region. Included are the R-substituted sulfonamide group in ortho, meta and para position. The R-group is represented by a group of either 4-(trifluoromethyl) phenyl (compound 14, 15 and 16) or pent-4-enyl (compound B17, 17 and 18). The P₃ capping group is represented by a Boc-group.

<table>
<thead>
<tr>
<th></th>
<th>ortho $K_i \pm SD$ (nM)</th>
<th>meta $K_i \pm SD$ (nM)</th>
<th>para $K_i \pm SD$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(compound)</td>
<td>(compound)</td>
<td>(compound)</td>
</tr>
<tr>
<td>4-(CF₃)phenyl</td>
<td>240 ± 50 (9)</td>
<td>58 ± 5.2 (10)</td>
<td>46 ± 5.3 (11)</td>
</tr>
<tr>
<td>5-ethylpent-4-enyl</td>
<td>190 ± 20 (A*)</td>
<td>210 ± 40 (12)</td>
<td>220 ± 30 (13)</td>
</tr>
</tbody>
</table>

* manuscript in production

In order to identify possible inhibitory effects of truncation different P₃ capping groups were analyzed. 4-(trifluoromethyl) phenyl in meta position was used as P₁' substituent. The result indicated a significant variation in inhibitory effect for the different capping variants, where more bulky groups tended to give more potent protease inhibitors (Table 8)
Table 8. Comparison of P$_2$ phenylglycine-based inhibitors with quinolinyl-oxy- and vinyl substituents spanning the P$_2$-P$_{1}'$ region with different N-capping groups. The 4-(trifluoromethyl) phenyl-substituted sulfonamide group is in meta position.

<table>
<thead>
<tr>
<th>Cap</th>
<th>Compound</th>
<th>Ki ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H (no protecting group)</td>
<td>19</td>
<td>1200 ± 220</td>
</tr>
<tr>
<td>([N-tert-butyl carbonate])</td>
<td>10</td>
<td>58 ± 5.2</td>
</tr>
<tr>
<td>([N-tert-butyl carbamoyl])</td>
<td>20</td>
<td>140 ± 20</td>
</tr>
<tr>
<td>([N-acetyl])</td>
<td>21</td>
<td>700 ± 170</td>
</tr>
<tr>
<td>([N-morpholino carbonyl])</td>
<td>22</td>
<td>590 ± 130</td>
</tr>
<tr>
<td>([N-nicotinyl carbonyl])</td>
<td>23</td>
<td>240 ± 50</td>
</tr>
<tr>
<td>([N-cyclopentyloxy carbonyl])</td>
<td>24</td>
<td>72 ± 10</td>
</tr>
</tbody>
</table>
Resistance profiling
Compounds 10 and 15 were also evaluated with respect to their susceptibility to NS3 resistant mutants. They retained inhibitory effect against the common A156T, D168V, and R155K mutants, as indicated by vitality values in the range 1-2.

Pharmacokinetic characterization
Compounds A, 9, 10, 11, 15, and 20 were evaluated based on their solubility, cell permeability and metabolic stability. These studies showed that 4-(trifluoromethyl) phenyl as sulfonamide substituent reduced inhibitor solubility as compared to pent-4-enyl. However, it increased the metabolic stability. An overall evaluation of P3 truncation indicates that inhibitors harboring N-tertbutil carbonate as capping group are stable in first pass metabolism, have good permeability and moderate solubility.

Conclusions
The results from the analysis of the phenylglycine analogues clearly indicate that vinylated P2 phenylglycine-based inhibitors have potential to inhibit the HCV NS3 protease. However, to improve the potential of these inhibitors there is a need for optimization. These studies also show that the hypothesis that alterations in the P2 position can reduce the susceptibility to resistance seems to be correct. Thus, it strengthens the alternative strategy to use phenylglycine-based inhibitors for the development of new HCV NS3 protease inhibitors with an improved resistance profile.

We have here highlighted several different molecular structures of importance and strategies for future drug design of NS3 protease inhibitors.

Analysis of serum protein interactions (Paper V)
The discovery of new drugs starts with the identification of target hits, which are optimized into lead and finally drug compounds. To reduce the rate of attrition due to bad ADME characteristics, compounds should preferably be evaluated early in the drug discovery process.

As a complement to the pharmacokinetic analyses performed in Paper IV, we were interested in looking also at the interactions between lead compounds and serum proteins. However, we were not satisfied with the conventional methods used for evaluation of serum protein interactions. These either use serum or isolated proteins, but are only studied under equilibrium conditions.
Interaction analysis

In Paper V a new method for analyzing SPR biosensor-based data for drug interactions with serum proteins was designed, with the aim to better understand the potential of a drug to be suitably distributed.

The evaluation was done by studying interactions with two of the most abundant proteins in blood serum, human serum albumin (HSA) and α1-acid glycoprotein (AGP). A set of inhibitors of HIV-1 protease, HIV-1 reverse transcriptase and cathepsin K was used for developing the new method.

The serum proteins were immobilized to the sensor surface and drug compounds were injected in concentration series over each protein. As expected and seen earlier for other compounds, the interaction kinetics was rapid, with both fast association and dissociation rates, resulting in moderate affinities [103]. This type of kinetics is advantageous for transport proteins but makes the analysis challenging.

Data interpretation

**Binding efficiency**

Interactions of this kind are not easily analyzed with conventionally used analytical methods (see introduction, p.31), and we therefore defined the concept of binding efficiency (BE). BE represents the slope of the initial linear part of the interaction saturation curve (**Figure 20**).

![Figure 20](image)

**Figure 20.** Illustration of the concept of binding efficiency for two different simulated interactions (1 and 2). The black lines represent the theoretical curves obtained with a 1:1 interaction model, where the binding efficiency (dashed line) is the slope of that line for low compound concentrations. The binding efficiency for interaction 1 (BE₁) is higher than for interaction 2 (BE₂), as indicated by the slope of the dashed line.
Figure 21. Ranking of BE-values for interactions with a) HSA and b) AGP. Compounds are represented by inhibitors of HIV-1 protease (black), HIV-1 reverse transcriptase (dark grey), cathepsin-K (white), and a set of reference compounds (light grey).
Binding efficiency was found to be a robust and easily reproducible parameter. In addition it could be determined for all compounds, which was not possible for any of the other kinetic constants. For the purpose of evaluating and characterizing serum protein interactions it was useful for qualitative ranking (Figure 21). By combining the set of inhibitors with reference compounds with known serum binding, qualitative information regarding their serum binding capacity was revealed.

**Discrimination of primary site interactions**

Since serum proteins are known to have multiple binding sites, another approach in the evaluation of serum protein interactions was to resolve the primary interaction with the “high affinity binding site” from the secondary interactions. This strategy was used in order to discriminate drug compounds with divergent secondary interactions, information contributing to the qualitative characterization using binding efficiency.

**Correlation analysis**

In order to determine experimental descriptors that can be used to predict serum protein binding, interaction data for the inhibitors of HIV-1 protease and HIV-1 reverse transcriptase was further correlated with data from a cell-based assay. Unfortunately, due to the small set of data and interaction complexities, no correlations were identified.

**Conclusions**

The study in Paper V is important for two reasons. First it illustrates a new strategy for analysis of serum protein interactions, which provides information that can be used to predict serum protein binding of drugs. The SPR-based assay can supply real-time data for the interactions, information which cannot be revealed using equilibrium-based methods, commonly used for evaluation of serum protein binding. However, considering the complexity of these types of interactions it is very difficult to identify experimental descriptors which correlate with cell-based systems. This supports the use of different analytical methods in the evaluation of interaction between lead compounds and serum protein.

In addition, this study shows the usefulness of this strategy for analyzing other rapid and low affinity interactions, perhaps less complex than serum protein interactions. In situations where kinetic data cannot be reliably determined, such as for low affinity interactions in early drug discovery screening, binding efficiency may provide useful information.
Concluding remarks and future perspectives

NS3 is one of the most attractive targets for HCV drugs. All information about the characteristics and function of this protein is therefore of great interest, however it needs to be correctly interpreted and adapted for the process of finding new drugs. Future HCV drug discovery therefore relies on extensive knowledge about the virus, as well as new strategies for the discovery of improved lead compounds.

This thesis provides an extensive characterization of HCV NS3 protease inhibitors, reflected by their inhibitory effects on enzyme activity and resistance profiling, as well as by their kinetic, structural, mechanistic, and functional properties, where both clinically relevant and novel inhibitors have been considered.

The project results show that

- Evaluation of the effect of inhibitors on NS3 protease activity is highly dependent on the catalytic properties of the enzyme as depicted by experimental conditions. Comparative analyses of inhibitory effects on genetic variants of NS3 should therefore account for differences in individually optimized catalytic efficiencies. This can be obtained by using vitality values.
- Interpretations for drug discovery, based on inhibition and interaction analyses, are affected by the enzyme model used.
• Differences in potency among NS3 protease inhibitors, explained by kinetic and mechanistic differences, can be revealed by time-resolved interaction analyses.
• Peptidomimetic design can be used to reduce the sensitivity of novel NS3 protease inhibitors to currently occurring resistant mutations.
• Binding efficiency is an analytical tool for characterization of low affinity interactions, which can be exemplified by serum protein interactions commonly interpreted in ADME profiling.

Future studies

The NS3 project has for a very long time been one of the major topics in our group and many questions have been answered throughout the years. The work presented in this thesis has provided new information and tools for further extension of the project.

In order to optimize peptidomimetic NS3 protease inhibitors and find new potential lead compounds the inhibitor evaluation should be extended and account for resistance mutations as well as different genotypes of NS3. The SPR biosensor-based approach can also preferably be used to add further information of mechanistic and kinetic properties of the inhibitors and also regarding their serum protein interactions.
Svensk sammanfattning

Det verkliga tecknet på intelligens är inte kunskap, utan fantasi

Albert Einstein

Biokemi kan enkelt förklaras som kemin bakom alla biologiska processer hos levande organismer. Den mänskliga kroppen är därfor ett mycket gott exempel på ett system fullt av biokemi.

För att lättare förstå vad som händer och sker i kroppen kan vi använda fantasin och föreställa oss ett tunnelbanesystem, med massa alternativa vägar att åka, och där av- och påstigning kan ske vid flera olika hållplatser. Tunnelbanan kryllar ofta av olika sorts människor; resenärer, musikanter, fattiga, rika, unga och gamla som alla samspelar och färdas runt i systemet. För att undvika irritation i denna blandning av människor är det viktigt att tågen går som de ska. Men som vi alla vet förekommer det ibland störningar som ställer till besvär.

Om vi nu tänker oss att tunnelbanesystemet istället är den mänskliga kroppen. Då skulle tunnlarna kunna representera blodkärlen, hjärtat skulle vara elen som får tåget att gå och hjärnan skulle kunna vara centralstationen. Alla människor skulle kunna representera proteiner, fetter och kolhydrater som transporterar runt i kroppen. Precis som tunnelbanan drabbas även kroppen ibland av störningar som måste åtgärdas för att systemet ska fungera.

Infektioner av virus och bakterier i kroppen kan i många fall läka ut av sig själva men ibland måste det till läkemedel för att hjälpa kroppens naturliga system. För att förstå hur man ska hjälpa kroppen behövs förstås läkare, men för att tillföra kunskap och bidra till att läkemedel utvecklas och blir bättre och mer effektiva behövs bl.a. biologer, kemister men även biokemister som förstår samspelet mellan biologin och kemin.

Proteiner och enzymer

Proteiner utgör tillsammans med fetter och kolhydrater till stor del de komponenter som behövs för att hålla kroppen vid liv. Det finns en mängd proteiner med olika funktioner i kroppen, t.ex. strukturella proteiner i hud, hår och...
naglar, reglerande proteiner såsom hormoner samt skyddande proteiner i form av antikroppar i kroppens försvar mot bakterier och virus. Andra proteiner av stor vikt är enzymerna, kroppens katalysatorer, vars uppgift är att påskynda mekanismer i kroppen som annars skulle ske mycket långsammare. Enzymer är involverade i en rad olika processer i kroppen, såsom nedbrytning och reglering. Men enzymer finns även i virus och bakterier, där de ofta har stor betydelse för fortfarande, och därmed spridning av sjukdomar.

**Läkemedelsutveckling**


**Hepatitis C**

Det finns idag behandling mot hepatit C, men denna har ofta svåra bieffekter, samtidigt som den är långvarig och kostsam. Dessutom fungerar inte behandlingen mot alla fall av hepatit C, vilket till stor del beror på att viruset finns i olika genetiska varianter.

En alternativ strategi för att behandla hepatit C är att utveckla antivirala läkemedel som direkt hämmar viruset. Det finns idag många olika hypoteser om huruvida man på bästa sätt kan förhindra spridning av viruset med hjälp av läkemedel. En av dem är att inhibiera ett eller flera av virusets egna proteiner, framför allt dess olika enzymer, och på så sätt förhindra nya virus från att utvecklas. Antivirala läkemedel har dock visat sig bidra till utvecklingen av resistenta virus som klarar av att stå emot läkemedelsbehandling. För att ta fram nya effektiva läkemedel mot hepatit C krävs därför en ökad förståelse för viruset och hur man kan förhindra dess spridning.

Min forskning

Det som jag valt att inriktas mig mot inom HCV-området är ett av virusets egna proteiner, NS3, ett enzym som har en grundläggande funktion för att kopiera av virusets gener kan ske. Om det genetiska materialet inte kan kopieras kan nya virus inte bildas och av denna anledning är NS3 ett bra mål för läkemedel. NS3 är också ett relativt enkelt protein att studera med hjälp av olika biokemiska metoder.

I min forskning har jag tillsammans med andra studerat interaktioner mellan NS3 och olika inhibitorer, med syfte att utvärdera inhibitorernas potential samt utveckla strategier och redskap för framtida läkemedelsutveckling. Detta har lett fram till fem olika delarbeten.

I det första delarbetet studerade vi olika inhibitorers effekt på flera genetiska varianter av NS3, med syfte att utvärdera och jämföra inhibitorernas kapacitet gentemot dessa. Våra försök visade att enzymvarianternas katalytiska förmåga var väldigt varierande, vilket var viktigt att ta hänsyn till när vi jämförde inhibitorernas potential. Resultaten visade också att de inhibitorska effekterna var olika beroende på vilken variant av NS3 som studerades. Det är därför av stor vikt att involvera olika genetiska enzymvarianter i utvecklingen av nya inhibitorer mot NS3. I det andra arbetet utvidgades studien från det första arbetet genom att använda en kompletterande metod som kunde ge oss interaktionsdata av annan karaktär. Dessa data innehöll information om interaktionens hastighet, styrka och mekanism, vilken visade sig kunna ge möjliga förklaringar till inhibitorernas olika effekt.

I de tredje och fjärde delarbetena studerade och utvärderade vi nya NS3 inhibitorer. Avsikten var här att, utifrån de olika inhibitorernas förmåga att påverka enzymets aktivitet, validera huruvida en ny struktur och modifieringar av denna är lämplig för framtidna NS3 inhibitorer. Resultaten från båda studierna var positiva, särskilt då inhibitorerna visade sig vara effektiva mot
läkemedelsresistenta varianter av NS3. I det fjärde arbetet gjordes även en utvärdering av olika läkemedelsegenskaper såsom löslighet och stabilitet, vilka visade sig vara fullt godkända för de nya inhibitorerna. Dessa resultat är lovande för framtida läkemedelsdesign.


Sammanfattningsvis kan man säga att min forskning har bidragit med viktig information för kommande utvecklingen av NS3 inhibitorer och förhoppningsvis också för framtida behandling av hepatit C.
First of all I would like to thank my supervisor Helena Danielson for giving me the opportunity to go through all this. I’m glad you decided to buy “grisen i säcken” when accepting me. We have shared a lot during these years and it’s been a pleasure to get to know you and to work with you. You are in many ways a true inspiration.

I would also like to thank Gunnar Johansson for being my co-supervisor during these years. Thanks for your support and input to my work and also for your trust in me when teaching your students.

Thanks to all my co-authors and collaborators. Without you I wouldn’t be here. You’ve done a great job!

Also a big THANK YOU to…

… The Department of Chemistry-BMC. The years at BMC would have been terrible without the nice working environment here. Good science starts in the coffee room!

…Past and present members of the HD group. Angelica, dearest “roommate”, I will miss your laughter forever. I’m so glad I met you! You know, this thesis wouldn’t contain HCV if it wasn’t for you and your dear sons… Thanks! Johan, you are a rock, always time to listen and with a helping hand (and of course a piece of candy…), New York shopping with you is unforgettable Tony, thesis writing would have been really boring without you, I really appreciated the personal gossip you finally shared with me, you deserve all bananas in the world Christian, thanks for the extra space on your lab bench and for excellent work in front of the camera, if not a chemist why not an actor Helena N, thanks for introducing me to the world below 1.8 m Eldar, my favorite ironic Uzbek, you are great Sara, I’m so glad you joined the group, we and especially I really needed some Norwegian spirit, Good
luck with everything! **Gun**, you’re a great source of knowledge, thanks for always being there and for everything you’ve taught me **Ikram**, it’s great to have you here **Göran**, what can I say… why keep it simple, HCV rules **Malin**, thanks for all the laughter, great discussions and nice company both in Boston and on our way to work **Tomas**, nice shoes! Thank you for everything and for always showing up with a smile **Matthias**, thanks for always helping out in SPR panic situations **Maria**, thanks for great talks and for letting me buy your sofa

…All students throughout the years: **Anja, Alex, Alexandra, Benjamin** and **Hilde**.

… Past and present colleagues at the 3rd floor, especially: **Bengt**, you are a great biochemist, **Marianne**, for creating a nice and organized lab, you go girl! **Francoise**, for nice discussions, **Birgit**, for great stories about bears and for keeping things in order in the lab, **Saga**, for sharing evenings (and for being social when I needed it the most…), **Olle**, thanks for a wonderful time in the course lab and nice dancing, **Emilia**, you and your smile will always be missed, there is no longer bird-time in the coffee room, thanks also to **Amino Acid Analysis Centre** for filling up our old lab, special thanks to **Anna T** for all nice and creative department-get-together (och vinslattar…)

… Everyone at the 4th floor (including the dark side), especially: **Sara**, you are a true talent, I owe you for all memorable photos **Christian**, for sharing general thoughts and wedding plans **Rikard**, for excellent discussions in the lunch room, enjoyed them all **Johanna**, “fika” and gossip moments would be nothing without you, I love that you know EVERYTHING **Cissi**, thanks for being the bad teacher so that I could be the nice one **Nisse**, good choice to come back, thanks for all the hair advise and for helping out with the reading of this thesis **Åsa**, for nice song writing and for struggling with the working environment **Micke**, I really enjoy lunch time and “fika” moments with the Head of the Department. Also thanks to the old and missed ones, **Ann**, a great member of “**Fikaklubben exclusive**” and **Diana**, always with a smile.

… The guys at the 5th floor **Gunnar S**, thanks for always smiling, for always being so helpful and for bringing “presents” almost every day **Bosse**, my computer would be nothing without you.

Finally, I would not be here if it wasn’t for my dearest friends and family. Thanks for caring and being supportive in all situations. You mean the world to me. A special thanks to…
…Lena & Torgny, you are really true friends. Thanks for all the support, and for being excellent with Oscar. I hope we stay this close forever. Torgny, thanks for improving my bicep!

…Daniel & Linda, I’m so glad I met Daniel so that I got to know you, thanks for great holidays and for all your support during these last months. Danne, nu har Dr And blivit Dr Svahn!

…Jimmy & Kattis, you are great. Jimmy, thank you for taking extra care of your student. I owe you for teaching me how to be crazy about the freezers, for trying to match make me with your friends (even the short ones), and for running 21 km.

…The girls from LiTH; Lena, Lisa, Sofie, Suss, Anna, Frida, Christina & Sara. We had a great time! It’s hard to forget all sweaty nights at Flamman, the cheap beer and nice toilet visits at HG and of course how to think big (jag tänker mig cellen…). Thank you for those years and all the years to come.

…Sanna, my first real friend – “Girls just wanna have fun” and we did have fun, in our way ☺. Thanks Tommy for taking care of her!

… Min nya utvidgade familj; Agneta, Roland & Unni, Anna. Tack för ni finns där för mig, Daniel & Oscar.

…Tommy & Elisabeth, inte så mycket plast, bara bonus!

…Hannah, tack för alla minnen.


References


97. Delang, L., et al., Comparative study of the genetic barriers and pathways towards resistance of selective inhibitors of hepatitis C


A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)