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MARGIT MUTSO

Different Approaches to Counteracting Hepatitis C Virus and Chikungunya Virus Infections

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Different Approaches to Counteracting Hepatitis C Virus and Chikungunya Virus Infections

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Supervisor: Eva Žusinaite, MD,
Senior Research Fellow in Applied Virology,
Institute of Technology, University of Tartu, Estonia

Co-supervisor: Andres Merits, PhD,
Professor of Applied Virology,
Institute of Technology, University of Tartu, Estonia

Reviewer: Reet Kurg, PhD,
Senior Research Scientist in Gene Technology
Institute of Technology, University of Tartu, Estonia

Opponent: Mark Harris, PhD
Professor of Virology
School of Molecular and Cellular Biology,
University of Leeds, UK

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to by their Roman numerals from this point forward.

I Karelson, M., Dobchev, D.A., Karelson, G., Tamm, T., Tämm, K., Nikonov, A., **Mutso, M.** and Merits, A. (2012) Fragment-based development of HCV protease inhibitors for the treatment of hepatitis C. *Current Computer Aided Drug Design*, **8**, 55–61.

II **Mutso, M.***, Nikonov, A.*, Pihlak, A*., Žusinaite, E., Viru, L., Selyutina, A., Reintamm, T., Kelve, M., Saarma, M., Karelson, M. and Merits, A. (2015). RNA interference-guided targeting of hepatitis C virus replication with antisense locked nucleic acid-based oligonucleotides containing 8-oxo-dG modifications. *PLoS One,* **10**, e0128686. doi: 10.1371/journal.pone.0128686.
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IV Thaa, B., Biasiotto, R., Eng, K., Neuvonen, M., Götte, B., Rheinemann, L., **Mutso, M.**, Utt, A., Varghese, F., Balistreri, G., Merits, A., Ahola, T. and McInerney, G. (2015). Differential PI3K-Akt-mTOR activation by Semliki Forest and chikungunya virus, dependent on nsP3 and connected to replication complex internalisation. *Journal of Virology*, **89**, in press doi:10.1128/JVI.01579-15

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This thesis also contains unpublished data.

Author's contribution:

I Designed and carried out cell culture based and cytotoxicity experiments. Wrote a part of the manuscript that describes cytotoxic properties of compounds.

II Designed and performed most of experiments, wrote final version of the manuscript.

III Preformed cell culture based virus stability assays and sequencing.

IV Performed infectivity and replication assays for viruses with swapped nsP3 regions.

LIST OF ABBREVIATIONS

aa	amino acid residue
ASO	antisense oligonucleotide
BPL	β-propiolactone
C	capsid protein
ccHCV	cell culture HCV
CHIKV	Chikungunya virus
CMC	carboxymethyl cellulose
CPV	cytopathic vacuole
CSE	conserved sequence element
DAA	directly acting antivirals
DMSO	dimethyl sulfoxide
dsRNA	double stranded RNA
EC50	effective concentration 50
EEEV	Eastern equine encephalitis virus
ER	endoplasmic reticulum
FCS	fetal calf serum
FQSAR	fragment – based quantitative structure–activity relationship method
GT	genotype
HAV	hepatitis A virus
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HTA	host targeting antiviral
HVD	hyper variable domain
ICA	infectious centre assay
IC50	inhibitory concentration 50
IFN	interferon
IRES	internal ribosome entry site
JEV	Japanese encephalitis virus
LD	lipid droplet
LNA	locked nucleic acid
MERS-CoV	Middle East respiratory syndrome coronavirus
miRNA	micro RNA
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
NS	non-structural protein (used for HCV)
nsP	non-structural protein (used for alphaviruses)
nt	nucleotide
NTPase	nucleoside triphosphatase
ON	oligonucleotide

ORF	open reading frame
peg	polyethylene glycol
pfu	plaque forming unit
PNA	peptide nucleic acid
PTO	phosphorothioate oligonucleotide
PV	poliovirus
RdRp	RNA dependent RNA polymerase
RNAi	RNA interference
RRV	Ross River virus
RSV	respiratory syncytial virus
SARS-CoV	severe acute respiratory syndrome coronavirus
SFV	Semliki Forest virus
SG	subgenomic
SINV	Sindbis virus
siRNA	small interfering RNA
SOC	standard of care
SVR	sustained virological response
TBEV	tick-borne encephalitis virus
Tm	melting temperature
UTR	untranslated region
VEEV	Venezuelan equine encephalitis virus
VLP	virus like particle
wt	wild type
ZBD	zinc binding domain

INTRODUCTION

Viruses are and will always be a part of every ecosystem, wildlife and human life. In the past, different viral outbreaks have left painful marks on humankind. Not surprisingly, countermeasures against different virus-caused epidemics also have a long history. It has been estimated that in 1000 AD, inoculations were already being used to prevent smallpox in China, Africa and Turkey. After some time, smallpox reached Europe and the Americas, where it caused a tremendous number of deaths. The older method – variolation – was replaced by the use of a vaccine against smallpox in 1796 by Edward Jenner. In 1885, Louis Pasteur introduced the rabies vaccine. Both of these vaccines pre-dated the discovery of viruses. In the 20th century, when actual viral pathogens were discovered, more modern methods became available, and a number of different vaccines were developed. The use of vaccines has greatly reduced both the number of infections and virus-caused pathology. Compared to the vaccines, the use of antiviral compounds (i.e., chemical inhibitors of the viral infection cycle) has a much shorter history.

Despite extensive research, some viruses and viral diseases still lack any good vaccine or treatment. Additionally, some viruses that currently have no importance or are efficiently controlled have the potential to become problems in the future. Viral features such as a high mutation rate, the ability to undergo recombination, diversity, the ability to escape immune systems and the complexity of the viral lifecycle are only some of the obstacles that must be overcome for antivirals and vaccines to be safe and efficient. Furthermore, vaccination (where available) currently only helps to prevent new infections. In the case of chronic infections, it is equally important to develop effective and affordable cures for the disease in order to reduce mortality and improve the quality of life of people who are already infected.

Hepatitis C virus (HCV) was discovered in 1989 (1), approximately 26 years ago, and yet there is no anti-HCV vaccine. Without an efficient vaccine, the options for combating HCV infection are limited to the use of antiviral drugs. In recent years, anti-HCV treatment achieved a real breakthrough. Nevertheless, HCV is still widespread and continues to produce disease. Different medications are needed to control HCV-induced pathology, spread and persistence. The first part of this dissertation is focused on the development of anti-HCV inhibitors using two different technological approaches.

Chikungunya virus (CHIKV) re-emerged in the past decade and is currently spreading around the world, affecting millions of people. This virus has gained the status of the causative agent of an acknowledged neglected tropical disease (2). The second part of this study is focused on the analysis of a laboratory-developed attenuated CHIKV vaccine strain, including the characterization of its genetic stability, and work that aims to reveal the importance of the introduced changes for the viral lifecycle.

1. REVIEW OF THE LITERATURE

1.1. New emerging viruses, outbreaks and epidemics

Viral outbreaks occur in different parts of the world every year. These outbreaks are frequently large enough to be called epidemics. Some of these outbreaks are predictable like influenza virus A; such epidemics occur every year, although the viral strains that cause these epidemics are generally different. Other viral outbreaks can be completely unexpected, such as the emergence of SARS-CoV in Asia in 2003 (3, 4) and the MERS-CoV outbreak that started in Saudi-Arabia in 2012 and has reached remote countries, such as South Korea and Thailand (5, 6). Filoviruses in Africa cause sporadic outbreaks, although a recent Ebola outbreak in West Africa demonstrated that under the right circumstances (for virus), even viruses that lack an efficient means of transmission can spread rapidly and get out of control (7). Increased travel simplifies the spread of viruses/diseases between continents. The international economy and transport of goods has resulted in the spread of viral transmission vectors, such as mosquitoes. Human pressure on the natural habitats of viruses has increased the possibility of human contact with potentially pathogenic viruses.

The viruses that have benefited most from these factors appear to have one thing in common: an RNA genome. In this group of viruses, the intrinsic abilities of the viruses to mutate and adapt rapidly are especially prominent. Often, first-line measures, such as the monitoring of pathogens and their outbreaks, are not efficient, and the precautions that are taken to minimize the spread of potential pathogens fail. Therefore, there is a growing need for the development of efficient antiviral strategies. Correspondingly, a number of basic questions must be addressed in order to succeed in the development of vaccines or antivirals. Current technologies are fast and efficient; a potent antiviral drug (or vaccine) can be produced rapidly, but only when we have a precise design for the antiviral (or vaccine). Otherwise, the powerful technology may turn out to be a fast track to failure. Thus, it is important to study different strategies of vaccine development (or different technological platforms for the development of antivirals), even if the developed compounds/vaccine candidates have a relatively small chance of actually being used against viral infections.

1.2. Current vaccines against viruses

All vaccines act through the immune system, and (with the exception of passive vaccination), they induce efficient and long-lasting (ideally lifelong) protection against pathogens. The immune system has been shaped to be efficient against pathogens, and a successful vaccine must safely mimic a pathogen. There are multiple ways to achieve this goal. Given the complexity of immune system/pathogen interactions, different approaches have different benefits and disadvantages.

1.2.1. Inactivated (killed) vaccines

The principle of inactivated vaccines is based on treating the pathogen in the way that it becomes unable to cause infection but maintains the ability to activate the immune system. This type of vaccine is known for its safety features. "Killed" pathogens can no longer replicate; therefore, no mutations or re-activation of the virus could occur. Other positive features of this type of vaccine include their inability to be transmitted. In addition to these biological properties, inactivated vaccines are easier to handle. They are not as demanding in terms of storage conditions (at least in comparison to live attenuated vaccines), making their transportation and distribution much more convenient. However, like all other types of vaccines, they have some disadvantages. Compared to live attenuated vaccines, inactivated vaccines stimulate a weaker immune response and sometimes provide poorly sustained protection. Therefore, multiple doses and/or boosts of inactivated vaccines are needed to achieve proper immunity. For instance, the vaccine against the tick-borne encephalitis virus (TBEV) requires three injections and subsequent booster vaccinations every three to five years to maintain protective immunity (8).

There are a number of strategies for inactivating pathogens for vaccine development purposes: gamma irradiation (9–11), ultraviolet treatment (12), heat (13) or the use of different chemicals. Inactivating chemicals include acids (14), ethylenimine derivatives (15), psoralens (16) and hydrogen peroxide (17, 18). Formaldehyde and β-propiolactone (BPL) are the main chemicals used in the production of licensed human vaccines. BPL inactivates the virus through interactions with viral nucleic acid (19); meanwhile, formaldehyde-mediated inactivation is based on crosslinking various amino acid residues in viral proteins (20, 21). For enveloped viruses, inactivation with mild detergents is commonly used (22).

Today, this type of vaccine is licensed (for human usage) against six viruses: poliovirus (PV) (23) hepatitis A virus (HAV), Japanese encephalitis virus (JEV), TBEV, rabies virus and influenza virus. There are also several vaccine candidates of this type in clinical development. The targeted viruses include Ross River virus (RRV) (24), CHIKV (25), respiratory syncytial virus (RSV) (26) and enterovirus 71 (27). Inactivated dengue virus vaccine research has been put on hold due to the lack of an equally protective immune response against all four serotypes of the virus and the risks of waning immunity (28). There are also inactivated vaccines for VEEV and EEEV that are licensed for horses but not for humans (29).

1.2.2. Subunit vaccines

In the case of subunit vaccines, only part of the viral genetic information is used to produce materials required to induce a protective immune response. Such vaccines are generally based on virus proteins that are generated using recombinant DNA and protein expression/purification technologies. Subunit vaccines have numerous advantages over traditional inactivated vaccines. These vaccines do not contain a pathogen; therefore, this approach lacks all concerns related to pathogen inactivation and the effects of such treatments on the antigenic properties of viral proteins. The disadvantages of this approach include the problem of insufficient immune activation and protection against a pathogen. In general, monomeric viral proteins are poor antigens. Therefore, most subunit vaccines represent artificial particles composed of viral proteins (virus-like particles or VLPs). Thus, the possibility of obtaining a good subunit vaccine also depends on the ability of viral proteins to form VLPs.

The first subunit vaccine was the vaccine against hepatitis B virus (HBV), which contains the viral surface antigen HBsAg produced in yeast. This antigen forms distinct particles that are very similar to the so-called 20 nm spheres produced in HBV-infected humans (30). This vaccine was licensed in 1986. Currently, there are some positive advances in HIV subunit vaccine development (31, 32), although the protective immunity generated by such vaccines is short-lived. Much work has been conducted towards the development of subunit vaccines for dengue virus; to date, promising results have been obtained using non-human primates (33). The subunit vaccines against Ebola virus have reached clinical trials (34). In addition, great success has been achieved in the development of subunit vaccines against oncogenic types of human papillomaviruses (35). These vaccines are very immunogenic and generate protective immune responses that do not fade over a period of nearly 10 years (36, 37) and, in all likelihood, will last much longer (currently, such data are not available, as these new vaccines were approved in 2006/2007).

1.2.3. Attenuated (live) vaccines and recombinant vaccines

Attenuated vaccines are viruses that are still able to replicate and cause infection but do not cause disease. Such vaccines are very effective in activating both humoral and cellular immune responses and typically produce long-lasting protection following a single-dose immunization. The negative sides of live vaccines are the risk of reversion of the attenuated virus back to a pathogenic version and the risk of transmission.

The first so-called attenuated vaccine was the smallpox vaccine developed by Jenner in 1796. The actual virus that was used as the vaccine was not an attenuated strain of variola virus but a related cowpox virus. The true attenuation of pathogenic viruses can be achieved using different approaches. Historically, pathogenic viral strains were passaged multiple times under unfavourable

conditions – in different hosts and/or at sub-optimal temperatures – until a non-pathogenic phenotype was obtained. For example, the attenuated polio vaccine was developed by Sabin after years-long passaging of the virus in different hosts after inoculation via different routes. This approach resulted in the development of a non-virulent PV strain that was licensed as a vaccine in 1960. A number of other attenuated vaccines were obtained using similar approaches, including the yellow fever virus vaccine (38), the measles and mumps vaccines (39, 40) and the rotavirus vaccine (41).

As we know now, under these conditions, certain attenuating mutations are generated in viral genomes. Current recombinant DNA technology and synthetic biology allow the direct introduction of all types of modifications into viral genomes. This approach is much faster and allows for the rapid analysis of modified viruses for pathogenicity, immune activation and stability in cell culture-based systems, in different animal models and ultimately in clinical trials. Intensive research has been carried out with the aim of developing attenuated vaccines for dengue virus, CHIKV and RSV using this modern approach.

In the case of recombinant (chimeric) vaccines, the antigen(s) of a pathogen of interest are inserted into a heterologous vector, which is usually derived from an approved vaccine strain of a different virus. This strategy is commonly used for modern veterinary vaccines, for which it has been found to be very efficient. In contrast, this approach is not yet widely used for the development of human vaccines. The only recombinant vaccine is the vaccine against JEV (ChimeriVax-JE), which represents a recombinant between the yellow fever vaccine (vector part) and the JEV (part encoding for envelope proteins) (42). The anti-rotavirus vaccine RotaTeq, which contains five human-bovine rotavirus hybrids, is also a chimeric virus-based vaccine. However, this vaccine is not a product of true recombination; instead, it was obtained via the re-assortment of genome segments from different viruses (43). The modern chimeric vaccine development approach has also been used to generate vaccine candidates against CHIKV (see 1.5.5.)

1.2.4. DNA vaccines (gene vaccines)

The production of naked DNA is much less complex and less expensive than the production of VLPs or proteins. Thus, the principle of this vaccine technology is introducing recombinant DNA that encodes the desired epitope(s), full proteins or even the full attenuated virus genome straight into the tissue. The peptide or protein is expressed *in situ* and should, at least in theory, produce an immune response against the desired pathogen. Similar to subunit vaccines, the pathogen itself is not present, and the safety concerns are primarily related to the possibility of unwanted integration of vaccine DNA into the human genome.

The first report of the effective introduction of plasmid DNA and subsequent antigen expression in an organism dates back to 1990 (44); as the technology appeared to be rather straightforward, many examples followed. In 1993, Ulmer

demonstrated that the injection of mice with naked DNA encoding an influenza virus protein can protect animals against challenge with wild-type (wt) influenza virus (45). The recent development of molecular engineering, including gene synthesis, provides practically unlimited possibilities for the construction of different DNA molecules. Together with an improved understanding of innate immune responses, this technology has boosted interest in the development of DNA vaccines.

Since 2005, at least three DNA vaccines have been licensed for veterinary use: one vaccine targeting West Nile virus in horses (46), another targeting haematopoietic necrosis virus in salmon and a third targeting canine malignant melanoma (47). Nevertheless, the progress of development of human DNA vaccines has been slow, partly because of delivery issues and differences in the innate immune response between humans and animal models and, most importantly, because of much longer trial periods and enhanced safety concerns. As a result, there is not yet a licensed DNA vaccine for human use, although multiple DNA vaccine candidates have reached clinical trials (48–50).

1.3. Antiviral drugs – principles and examples of different approaches

As mentioned above, infections caused by many medically important viruses, including HIV-1 and HCV, cannot currently be prevented by vaccination. Similarly, the currently available vaccines cannot eliminate established infections. Therefore, treatments that can inhibit (or prevent) virus-induced pathogenesis, limit virus spread and ideally eliminate the virus from infected organisms are needed.

Not long ago, only different natural substances were used to treat diseases. Some of these natural medicines have indeed been proven to possess antiviral properties. However, the effects of these substances are rarely virus-specific, and their mechanisms of action often remain unknown. Nevertheless, the existence of such medicines has provided inspiration for the development of synthetic antiviral drugs. Most of these substances directly target viral components (proteins or genomes); hence, these substances are called directly acting antivirals (DAA). Such substances (or, more commonly, their parental compounds, which are called “hits”) are typically obtained by screening large libraries of chemical compounds. Alternatively, the structures of potential hit compounds can be designed rationally by taking into account the known structures of viral macromolecules (drug targets) and multiple additional factors. Finally, instead of targeting the virus, antiviral compounds can target host factors that are essential for virus replication (host targeting antivirals, HTA). Antiviral compounds can target different steps of the viral lifecycle, including entry, replication, protein synthesis, maturation or the release of new viral particles. Currently, approximately 50 antivirals are licensed against major viral pathogens; approxi-

mately half of these antivirals are against HIV-1. However, the number of substances with known antiviral effects is much larger. Thus, it is impossible to cover all of these substances in a single review. Therefore, only a brief description of nucleoside/nucleotide analogues and some non-nucleoside inhibitors is provided as an example. Antisense oligonucleotide-based drugs and drug candidates are reviewed more extensively. Drugs that inhibit HCV are reviewed in chapters 1.4.5 and 1.4.6, as these drugs are most relevant to the topic of the current thesis.

1.3.1. Nucleoside/nucleotide analogues

Nucleoside/nucleotide analogues mimic substrates of viral polymerases and generally act as terminators of DNA or RNA synthesis, abolishing genome replication (51). Such inhibitors tend to be rather specific (effective against one virus or a narrow group of viruses) and have been developed and licensed for use against herpesviruses (acyclovir) (52, 53), HIV-1 (AZT) (54, 55), HBV (telbivudine) (56) and now also against HCV (sofosbuvir) (57).

Some other nucleoside inhibitors (for example, ribavirin and favipiravir) have a broad antiviral spectrum. Ribavirin is a synthetic guanosine analogue and is used in the clinical treatment of HCV, RSV and influenza virus infections and also for the treatment of infections caused by other (often rare and/or lacking specific inhibitors) viruses, such as Lassa virus and CHIKV. Depending on the targeted virus, ribavirin may be used with or without polyethylene glycol-conjugated interferon-alpha (pegIFN-α) and/or DAAs (58, 59). Ribavirin has other activities in addition to being a substrate for viral polymerase. For example it depletes the cellular pool of guanosine nucleotides, thus affecting viral replication/transcription (60, 61). Favipiravir is licensed (in some countries) for the treatment of influenza but has also been shown to inhibit several other viruses, including hantaviruses, Ebola virus and CHIKV (62–64).

1.3.2. Non-nucleotide inhibitors

Non-nucleotide inhibitors are extremely diverse in terms of structure, function and mode of action. The structure of such inhibitors may vary from small molecules to proteins and the functionality may vary form very specific to very general inhibitors. Some of these compounds block viral enzymatic activities by acting as analogues of substrates or as allosteric inhibitors. Other compounds block virion internalization and unpacking, inhibit the release of new generation of particles or target host factors that are critical for the viral life cycle. Many of these compounds, such as allosteric inhibitors or substrate analogues, have high activity and narrow specificity. In these cases, the use of compounds with different mechanisms of action is needed for the efficient treatment of viruses that are capable of developing resistance against specific inhibitors.

Some non-nucleoside inhibitors are active against a broad spectrum of viruses. For example, chloroquine, which is an anti-malaria drug, has broad antiviral activity and acts by disrupting virus entry. In *in vitro* studies, chloroquine inhibited HIV-1, SARS-CoV, alphaviruses, influenza A virus and Ebola virus; however, in *in vivo* studies, chloroquine has generally failed to demonstrate its effectiveness (65–69). Different immunomodulators, such as IFN-α, retinoic acid-inducible gene 1 (RIG-I) agonists and poly(I:C), act by boosting the immune system. Type I IFNs represent the first line of natural defence against virus infection and are therefore active against many viruses. IFNs activate the production of other cytokines and antiviral proteins and also activate cells of the immune system. Currently, IFN-α (typically in the form of pegIFN-α) is used for the treatment of chronic HCV and HBV infections (70). As a key part of innate immunity, IFN-α is also responsible for the development of multiple side effects.

1.3.3. Antisense oligonucleotides and antiviral siRNA

Antisense oligonucleotides (ASOs), which are used as inhibitors of viral infection, are DNA, RNA or DNA/RNA mixomer (and different modified versions of these) molecules that are typically 18-25 nucleotides in length. These oligonucleotides are primarily designed to target viral mRNAs (in the case of positive-strand RNA viruses, these compounds target viral genomes that function as mRNAs). Depending on their composition, ASOs may activate different downstream pathways that lead to targeted RNA degradation and/or cause the steric blockade of mRNA translation (for viral genomes, also replication) by binding to their target sites in RNA molecules and interfering with the cellular translation machinery (71) and/or viral replicase.

ASOs are designed to bind to specific positions in viral mRNAs (or genomes), mRNAs that encode proviral host factors or cellular micro RNA (miRNA). This feature ensures the specificity of the effect but also represents an intrinsic weakness of ASOs. To be highly effective, the sequence of an ASO must have a perfect or nearly perfect match with its target sequence. For host targets that have conserved sequences, this requirement is relatively easy to achieve. In contrast, RNA viruses are genetically very variable; such viruses often have different genotypes (GT) and each GT exists in the form of quasi-species. Hence, such virus populations have some pre-existing resistance to ASOs and can acquire additional mutations very rapidly. When resistant variants of genomes become prominent, the antiviral efficiency of ASOs can be lost or severely diminished.

The simplest ASOs are DNA oligonucleotides. Such ASOs bind to the target RNA and form DNA:RNA heteroduplexes. Such duplexes cause translational arrest but can also be recognized and then degraded by cellular RNase H1 (71). Hence, ASO-mediated inhibition depends on the functions of the target site. To cause translational arrest, the target of an ASO must overlap with sequences that

are essential for the initiation of translation. Furthermore, the probability of ASO binding greatly depends on the secondary structure of the target RNA (72). The actual structures of viral RNAs are complex, especially in cells, where they are dynamic and difficult to analyse. When designing an ASO, it is important to take into account these structures; regions that are actually accessible (and not hidden by secondary structures) should be targeted (71, 73). Different computer programs are designed to predict RNA secondary structures, but these predictions are often not supported by experimental data. The more accurate methods, such as SHAPE (selective 2'-hydroxyl acylation analysed by primer extension), can provide better predictions (74), but such approaches are also more time- and resource-demanding. In addition, all viral RNAs form RNA:protein complexes, which may hide potential ASO target sites. The effect(s) of such complex formation on the secondary structure of RNA is hard to predict. Thus, the design of efficient ASOs is not as simple as it may look. Finally, even if the issues with target site selection are solved, DNA ASOs remain nearly useless due to poor cellular uptake, low stability against enzymatic degradation, unwanted side effects and low binding efficiency. Many of these disadvantages of DNA ASOs can be overcome or alleviated by including different modifications in the ASO design.

To date, numerous modifications that enhance the inhibitory properties of ASOs have been described. Phosphorothioate oligonucleotides (PTOs), where a non-bridging oxygen on the phosphate backbone is replaced by a sulfur atom, are commonly used. This modification renders the internucleotide linkage resistant to nuclease degradation. The downside of this modification is a reduced melting temperature (Tm) and reduced stability of the ASO:RNA duplex (75). To further enhance ASO stability and improve binding properties, other modifications, such as 2′-O-Methyl (2′-OMe) or 2’-O-methoxyethyl RNAs (76), peptide nucleic acids (PNA) (77), tricyclo-DNA (78), boranophosphate- (79), oxepane- (80), cyclohexene- (81), fluoro-arabino (FANA)-modified ASOs (82), N3’,P5’ –phosphoramidates (83), morpholinos (84), and/or 2’-O-methyl locked nucleic acids (LNA) (85), have been developed and used.

Although most of these modifications increase the binding affinity of ASOs for the target RNA and enhance the stability of the formed duplex, they often cause problems. Excessively strong binding to the target can result in increased binding to non-targeted RNAs; effects that result from such interactions are termed off-target effects. In addition, RNase H-mediated degradation requires a duplex containing 6 or more DNA:RNA base pairs that are not interrupted by LNA, PNA, morpholino or 2’OMe modifications (71). Thus, when including different modifications in ASOs, it is important to know the mechanism(s) by which these modifications contribute to improved inhibitory effects and potential disadvantages associated with these modifications. The questions of where, how many and which type of modifications should be included are clearly crucial for efficient ASO design. As a result, the current situation with ASOs is quite similar to the situation with other types of antiviral compounds. There are

many reports of the successful application of ASOs against different viruses in cell culture. The viruses that have been targeted include HCV (86) and CHIKV (87). In some cases, antiviral effects have also been observed in *in vivo* models (87, 88). However, only a few ASOs that are designed to target viruses (including influenza virus, Ebola virus and HCV) have reached clinical trials (89). To date, the only ASO-based antiviral drug that is licensed for clinical use is the intraocular drug fomivirsen, which was used for the treatment of human cytomegalovirus (HCMV)-caused retinitis (90). This drug was approved in 1998 and withdrawn a few years later due to poor demand.

Small interfering RNAs (siRNA) are dsRNA molecules that induce RNA interference (RNAi), which leads to the degradation of the targeted RNA. siRNAs have many similarities to ASOs (including a similar size – typically 21 nucleotides), but unlike ASOs, siRNAs are components of natural regulatory systems, and even when introduced into cells artificially, siRNAs still act via complicated pre-existing molecular machinery. Briefly, one strand (called the guide strand) of the siRNA molecule is loaded into the RNA-induced silencing complex (RISC) and is bound to the complement target RNA, leading to its degradation (91). This pathway is used extensively in molecular biology studies as an easy option for down-regulating specific genes or inhibiting viruses in *in vitro* systems. In *in vivo* models and especially in potential therapeutic applications, many unsolved problems, such as inefficient delivery, poor target organ/tissue specificity, low stability of the compound and off-target effects, limit the use of siRNAs as antivirals (reviewed in (92)).

1.4. Hepatitis C virus (family *Flaviviridae*)

Hepatitis C virus (HCV) belongs to the family *Flaviviridae* and is currently the only recognized member of the genus *Hepacivirus*. This status will certainly change in the future, as similar viruses were recently discovered in horses, dogs, bats and rodents (93–97). It is estimated that approximately 3% of the world population is chronically infected with HCV. Although this infection is often asymptomatic, chronic HCV infection may lead to severe liver diseases, such as fibrosis, cirrhosis and finally hepatocellular carcinoma (HCC). HCV is associated with significant mortality resulting from these liver-associated diseases but has also been linked to several metabolic, cardiovascular, neurological and immunological disorders (98, 99). Due to its medical importance, HCV is currently the third most studied virus in the world (after HIV and influenza viruses).

1.4.1. HCV genotypes, distribution and HCV-induced diseases

HCV has very high genetic diversity. Seven different HCV GTs are currently recognized, with additional grouping into subtypes. Strictly speaking, the classification is justified only from the medical point of view since different HCV

GTs cause similar clinical diseases. From the perspective of virus systematics, this classification is misleading. The similarity of nucleic acid sequences between different HCV GTs is as low as 65%. In the case of almost any other group of viruses, the current HCV GTs would be considered as different virus species (rather than variants of a single virus). In addition, different HCV GTs also exhibit different levels of responsiveness to antiviral treatment.

HCV prevalence and GT distribution vary around the world (Fig. 1). Globally, the most common and prevalent GT is GT1 (46% of all HCV cases), followed by GT3 (30%), GT2 and GT4. GT1 is distributed most widely and is prominent in Europe, Australia, South America and North America. GT3 is distributed mainly in Asia but is also rather common in Australia and South America. In Africa and the Middle East, the most prominent GT is GT4 (100–102).

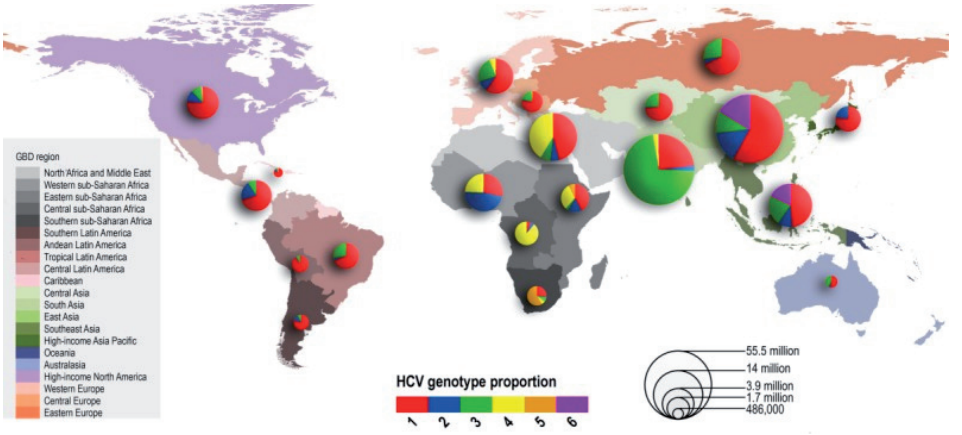

Figure 1. Distribution and prevalence of HCV GTs. The map is derived from Messina et al. 2015 "Global distribution and prevalence of hepatitis C virus genotypes." Hepatology, 61: 77–87. doi: 10.1002/hep.27259 (102).

HCV is a blood-transmitted virus. Virus-containing blood is highly infectious; therefore, transmission via contaminated blood is very efficient. Primary HCV infection is usually asymptomatic or is associated with very mild symptoms and is therefore typically not recognized and diagnosed. Only 15–45% of infected patients clear the virus, while in 55–85% of patients, primary infections are followed by chronic HCV infection (103, 104). The likelihood of clearing the primary infection depends both on the HCV GT (being lowest in the case of GT1 (103)) and on host factors. The first and the most prominent determinant that is known to affect the probability of clearing HCV infection (and also the efficiency of anti-HCV treatment) is the single nucleotide polymorphism rs12979860, which is located 3 kb upstream of the IL28B (interferon λ3) gene (105–107).

HCV has been shown to infect monocytes (108), lymphocytes (109) and some other cell types (110); however, the main target of the virus is the liver and hepatocytes (111). Chronic HCV infection in the liver leads to fibrosis (112) and cirrhosis and can result in HCC. It is estimated that more than 350,000 people worldwide die from HCV-related liver diseases each year, and HCV-induced cirrhosis is the major reason for liver transplantation in Western countries. The mechanisms by which HCV induces these diseases are not obvious, as HCV infection itself is not cytotoxic. However, chronic HCV infection is associated with an inflammatory response in the liver. HCV infection and disease progression can be hidden for decades until the liver structure is seriously damaged and the disease culminates with cirrhosis. The other liver disease that is associated with HCV infection is steatosis (reviewed in (113)). Steatosis is often correlated with the progression of fibrosis and the development of HCC. The molecular basis of HCV-induced steatosis is in the tight connection of HCV infection with lipid metabolism. Thus, HCV infection leads to changes in cellular metabolism that promote the accumulation of lipid droplets (LD). The triglycerides and cholesterol esters that are found in the HCV-infected liver differ from those of obese patients, suggesting the existence of a different course of steatosis (114). HCV infection is also linked to insulin resistance and oxidative stress. Moreover, due to the ability of HCV to interfere with different immune and metabolic pathways, HCV infection is also associated with a number of extrahepatic manifestations, including neuropsychiatric symptoms, cardiovascular diseases, fatigue and autoimmune syndromes (99).

1.4.2. HCV virion and genome

The structural characterization of infectious HCV particles has faced many obstacles. Different studies have indicated the diversity of HCV particles circulating in human serum. These particles can vary in size (40–100 nm) and density (1.03 to 1.25 g/ml), and the glycoprotein spikes are often barely distinguishable. The analysis of cell culture-produced HCV virions (ccHCV) also has problems, as the morphologically uniform particles have a different density than the infectious particles (115). Similarly, the composition of HCV particles depends on the source from which the particles were obtained. Nevertheless, the basic structure of the HCV virion is now reasonably well known (Fig. 2A). The HCV virion contains an RNA genome that is packed into a shell made of capsid protein. This nucleocapsid is surrounded by the endoplasmic reticulum (ER)-derived envelope that contains HCV E1/E2 glycoprotein heterodimers and cellular ApoE proteins. Structural analyses carried out using EM have shown the presence of a non-continuous lipid bilayer that might be caused by the embedding of lipoproteins into HCV particles (115, 116).

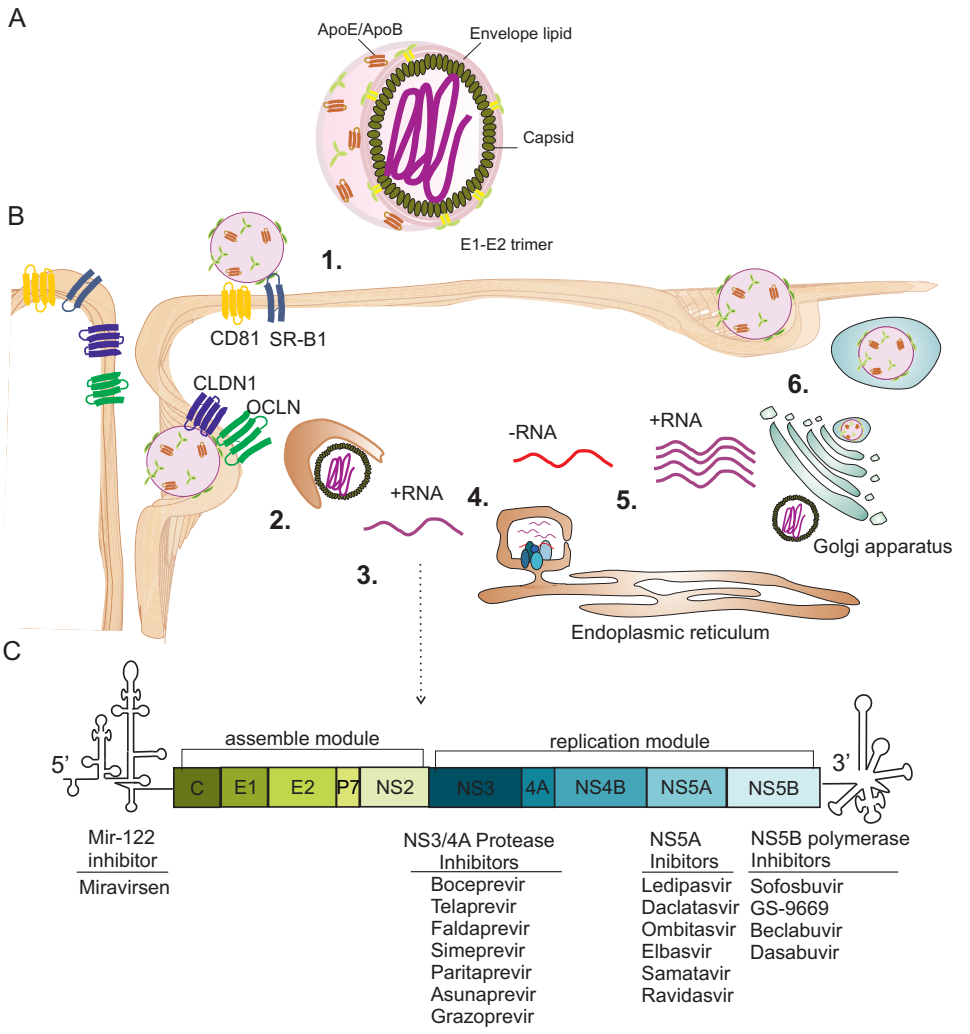

Figure 2. A. Schematic presentation of an HCV virion. **B.** HCV lifecycle: 1. Receptor-mediated virion binding 2. Virion internalization and nucleocapsid release into the cytoplasm 3. Genome translation 4. Replication complex formation on ER-derived membranes 5. Replication of the viral genome and nucleocapsid formation 6. Virion maturation at the Golgi complex and exit by exocytosis **C.** Schematic presentation of the HCV genome and the virus-encoded polyprotein. Anti-HCV DAAs (clinically approved compounds and compounds in late stages of clinical trials) are listed below their targets.

The HCV genome is positive-strand RNA approximately 9.6 kb in length. The genome contains one long major open reading frame (ORF) (Fig. 2C) and at least one overlapping shorter ORF. The HCV genome lacks a 5' cap and 3' poly(A) structures, but its 5' and 3' untranslated regions (UTRs) are highly structured and contain crucial elements that are required for genome translation and replication:

- The 5’ UTR contains sites for binding the cellular miRNA mir-122; these sites are essential for HCV genome translation and replication. As mir-122 is expressed primarily in the liver, the presence of such sites contributes to the hepatotropism of HCV (117). Another essential structure that is located in the 5’ UTR is an internal ribosome entry site (IRES) that has two well-described functions. This site acts as an initiator of translation and participates in the regulation of negative-strand RNA synthesis (118, 119).
- The 3’ UTR consists of a variable domain, a poly(U) tail and a conserved X tail that enhance genome translation and are crucial for the synthesis of negative-strand RNA (120–124).

IRES-mediated translation results in the synthesis of a large (~3000 amino acid (aa) residues) precursor polyprotein (Fig. 2C). During its synthesis, the N-terminal part of this polyprotein interacts with ER membranes. This polyprotein is co- and post-transnationally processed by cellular and viral proteases into 10 mature proteins. Of these proteins, C (capsid protein), E1 and E2 are structural proteins, and the remaining proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are non-structural proteins (Fig. 2C) (123–125).

1.4.3. HCV structural and non-structural proteins

Together with the RNA genome, **Capsid** protein (**C**, also known as **Core** protein) forms the nucleocapsid of the HCV virion. Capsid protein is first released from the polyprotein in its immature form (191 aa) and is further processed by a signal peptidase to generate the mature protein of 177 aa (126). The N-terminal part of the protein contains an RNA binding domain and is important for the formation of homo-oligomers. The C-terminal part of the protein mediates the association with LD and has also been shown to induce LD formation (127). The interaction of the capsid protein with NS5A and LD is critical for the assembly of HCV particles (128, 129).

E1 and **E2** are the envelope glycoproteins that are essential for virion attachment, internalization, membrane fusion and the maturation of new virions. E1 and E2 are highly glycosylated and occur in the form of heterodimers. The E1 and E2 heterodimer is stabilized by disulphide bonds and is arranged to the membranes of budding virions (130). E2 is also the major HCV antigen and facilitates the receptor-binding step in virus attachment to cells. The structure of the E2 core domain was recently resolved by two research teams (131, 132). Despite the fact that E2 was also considered to mediate membrane fusion both of these studies failed to reveal the structural hallmarks of the fusion protein (132, 133). Similarly, the partial 3D structure of the E1 protein (in the form of a homodimer) lacks folding characteristic of class II fusion proteins (134). It was only recently found that during virion assembly, E1/E2 heterodimers are

rearranged, and E1 trimers are formed. This re-arrangement is consistent with a possible role of E1 as an HCV fusion protein (135).

P7 is a small transmembrane protein with both termini orientated to the ER lumen. P7 is essential for virion assembly and release both *in vitro* and *in vivo* (136, 137), probably because it is needed for the final steps of capsid assembly and envelopment (138). P7 is a viroporin and forms hexamers or heptamers with cation-channel activities. It has been hypothesized that these ion channels prevent acidification in intracellular compartments that normally have acidic environments. This loss of acidification is required for productive HCV infection, possibly by protecting nascent virus particles during the maturation process (139).

NS2 is a membrane–associated protein with two distinct functions. First, the highly conserved (between different HCV GTs) C-terminal part of NS2 (aa residues 94–217) contains a cysteine protease domain (140). The protease activity of NS2 is enhanced by the N-terminal part of NS3 and is required for the processing of the site between NS2 and NS3. This cleavage is essential for obtaining free NS3, which is in turn required for HCV genome replication. Interestingly, NS2 itself is not required for HCV RNA replication (141). Second, NS2 plays a central role in virion assembly. It is hypothesized that for this function, the membrane binding domain of NS2 and complex protein-protein interactions mediated by NS2 are required. However, the exact mechanisms by which NS2 participates in infectious particle production are not yet known (142–146).

NS3 is a large protein that consists of N-terminal protease and C-terminal NTPase/RNA helicase domains. These enzymatic activities have been shown to work independently of each other, although they are enhanced by the presence of both domains of NS3.

The NTPase/RNA helicase domain is a very well-characterized member of helicase superfamily II. This domain is essential for viral RNA replication and probably acts by unwinding RNA secondary structures and dsRNA replication intermediates. NS3 does not discriminate between its natural substrate (RNA) and DNA and is also able to bind and very efficiently unwind DNA (147). Biochemical assays of NS3 helicase activity have shown that the pH optimum for the enzyme is ~6.4. Relatively low pH values likely promote more efficient conformational changes of NS3, resulting in higher enzymatic efficiency (148).

NS3 is also a serine protease; the protease fold covers the N-terminal third of the protein. The catalytic triad consists of the His57, Asp81, and Ser139 residues. For optimal protease activity, the **NS4A** protein is needed as a cofactor. Together, these proteins form the fully active **NS3/4A** serine protease that performes the processing of the non-structural part of the HCV polyprotein (149–153). The 3D structure of NS3/4A reveals that two β-barrel subdomains of NS3 and the central portion of NS4A form a chymotrypsin-like fold, which is stabilized by Zn^{2+} ions. The N-terminal part of NS4A forms a transmembrane α-helix that mediates membrane binding of NS3/4A, while the C-terminal domain

of NS4A interacts with other replicase components. It has been shown that the protease activity of NS3/4A depends on the positioning of its active site with respect to membranes. The conformational flexibility of the enzyme is achieved via a linker segment located between the helicase and protease domains of NS3 (154).

The substrate binding site of the NS3/4A protease can accommodate peptides with a length of 6 aa, but the most efficient cleavage is obtained for longer, 10 aa substrates. The consensus sequence of the substrate is D/E-X-X-X-X-C/T↓S/A-X-X-X (155, 156). This consensus sequence is not only present in the viral polyprotein but can also be found in a variety of host proteins. One of the best known host targets of the NS3/4A protease is the adaptor protein MAVS (156). NS3/4A cleaves this protein and thus interferes with innate immune responses mediated by the RIG-I/MDA-5 pathway. However, MAVS is not the only host protein involved in pathogen recognition that is cleaved by NS3/4A. The enzyme is also known to cleave TRIF, which is an adaptor for the TLR3 (155), DDB1 (157) and GPx8 (158) proteins.

NS4B has four transmembrane segments and forms three different domains (159). NS4B harbours NTPase activity and has been shown to bind RNA (160, 161). This protein induces the formation of a membranous web, which is critical for the assembly of HCV replication complexes (162). The N-terminal domain of NS4B mediates protein-protein interactions, and its topology is dependent on other replicase proteins (163). The C-terminal part of NS4B contains two α-helixes and is palmitoylated (164). This domain is also involved in protein-protein interactions and is important for the oligomerization of NS4B molecules (165). NS4B oligomerization appears to be the key determinant for the induction of vesicle formation (166).

NS5A is a multifunctional protein that is anchored to membranes by its N-terminal part. NS5A contains three domains (D1, D2, and D3) that are separated by low complexity sequences (167). The D1 domain forms dimers and can facilitate RNA binding (168); this domain also can bind Zn^{2+} ions via cysteine residues (167). The D1 domain is also involved in binding to LDs, and together with the D2 domain, this domain is important in RNA replication (169). The D2 and D3 domains are thought to facilitate interactions with host proteins. To date, NS5A has been shown to interact with more than 130 host proteins. It has been hypothesized that this large number of interactions originates from the ability of NS5A to be present in different states of phosphorylation and from the abilities of D2 and D3 to acquire different conformations. Indeed, the D2 and D3 domains contain serine residues that can be phosphorylated; both basally and hyper-phosphorylated forms of NS5A can be found in infected cells (170, 171). The D3 domain also interacts with the capsid protein, indicating its role in virion formation (129).

NS5B is an RNA-dependent RNA polymerase (RdRp). The large catalytic domain is located in its N-terminal part (530 aa) and is followed by a linker sequence and a C-terminal peptide (21 aa), which serves as a membrane anchor.

The 3D-structure of the catalytic domain of NS5B shows a classical "right hand" organization, with finger, thumb and palm domains (172). The finger and thumb domains provide contacts with RNA, and the palm region contains the catalytic site. For *de novo* and primer-dependent synthesis, the RdRp needs divalent metals as cofactors. The primary conformation of NS5B appears to be the "closed hand," in which the template binding groove fits only ssRNA and uses nucleotide for priming (173). This conformation is used in the initiation of the synthesis of HCV negative-strand RNA. The subsequent elongation step requires the open conformation of NS5B, as the groove must accommodate the primer-template RNA complex (174, 175). NS5B also appears to have a specific role in replication complex assembly that is distinct from its RdRp activity (176).

1.4.4. Hepatitis C virus infection cycle

Hepatocytes are the main targets of HCV. The infection starts with the attachment of viral particles to specific cell receptors, primarily via E2 (Fig. 2B). The entry of HCV is a very complex process and involves interactions with many different host factors. Binding is initiated by attachment to glycosaminoglycans and/or lipoprotein receptors (177, 178). This attachment is followed by coordinated interactions of HCV particles with the scavenger receptor class B type I (179) and a major receptor of high-density lipoprotein – CD81 tetraspanin (180). These interactions trigger subsequent interactions of HCV with the tight junction proteins claudin-1 (181) and occludin (182). This set of particle-receptor interactions ultimately leads to the uptake and cellular internalization of HCV through clathrin-dependent endocytosis. The precise mechanism of membrane fusion and particle uncoating remains to be discovered.

After fusion, the RNA genome is released into the cytoplasm of the cells and translated into a viral polyprotein in a cap-independent manner. Translation is mediated by an IRES structure located in the 5' UTR of the RNA genome (183, 184). In addition to the basic translation machinery, additional cellular factors are known to stimulate IRES activity. During synthesis, the polyprotein binds to the ER membrane (Fig. 2B) and is co- and post-translationally cleaved by viral (NS2/3 and NS3/4A) and host proteases into mature proteins (185).

Viral non-structural proteins are needed for the initiation of HCV RNA replication (149, 151, 152, 185), and their accumulation initiates the formation of multi-membrane vesicles. The HCV replication complexes are most likely ER-derived "double-membrane vesicles" (186). The morphology and exact composition of these complexes are poorly understood. The newly formed replication complex uses the viral genome as a template for negative-strand RNA synthesis. As with other positive-strand RNA viruses of eukaryotes, the negative-strand RNA likely forms a duplex with the complementary positive strand. For the synthesis of new genomes, the viral RdRp uses the negative-strand RNA of

the duplex as a template. Newly synthesized positive-strand RNAs either re-enter the replication cycle or are directed to viral particle formation (187, 188).

HCV particle formation is a complicated and incompletely understood process. This process is tightly linked to lipid metabolism. Interestingly, many NS-proteins of HCV take part in particle assembly, indicating links with RNA replication. Particle formation is started by the interaction of genomic RNA with the capsid protein (189). Locating genomic RNA into LD and the smooth ER is crucial for assembly (Fig. 2B). HCV particles form by budding to the luminal side of smooth ER membranes containing E1/E2 heterodimers. The particles are transported to the plasma membrane via the very low-density lipoprotein synthesis/secretion pathway. During this process, complex modifications of formed particles occur. Infectious particles are released by exocytosis (190–192). It is also known that HCV can infect neighbouring cells without actually exiting to the extracellular space (Fig. 2B). The mechanism that underlies this cell-to-cell spread is not yet known; however, it has been shown that this type of spreading is resistant to the presence of neutralizing monoclonal antibodies (193–195).

1.4.5. Historical and current therapies for HCV infection

The aim of anti-HCV treatment is to reach a sustained virological response (SVR). An SVR is defined as the condition in which 24 weeks after the end of treatment, no virus can be detected in the patient's blood. Until 2011, the main standard of care (SOC) for chronic HCV infection was based on combined treatment with IFN-α (or pegIFN-α) and ribavirin. Depending on the HCV GT, the SOC treatment lasted from 24 to 48 weeks (196, 197), and its efficiency depended on host genetics (105) and on the HCV GT. In general, an SVR was achieved for only 40–50% of GT1 treatment-naïve patients; in contrast, an SVR was achieved in approximately 80% of infections with other GTs. PegIFN-α and ribavirin are broad-spectrum antivirals (see 1.3.1, 1.3.2), and their use is associated with various side effects.

Boceprevir and telaprevir, which are inhibitors of the HCV GT1 NS3/4A protease (mimic the substrate of the enzyme), became the first approved DAAs against HCV. The approval of these inhibitors in 2011 allowed the use of triple therapy: the new inhibitors were used in combination with the former SOC. The use of these inhibitors raised the SVR rate to 60% in the treatment of experienced patients compared to a 20% SVR for the control group (198, 199). In the case of treatment-naïve HCV GT1 patients, the rate of SVR achieved by triple therapy was approximately 70–80%. The use of these DAAs did not alleviate the side effects of the SOC; instead, new adverse effects (such as rash in the case of telaprevir) were observed. Thus, the first DAAs resulted in a clear improvement of HCV treatment but not a breakthrough.

The real breakthrough – IFN-free treatment with oral drugs with pan-genotype activity – was achieved recently. The cornerstone of this therapy is the

nucleotide analogue NS5B polymerase inhibitor sofosbuvir, which was developed by Pharmasset/Gilead and is marketed as a mixture with ribavirin under the brand name SOVALDI® (57). Unlike the first-generation protease inhibitors, sofosbuvir is active against all HCV GTs (although its activity against GT3 is somewhat lower). Most importantly, sofosbuvir shows a very high barrier of genetic resistance. Mutants resistant to this drug are rare, and such mutations are associated with a great fitness cost. Furthermore, the treatment is considerably shorter (24 weeks for GT3 and 12 weeks for other GTs) and can be applied both to treatment-naïve and experienced patients, with or without liver fibrosis/cirrhosis (57, 200).

In the last two years, a few more DAAs targeting HCV have received approval for clinical use. In addition to the NS3/4A protease and the NS5B polymerase, the NS5A protein has been successfully targeted (Fig. 2C). The list of approved DAAs (as October, 2015) includes nine compounds:

- Sofosbuvir, a nucleotide analogue inhibitor of NS5B
- Dasabuvir, a non-nucleoside inhibitor of NS5B

Thus, there are two classes of NS5B inhibitors with different mechanisms of action. Nucleotide inhibitors act as pseudo-substrates of the enzyme, while non-nucleoside inhibitors act by creating unfavourable conformational changes in the proteins (201–203).

- Boceprevir and telaprevir, which are first-generation NS3/4A inhibitors. These compounds are linear peptidomimetics that bind covalently to the NS3/4A protease and block the substrate binding sites (204, 205).
- Simeprevir and paritaprevir, which are second-generation protease inhibitors. These inhibitors are macrocyclic compounds that do not bind covalently to the enzyme (206, 207).
- Ledipasvir, ombitasvir and daclatasvir target NS5A. These compounds have demonstrated pan-genotypic activity and a relatively high barrier to resistance. Curiously, the precise mechanism of action of these inhibitors is not known. It is proposed that these inhibitors can either regulate NS5A phosphorylation and/or regulate lipid metabolism. Regardless of the mechanism, these compounds are among the most efficient inhibitors and greatly reduce HCV replication (208).

As the number of approved DAAs has increased rapidly, precise DAA combinations have been developed to achieve the safest and most efficient outcomes (209). The currently approved combinations include:

- HARVONI®: a mixture of sofosbuvir and ledipasvir (210);
- VIEKIRA®: a mixture of ombitasvir, paritaprevir, dasabuvir and ritonavir (not an HCV inhibitor; instead, it is an inhibitor of CYP3A4 enzymes) (211).

In addition, some new DAAs can be used in combination with pegIFN-α and/or ribavirin. Examples of such DAAs are simeprevir (212) and daclatasvir; these

drugs can also be combined with sofosbuvir. Many of these combinations exhibit SVR rates of up to 99%, and in the case of IFN-free combinations, the side-effects are drastically reduced (100). With new drugs in the pipeline (see 1.4.6), the number of successful drugs/drug combinations will continue to increase. However, the persisting problem is the cost of treatment, which has increased considerably (~20,000–30,000 US dollars in 2010 compared to 84,000–168,000 US dollars in 2015). This increase has boosted interest in the development of more affordable treatments for HCV infection.

1.4.6. Further development of new anti-HCV therapies

As reviewed above, the progress in HCV treatment in the last 5 years has been tremendous. New DAAs have brought SVR rates near 100% and reduced the side effects caused by pegIFN-α and ribavirin. Nevertheless, there are still multiple aspects to resolve and improve, such as the increased costs of treatment. The development of DAAs has also been biased towards the treatment of GT1 patients; consequently, the treatment efficiencies for patients infected with other GTs are lower. Patients with severe cirrhosis and post-transplantation patients are still in need of improved treatment. Another aspect is the acquired resistance of HCV to DAAs; due to the relatively short history of these treatments, the possible magnitude of the problem is not yet known. Therefore, further research and development in the area of HCV inhibitors is still needed.

Most new inhibitors in the late stages of clinical trials target the same proteins that are targeted by already approved DAAs (Fig. 2C).

- there are several promising non-nucleoside inhibitors of NS5B, including beclabuvir (phase III) and GS-9669 (phase II);
- grazoprevir is a new macrocyclic NS3/4A inhibitor that has entered phase III trials; the same is true for the non-macrocyclic (tripeptide) NS3/4A inhibitors faldaprevir and asunaprevir;
- of the new NS5A inhibitors, elbasvir has entered phase III trials; samatasvir and ravidasvir (PPI-668) have been tested in phase II trials.

Based on these (possibly incomplete) data, it is not hard to predict that the number of anti-HCV DAAs will increase. However, the list of targets does not appear to expand. There are no approved drugs (or compounds in late stages of development) that target the NS4B and p7 proteins or the helicase activity of NS3. Multiple *in vitro* studies have shown that these proteins can be targeted to reduce the release of HCV particles. Thus, clemizole and its analogues target NS4B RNA binding activity. Anguizole and structurally related compounds bind NS4B and inhibit the lipid vesicle aggregation that is characteristic of HCV infection. These are only some examples of compounds targeting HCV NS4B (213).

One group of antivirals that has received relatively little attention to date are host targeting antivirals (HTA), which target factors with proviral effects in the HCV infection cycle. Only a few such inhibitors have been tested in clinical trials. Examples of such compounds are cyclophilin inhibitors (alisporivir) (214) and ASOs that target liver-specific mir-122 (miravirsen) (215). These inhibitors are able to supress different HCV GTs and possess high resistance barriers. Other types of HTAs include antibodies that target the extracellular domains of claudin (216) and compounds that inhibit different steps of the cholesterol biosynthesis pathway (217).

1.4.7. Status and perspectives of anti-HCV vaccine development

The genetic diversity of HCV and its ability to escape the immune system have hampered vaccine development; as a result, no vaccine is available for HCV. Another obstacle is that in natural infection, HCV generally does not activate an immune response to an extent that allows the efficient elimination of the virus. Based on the experience acquired from other antiviral vaccines, it is clear that successful vaccination should induce a strong neutralizing antibody and (preferably) also T-cell responses. Ideally, these responses should target all HCV GTs or at least a range of HCV GTs. However, given the very low sequence conservation, a pan-genotypic HCV vaccine may not be possible. Despite all of these difficulties, several anti-HCV vaccine candidates have reached clinical trials.

In contrast to the majority of viral vaccines, most HCV vaccines have been designed not as prophylactic vaccines but as therapeutic vaccines. Such vaccines aim to eradicate already established chronic HCV infection and are often tested in combination with antiviral therapy (218, 219). In addition, the use of therapeutic vaccines may result in long-lasting immunity that will prevent (or reduce) re-infection. Given the spread of HCV in certain risk groups (such as intravenous drug users), long-lasting immune protection may represent an important benefit, as the probability of re-infection (with the same or different HCV GT) is high. One of these vaccine candidates, E1E2/MF59, contains the E1 and E2 proteins together with the adjuvant MF59C.1 (oil (squalene)-in-water emulsion). A clinical trial of this vaccine in combination with pegIFN-α/ribavirin treatment resulted in a reduced viral load (220).

The progress achieved in the area of anti-HCV drug development has not reduced the importance of preventing HCV infection. First, as primary HCV infections generally do not cause symptoms, they are usually unnoticed and patients turn to doctors only when serious liver damage has already occurred. Second, the prevention of infection is likely to be a much less expensive option than the treatment of chronic HCV infection. Finally, at least in risk groups, there is a possibility of re-infection among previously treated patients. Therefore, the development of a prophylactic vaccine has clearly maintained its importance. However, to date, few vaccine candidates of this type have been

generated and tested in clinical trials. One example of this strategy is a vaccine that aims to induce cellular immunity by using heterologous virus vectors that express parts of the HCV polyprotein. Such vaccine candidates are based on a replication-defective simian adenoviral vector (ChAd3) and modified vaccinia Ankara based vectors expressing the NS3, NS4, NS5A, and NS5B proteins of HCV GT1b. Clinical trials have shown that this vaccine induces durable, broad, sustained, and balanced T cell responses (221). These findings are encouraging and indicate the potential of prophylactic HCV vaccines.

1.5. Chikungunya virus (family *Togaviridae*)

Chikungunya virus (CHIKV) belongs to the genus *Alphavirus* family *Togaviridae*. To the date approximately 30 different alphaviruses have been described. These viruses are found on every continent and infect a broad range of hosts, from invertebrates to humans. Alphaviruses are mainly arboviruses, and they spread between vertebrate hosts by using invertebrate vectors (mainly mosquitos) (222, 223). Many alphaviruses infect humans and can cause diseases; however, before 2005, these viruses were not considered to have significant medical importance. This view changed during the last decade, as one alphavirus, CHIKV, has spread rapidly and travelled beyond its usual hotspots, affecting millions of people (224, 225). There are three genotypes (sometimes referred to as lineages) of CHIKV: East Central South African (ECSA), Asian, and West African (222). The closest relative of CHIKV is o'nyong'nyong virus (ONNV) (222). Other alphaviruses that are pathogenic to humans and cause fever, rash, arthritis and myalgia (typical symptoms of CHIKV, see 1.5.4) are Ross River virus (RRV) and Barmah Forest virus (BFV) in Australia/Oceania and the widespread Sindbis virus (SINV) (226). New World alphaviruses, such as Western, Venezuelan and Eastern equine encephalitis viruses (WEEV, VEEV and EEEV), primarily cause encephalitis (227).

CHIKV is transmitted to humans by *Aedes* mosquitoes. The mosquitoes acquire infection by feeding on infected humans (or animals). CHIKV infection in mosquitoes is persistent and is thought to be non-pathogenic (or largely non-pathogenic) (228). The most common vector for CHIKV is the tropical mosquito *Aedes aegypti*. *Aedes albopictus* mosquitoes can also transmit CHIKV, albeit typically less efficiently. However, *Aedes albopictus* tolerates colder climates and is more widespread in urban areas, and the area of this mosquito has rapidly expanded due to climate changes and the globalized economy (transport of different goods) (229). Furthermore, as highlighted below, CHIKV of the ECSA genotype can specifically adapt to this vector.

CHIKV was first isolated in Tanzania in 1952 (230), and for nearly half of a century, the virus remained largely unstudied. An outbreak of unprecedented magnitude started in 2004/2005. This outbreak was caused by the ECSA genotype of the virus (231), which acquired a single aa change in the virus envelope

glycoprotein E1 (Ala226 to Val) during the outbreak. This change greately increased the infectivity of the virus for *Aedes albopictus* midgut cells (232). Additional mutations that facilitate infectivity of CHIKV for *Aedes albopictus* even further were also documented (229, 233). These changes were sufficient to allow a vector switch, which led to epidemics on Indian Ocean islands (including La Reunion) in 2005 (234, 235) and subsequently in South-East Asia. Due to increased travel, smaller outbreaks also occurred in Europe: in 2007 in Italy (236) and in 2011 in France (237). In 2013, CHIKV reached the Caribbean islands and Central America, and local circulation of the virus was established (238–240). The Asian genotype of CHIKV, which is responsible for this outbreak (241), cannot adapt to *Aedes albopictus* as easily as the ECSA genotype (242). Consequently, CHIKV transmission in the region occurs mostly via *Aedes aegypti* mosquitoes. The current worldwide distribution of CHIKV is shown in Fig. 3.

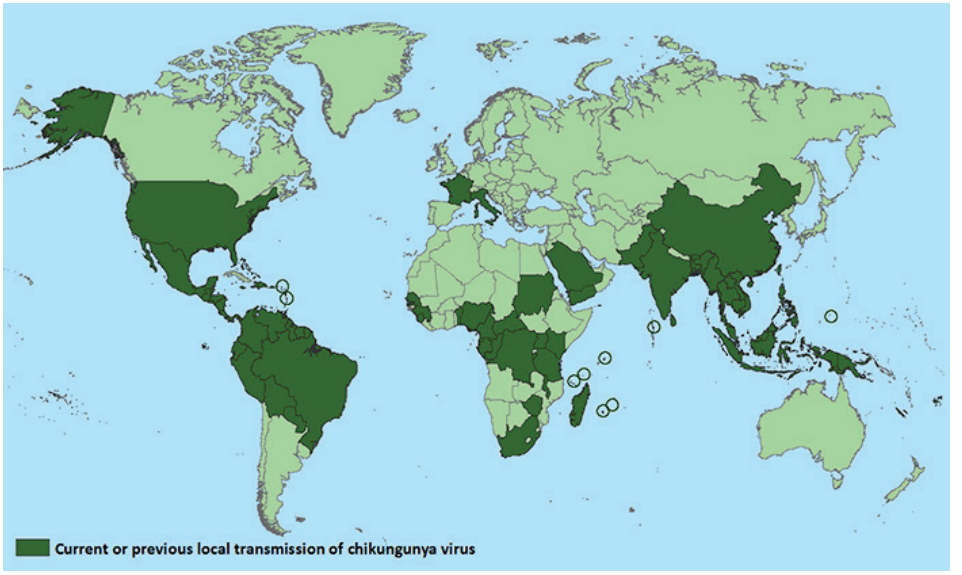

Figure 3. Global distribution of CHIKV as of October 2015. Countries and territories with local transmission are shown in dark green.
(http://www.cdc.gov/chikungunya/geo/index.html Permission obtained from the Centers for Disease Control and Prevention.)

1.5.1. CHIKV virion and genome organization

Alphaviruses have enveloped virions with very regular structures. The external virion diameter is approximately 65 nm and it contains an inner nucleocapsid with a diameter of approximately 40 nm (243). The nucleocapsid contains a single RNA genome and 240 copies of the capsid protein (T4 symmetry). The

nucleocapsid is surrounded by a lipid bilayer derived from the cellular plasma membrane (Fig. 4A). The envelope is tightly packed with viral glycoproteins that form 80 spikes. Each spike consists of three E1-E2 heterodimers. Thus, similar to the capsid, the outer glycoprotein layer also exhibits T4 icosahedral symmetry (244). The 3D structure of E1-E2 dimers of CHIKV has been resolved and in combination with excellent cryo-EM data, has resulted in reconstruction of a high-resolution structure of CHIKV virions (245).

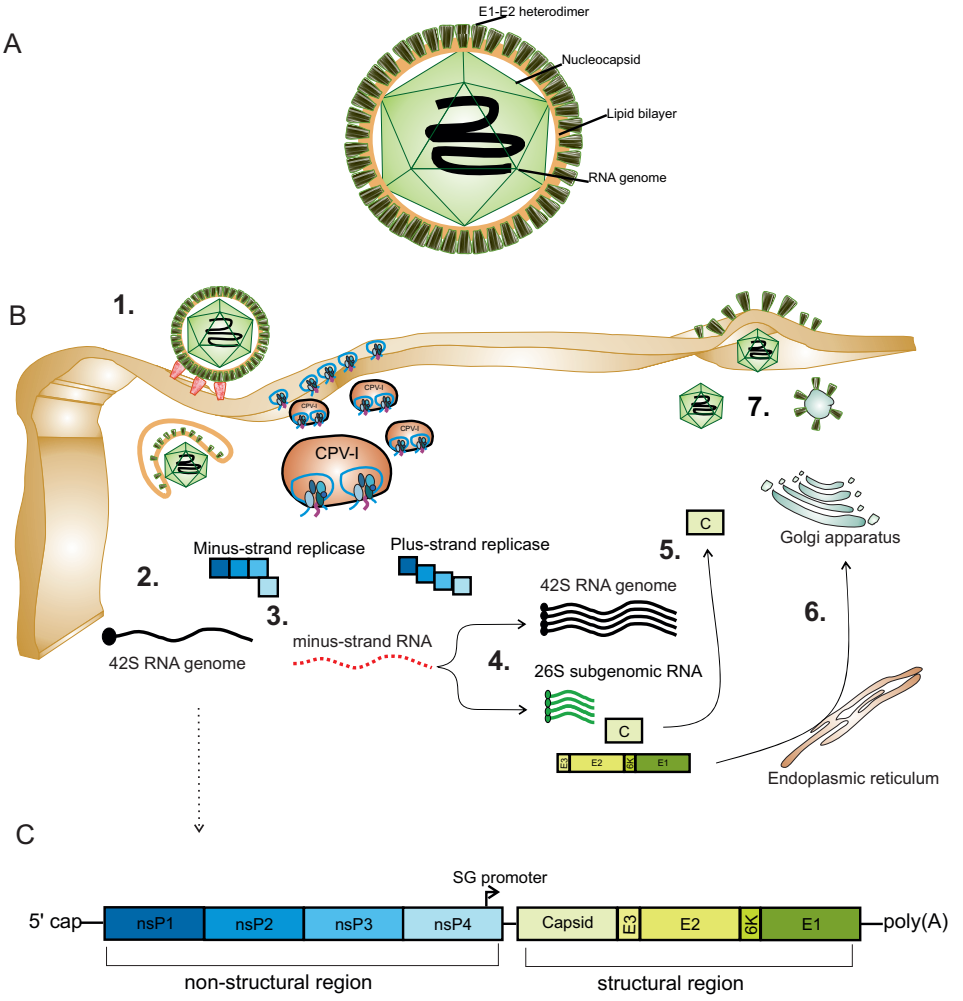

Figure 4. A. Schematic representation of a CHIKV virion. **B.** CHIKV lifecycle: 1. Binding to the receptor and internalization of the virion via clathrin-dependent endocytosis 2. Nucleocapsid release into the cytoplasm, followed by the synthesis of the non-structural polyprotein 3. Replication complex (spherule) formation coincides with negative-strand RNA synthesis. Replication complexes are internalized, and CPV-I structures are formed 4. Synthesis of genomic (42S) and subgenomic (26S) RNAs 5.

Nucleocapsid assembly 6. Processing and maturation of virion glycoproteins. 7. Budding of new virions **C.** Schematic presentation of the CHIKV genome.

The CHIKV genome has a length of approximately 12 kb. Similar to cellular mRNA, the genome contains a cap structure at its 5' end and a 3' poly(A) tail (Fig. 4C). The genome contains two ORFs. The 5' ORF (roughly 2/3 of the CHIKV genome) encodes precursor(s) of the viral non-structural proteins (nsPs), while the 3' ORF encodes a precursor of the viral structural proteins. The CHIKV genome has a short 5' UTR, while the 3' UTR contains a variable number of repeated motifs and therefore its length varies considerably between different CHIKV genotypes and isolates (246). The third non-coding region is located between two ORFs. This genome organization is shared by all alphaviruses. There are four conserved sequence elements (CSE) that are essential for genome replication and transcription and are highly conserved in all alphaviruses. CSE1 is located in the 5' UTR and is essential for the synthesis of negative- and positive-strand RNAs (247). CSE2 (also known as 51 b element) is located in the region that encodes the N-terminal part of nsP1. This element contains two hairpin structures, which are dispensable for replication in mammalian cells but crucial for replication in insect cells (248, 249). CSE3 is located at the end of the coding region of nsP4 and is included in the subgenomic (SG) RNA promoter that is used for the synthesis of mRNA for structural proteins (250, 251). CSE4 is located before the poly(A)tail and contains the start site for negative-strand RNA synthesis (252, 253).

1.5.2. Functions of CHIKV-encoded proteins

Current knowledge about CHIKV protein functions is fragmented. Multiple functions have been demonstrated for proteins of other alphaviruses, and it is often assumed that CHIKV proteins carry the same activities and functions. This assumption is likely to be true for conserved enzymatic or structural properties. Other functions, such as interactions with the host, may not be conserved. In the brief review below, I will concentrate on the functions that have either been directly demonstrated for CHIKV proteins or have been revealed for proteins of other alphaviruses but almost certainly also apply to their CHIKV counterparts.

Non-structural protein 1 (nsP1) is essential for the synthesis of negative-strand RNAs (254). This protein has methyltransferase and guanylyltransferase activities that are required for the synthesis of the cap structure at the 5' end of the virus genome and SG mRNA using unique (but common for alphavirus-like RNA viruses) pathway (255–257). Alphavirus replication complexes are anchored to cellular membranes via an amphipathic helix located in the central part of nsP1 (255, 258–260). Palmitoylation of nsP1 enhances its binding to cellular membranes (261). In infected cells, part of nsP1 is located in the plasma membrane, where it counteracts the effects of the cellular antiviral protein tetherin (262, 263).

Non-structural protein 2 (nsP2) is a multifunctional protein that performs different tasks. The N-terminal region (aa residues 1-470) of nsP2 possesses NTPase and 5'-RNA triphosphatase activities. The full-length nsP2 also has RNA helicase activity that unwinds double-stranded RNA molecules in the 5'-3' direction. The significance of this activity for virus replication is not precisely known. nsP2 also has an opposite, RNA matchmaker activity (264, 265). The C-terminal region of nsP2 is a cysteine papain-like protease that is responsible for the processing of the viral non structural polyprotein (266). nsP2 is also a key regulator of virus-host interactions. Part of nsP2 is transported to the nucleus, where (in the case of vertebrate cells) it causes the degradation of cellular RNA polymerase II and shuts down host cell transcription (267). Different mutations in nsP2 have been shown to reduce the cytotoxicity of CHIKV infection (268, 269). In addition, nsP2 interferes with interferon-induced signalling and the unfolded protein response (270, 271).

Non-structural protein 3 (nsP3) is the least studied replicase protein of alphaviruses. This protein contains three domains: the macro domain, the Zn binding domain (ZBD) and the C-terminal hypervariable domain (HVD). The macro- and Zn binding domains are conserved between alphaviruses, and the crystal structures of these domains have been resolved (272, 273). The macro domain of some (but not all) alphaviruses has weak adenosine di-phosphoribose 1"-phosphate phosphatase activity (272), which unlikely has any significance for alphavirus infection. Most likely this activity reflects another, yet to be discovered, enzymatic activity of this domain. The ability of the macro domain (and the Zn binding domain) to bind RNA is also important for the virus (273). The macro domain can also bind ADP-ribose, poly(ADP-ribose) and poly(ADP-ribose) polymerase 1 (274, 275). This property suggests a role of nsP3 in the suppression of the cellular anti-viral response, as IFN-stimulated poly(ADP-ribose) polymerases are powerful inhibitors of alphavirus infection (276, 277).

The HVD of nsP3 is intrinsically disordered. With the exception of several conserved motifs, no sequence similarity between corresponding domains from different alphaviruses can be observed (223). The HVDs of Semliki Forest virus (SFV) and SINV are heavily phosphorylated. It has been established that in the case of the nsP3 of SFV, approximately 16 aa residues, which are located at the beginning of HVD, are phosphorylated. For SINV, it has been shown that the reduction of nsP3 phosphorylation decreases the replication of viral RNA. Moreover, in the case of SFV, the deletion of the phosphorylated region results in a loss of neurovirulence (278). Interestingly, the phosphorylation of nsP3 has not yet been experimentally demonstrated for CHIKV. Most likely, this function exists, but its significance for different viruses is not necessarily the same.

There are also other functions of HVD that are similar between CHIKV and at least some other alphaviruses. Thus, the HVDs from SFV and CHIKV interact with cellular G3BP proteins. This interaction interrupts the formation of stress granules and is also important for the initial stages of CHIKV infection (279–284). It is reasonable to assume that the HVD also contains motifs re-

quired for interactions with other cellular proteins. Some of these interactions may be conserved for all alphaviruses, other interactions may be conserved for some alphaviruses and some interactions may be unique to a specific virus. Several of these interactions have been revealed, and in a few cases, their biological significance has been demonstrated (285, 286). Taken together, even though the precise functions of nsP3 remain largely unrevealed, it is clear that this protein plays a critical role in the correct formation and localization of replication complexes (259) and is central for alphavirus-host interactions.

Non-structural protein 4 (nsP4) is highly conserved among alphaviruses. Very little is known about the functions of CHIKV nsP4, because in infected cells, this protein is present in very low amounts. Upon expression in the form of a recombinant protein, nsP4 aggregates and is extremely hard to solubilize (unpublished observation of our lab). Therefore, most of the available information regarding the functions of alphavirus nsP4 originates from studies of SINV. It has been demonstrated that nsP4 is an RNA-dependent RNA polymerase (RdRp) of alphaviruses that is responsible for viral RNA replication (223, 287). This protein also possesses terminal adenylyl-transferase activity that is essential for the synthesis/repair of the poly(A) tails of viral positive-strand RNAs (288). This protein functions together with other ns proteins (259, 289, 290) and host proteins (259, 291). nsP4 has also been shown to have non-replicative functions. It was recently shown that nsP4 of CHIKV is essential for the suppression of the ER stress response that limits virus replication (292).

Alphavirus structural proteins are translated from SG RNA in the form of a structural polyprotein (C-E3-E2-6K-E1). This precursor is cleaved into mature proteins by virus- and host-encoded proteases. The **capsid protein** has protease activity and cleaves itself from the rest of the structural polyprotein. In addition to the binding of the viral genome and the formation of the nucleocapsid, the capsid protein participates in the acquisition of the virion envelope by binding to the E2 glycoprotein (293, 294). **E1, E2** and **E3** are all glycosylated membrane proteins. E1 and E2 form spikes on the virion surface and are essential for virion assembly. E3 is not a transmembrane protein and remains associated with mature virions of some (such as SFV) alphaviruses. E2 is a palmitoylated transmembrane protein (295–297) and is responsible for the binding of the cellular receptor (298). E1 is the fusion protein of alphaviruses (299). **6K** is also included in virions, but the precise functions of this protein are not known (300). The final structural protein, **transframe (TF),** is produced by a ribosomal frame-shift that occurs in the region encoding the 6K peptide. This protein affects the pathogenicity of SINV infection and, to some extent, virus production in cell culture. However, the functions of this protein remain largely unknown (301, 302).

1.5.3. CHIKV replication cycle

The CHIKV replication cycle is similar to that of other alphaviruses (Fig. 4B). Infection starts with E2-mediated binding of the virion to an unknown cellular receptor (or receptors). Binding is followed by rapid internalization of the virion into endosomal vacuoles. Due to the low pH in the endosomes, E1-E2 heterodimers dissociate and the viral E1 protein undergoes irreversible conformational changes that allow fusion between the endosome and the virion membranes (245). After membrane fusion, a free nucleocapsid enters the cytoplasm and becomes disassembled, and the genomic RNA is released.

After entering the cell, the 5' two-thirds of the genome is translated into the non structural polyprotein (P1234). P1234 attaches itself to the inner surface of the plasma membrane. At the same time, the switch from genome translation to replication should occur. The mechanism of this process is unknown, although it has been suggested that this process may be mediated by the action of host proteins, possibly G3BPs (283). RNA replication is associated with tightly regulated processing of P1234; interruption of this process will result in the loss of infectivity or reduced viral fitness. First, nsP4 is cleaved from P1234 by the protease activity of the nsP2 region. The products of this reaction (P123 and nsP4) form an early replication complex, which synthesizes negative-strand RNA (anti-genome). Most likely, only one negative-strand RNA is made and it forms a duplex with the genomic RNA (303). This step must occur in the vicinity of the plasma membrane and coincides with formation of specific structures, membrane invaginations (called spherules), which contain viral RNA and replicase proteins (304, 305). After negative-strand RNA synthesis is completed, P123 is processed into nsP1 and the P23 polyprotein, and the final cleavage of P23 into nsP2 and nsP3 follows almost instantly. This cleavage results in the formation of a late replicase (nsP1+nsP2+nsP3+nsP4) that uses negative-strand RNA (or, more likely, double-stranded RNA) as a template and synthesizes new genomes and SG RNAs (303). SG RNAs are made in excess in comparison to viral genomes and are used for the synthesis of structural proteins. During the early stages of infection, the newly made genomic strand enters the replication cycle, and the number of functional replicase complexes increases. During the later stage of infection, the accumulation of free nsP2 results in a change in the P1234 processing pathway, preventing further formation of early (and consequently also late) replicase complexes (306). The already formed replicase complexes remain active, become internalized via phosphatidylinositol-3-kinase-, actin-, and microtubule-dependent transport (305) and are ultimately located in virus replication organelles – cytopathic vacuoles type I (CPV-I).

The packaging signal of CHIKV RNA is located in the region that encodes the protease domain of nsP2 (307). The interaction of capsid proteins and genomic RNAs results in the formation of nucleocapsids, which are then transported to the cell membrane. Budding is triggered by the interaction between the capsid protein and the E2 envelope protein (308, 309).

1.5.4. Pathology associated with CHIKV infection

CHIKV is transmitted via a mosquito bite and typically has an incubation time of between two and six days. Only 15% of CHIKV infections are asymptomatic. The typical symptoms of infection include high fever (~40°C), headache, back pain, myalgia, vomiting, nausea, conjunctivitis, arthralgia and rash (310, 311). CHIKV infection is characterized by high viremia, with viral titres ranging from 10^5 to 10^{12} pfu/ml (plaque forming units per millilitre). High viremia is a pre-requisite for mosquito transmission and usually lasts for up to five days (only then can mosquitoes acquire the virus). At this stage, viral replication is controlled by the innate immune response, and the severity of infection depends on the expression of several antiviral genes, including viperin and TLR3 (312, 313). Most symptoms of acute infection are cleared after several weeks, but in many cases, chronic symptoms (most commonly arthralgia/ arthritis) of different severity may occur. Alphavirus-induced arthritis primarily affects peripheral joints: fingers, wrists, elbows, toes, ankles and knees (311, 314). For CHIKV infection, it has been estimated that approximately 40% of patients develop a chronic disease (315). Chronic arthritis is more likely to occur in elderly people and in patients who have higher viral loads during the acute phase of infection (316). Additionally, patients with a previous history of rheumatic arthritis or joint injuries are more prone to suffer from severe CHIKV infection and subsequent chronic arthritis (317).

1.5.5. Development of antivirals and vaccines against CHIKV

No licensed vaccines or specific antivirals against CHIKV infection are available. Therefore, the treatment of CHIKV infection aims to relieve symptoms. There are also some broad-spectrum antivirals that can be used for the treatment of CHIKV infection. Some compounds that were developed for the treatment of other viral infections have also shown some antiviral efficiency towards CHIKV. However, the achieved benefit in the treatment of real infections (in patients) has been low, and in most cases, the mechanism that underlies the observed antiviral activity is unknown. The exception to this rule is the polymerase inhibitor favipiravir, which shows anti-CHIKV activity and clearly acts as an inhibitor of nsP4 (62). Other active agents that exhibit activities towards CHIKV are IFN-α and 6-azauridine (318). Ribavirin can act both as a nucleotide analogue and as an inhibitor of purine nucleotide biosynthesis. It has been hypothesized that reduced intracellular levels of GTP may impair the guanylyl transferase function of nsP1 (318). Chloroquine (68) and arbidol are thought to disrupt CHIKV entry (319).

The screening of libraries of synthetic and natural compounds has resulted in the identification of multiple hits with anti-CHIKV activity. The mechanisms of action of these compounds are typically unknown; at best, educated guesses concerning the possible targets can be made (based on molecular docking). For

example, several compounds that presumably target the nsP2 protease have been discovered (320, 321) or, in one case, specifically designed (322). The mechanisms of action of natural compounds with anti-CHIKV activity, such as silymarin (323), harringtonine (324), suramin (325) and trigocherrierin A (326), are not known, although it is possible that these compounds are HTAs. Few other compounds that target essential host factors for CHIKV infection have been described (291, 327).

Although no licensed vaccine is available for CHIKV, the existing data strongly suggest that the development of an efficient vaccine for this virus is not only possible but also achievable in the relatively near future. The first attempts to generate formalin-inactivated CHIKV vaccines date back to 1971 (328). Live-attenuated vaccine development was performed by the US Army. The vaccine was obtained from a CHIKV strain isolated in Thailand. Propagation of the virus in cell culture (unfortunately, the exact passage history is unknown) resulted in an attenuated virus that is designated as CHIKV TSI-GSD-218. This vaccine was found to be efficient, but immunized volunteers suffered from side effects (mild to moderate arthralgia) (329). It was subsequently confirmed that the attenuation was based on only two mutations in the E2 protein and that the "side effects" may have in fact been caused by viruses acquiring reversions at these positions (330, 331). However, many novel CHIKV vaccine candidates have been tested in animal models (mostly in mice, fewer in non-human primates), and a few of these candidates are in clinical trials.

Multiple approaches have been used to generate new CHIKV vaccine candidates, from classical inactivated vaccines to the development of modern attenuated, DNA, subunit and chimeric vaccines. In one approach, the SG promoter of CHIKV was substituted with the IRES element from encephalomyocarditis virus positioned between two ORFs in such a way as to trigger the expression of structural proteins (332, 333). Recombinant viruses, which lack regions encoding the 6K protein, a part of the nsP3 region (334) or the transmembrane part of the E2 protein (335), have been constructed and tested. DNA vaccines have been designed to express the complete virus genome, the virus genome lacking the capsid protein region or just one or several structural proteins of CHIKV (334, 336). Subunit vaccines based on the E1 and E2 proteins or on CHIKV VLPs assembled in mammalian or insect cells have been used (243, 336, 337). Additionally, chimeric viruses expressing CHIKV antigens have been constructed using measles virus (338), adenovirus (339), VSV (340), MVA (341–343) or other alphaviruses (344) as vectors. With rare exceptions (mostly observed when single proteins or constructs expressing single proteins have been used), all of these vaccine candidates elicit strong anti-CHIKV immune responses. Furthermore, in most cases, the antibody response generated by the vaccine candidates is neutralizing and protects against challenge with wild-type CHIKV of the same (most of these vaccine candidates are based on the ECSA genotype) or different genotype. Progress in the development of

novel anti-CHIKV vaccine candidates has also been recently reviewed (reviewed in (319)).

Taken together, the differences from the area of anti-HCV vaccine development (see 1.4.7) are striking. There are many (maybe too many) promising vaccine candidates for CHIKV, and the main issues that remain are the costs of clinical development and the ever-present question of rational use of the vaccine, as the unpredictable nature of CHIKV outbreaks makes it very difficult to estimate where and when the virus will strike next.

2. OBJECTIVES

HCV and CHIKV represent serious threats to human health and wellbeing. CHIKV is spreading rapidly and affects millions of people. There is currently no vaccine to prevent CHIKV infection. HCV causes chronic infection in more than one hundred million people. Even with revolutions in anti-HCV therapy, additional and affordable treatments are needed. It remains essential to develop novel and efficient vaccine/antiviral strategies to counteract the emerging resistance of chronic infections and infections caused by new viruses.

The objectives of the studies included into this thesis were:

1. To provide experimental verification of the efficiency and to analyse the side effects of novel anti-HCV drug candidates, which are low molecular weight inhibitors of the HCV NS3/4A serine protease that were selected by *in silico* screening.
2. To experimentally validate an ASO-based antiviral strategy targeting the HCV coding region. The aspects to be analysed included the development of a method for ASO target selection, the experimental validation of the impact of 8-oxo-dG modification on the basic properties of ASOs and antiviral activity and to reveal the molecular basis of their efficacy (or lack of efficacy).

   These objectives were oriented to validate and improve novel approaches and technologies, confirm their applicability and/or to highlight problems associated with these approaches.
3. To test the genetic stability of novel attenuated vaccine candidates developed against CHIKV. To analyse the molecular basis of virus attenuation that was achieved by introducing deletions in the HVD of the nsP3 and to study the biological functions of this region in CHIKV-infected cells.

3. MATERIALS AND METHODS

The evaluation of the cytotoxicity of seven *in silico* predicted compounds using the xCELLigence system is explained in publication **I**, Supplementary Material 3. The materials and methods for experiments conducted with ASOs targeting HCV in a cell culture system and using cell-free assays are described in publication **II.** Detailed descriptions of the materials and methods used in CHIKV studies are provided in publications **III** and **IV**. The methods used to obtain unpublished data are provided below.

HCV inhibition and cell cytotoxicity assay for *in silico* designed compounds
All analysed compounds (19145, 32387, 24757, 32385, 23330, 23332, and 31356) were dissolved in dimethyl sulfoxide (DMSO). DMSO is a widely used solvent and has been shown to be non-toxic (depending on the cell line) at final concentrations of 0.1–0.5%. In the case of the Huh-luc/neo-ET cell line, we found that the well-tolerated (no cytotoxic or cytostatic effect was observed) concentration of DMSO was 0.2%. Therefore, 0.2% DMSO in growth medium was used as a vehicle control, and all compounds were applied to cells at a final concentration of 0.2% DMSO.

To analyse anti-HCV properties, the dissolved compounds were diluted in growth medium. We also ensured that the compounds did not precipitate during the preparation of the dilutions. Medium containing the compounds was then applied to Huh-luc/neo-ET cells. At the selected time points, the cells were lysed, and luciferase activity and total protein concentrations were measured and normalized to cells treated with the vehicle control.

The cytotoxicity of the compounds was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. Inhibitors were added to the cells (grown in the wells of a 96-well cell culture plate) at the indicated concentration, and the cells were incubated in the medium for 48 h. The MTT reagent was added to the wells at a final concentration of 0.5 mg/ml, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for 2 h. Subsequently, the supernatant was removed, 100 µl of DMSO were added per well and the plates were shaken gently for 15 min. The optical densities were measured at 540 nm using a microplate reader (Sunrise Tecan, Magellan™).

Virological methods
For infectious centre assay (ICA), 1 µg of *in vitro* synthesized RNA was electroporated into 8×10^6 BHK-21 cells. Ten-fold serial dilutions of electroporated cells were seeded into six-well tissue culture plates that contained 1.5×10^6 BHK-21 cells per well. After incubation for 2 h at 37°C, the cell culture medium was aspirated, and the cells were overlaid with 2 ml of carboxymethyl cellulose (CMC)-containing growth medium (final concentration of 0.8% CMC) supplemented with 2% fetal calf serum (FCS). Plaques were stained with crystal violet after 2–3 days of incubation at 37°C.

The plaque assay, combined with an analysis of the genetic homogeneity of the viral stocks and the rescued viruses, was carried out as follows. Monolayers of BHK-21 cells were grown in 6-cm cell culture plates and infected with 20–30 pfu of the collected viruses. After 1 h of incubation at 37°C, the inoculum was removed, and the cells were covered with growth medium containing 1% agarose and 2% FCS. At 48 h p.i., another layer of 1% agarose, which contained a neutral red staining solution, was added. Twelve hours later, 5 plaques were isolated. The viruses were eluted with 0.5 ml of growth medium and used to infect new BHK-21 cells in a 24-well plate. At 24 h p.i., the total RNA was extracted from each well infected with the obtained virus using the TRIzol reagent (Life Technologies). Reverse transcription was carried out using a first-strand cDNA synthesis kit (Thermo Scientific). CHIKV- or SFV-specific cDNA fragments were PCR-amplified using pairs of primers that matched the non-structural part of the virus genomes and sequenced.

4. RESULTS AND DISCUSSION

4.1. Novel technologies for the development of antiviral drugs

The first anti-HCV DAAs were approved 4 years ago (22 years after the discovery of the virus). Although those DAAs were promising, they were accompanied by side effects and virus-acquired resistance. The real breakthrough in anti-HCV therapy was achieved recently with new DAAs that have minimal side effects and in some cases, a high barrier to resistance. These new therapies are expensive, and the appearance of new resistant HCV strains remains in question. Therefore, new efficient and cost-effective treatments are still needed. Approaches that could speed up the development of novel antivirals would be beneficial for novel anti-HCV drugs and drugs against other viral pathogens.

4.1.1. Analysis of novel HCV NS3/4A protease inhibitors designed using the fragment-based drug design approach (I, unpublished data)

All currently approved HCV NS3/4A protease inhibitors (and compounds in late stages of clinical trials) are based on the structures of modified peptide substrates. Boceprevir and telaprevir are peptidomimetics, and other inhibitors are either tripeptide or macrocyclic compounds. Their structures are complex, and they have relatively high molecular masses, from ~520 Da (boceprevir) to ~870 Da (faldaprevir). All of these compounds have a long history of development. Here, we tested whether a simpler rational drug design approach can be used to develop compounds that target the NS3/4A protease and inhibit HCV replication.

4.1.1.1. Fragment-based design, assessment and selection of compounds

Rational design and selection is one approach obtaining new antivirals. Computational technology, which is based on the fragmentation of chemical compounds, can estimate the activities of new inhibitor candidates. Here, this technology was applied to generate new potential inhibitors targeting the HCV NS3/4A protease. The employed data set consisted of 102 chemical compounds that are known to inhibit the HCV NS3/4A protease. The compounds used in the calculations included boceprevir (then known as SCH503034), telaprevir (VX-950) and the failed drug Ciluprevir (BILN2061), with different modifications (345–350). All of these compounds are relatively large molecules and have different possible configurations. Using the Fragment–based Quantitative Structure–Activity Relationship (FQSAR) method, these substances were sepa-

rated into three fragments, resulting in a set of 103 different structural fragments. *In silico* combination of these fragments and selection using the FQSAR approach generated a list that consisted of 20 new potential HCV inhibitors with high predicted Log (1/Ki) values.

The downside of this type of *in silico* design is that the predicted compounds are not typically available; in most cases they simply do not exist. The chemical synthesis of novel compounds is costly and time consuming. To circumvent this problem, a search of commercially available compound databases was performed to find compounds that are structurally similar to the 20 potential inhibitors found using the FQSAR calculations. This approach is not ideal, as even minor changes in the compound structure can alter (or eliminate) its inhibitory properties. This search resulted in seven compounds that were selected for further experiments (hereafter referred to as compounds 19145, 31356, 24757, 23330, 233332, 32385, and 32787; **I**; Supplementary Material 1, Fig. S1).

4.1.1.2. Analysis of the cytotoxicity of selected NS3/4A protease inhibitor candidates

The compounds that were selected via rational *in silico* design were predicted to inhibit HCV replication by inhibiting NS3/4A protease activity. To test this hypothesis, we decided to use a cell-based assay using the Huh-luc/neo-ET cell line (licensed from ReBlikon GmbH). This stable cell line harbours an HCV replicon containing the firefly luciferase (Luc) reporter gene to simplify measurements of HCV replication (**II**, Fig. 2A).

In cell-based assays, the inhibition of virus replication may also result from indirect effects of the compounds. Therefore, cytotoxicity is among the first properties of any novel antiviral compound to be tested. For this purpose, different cytotoxicity assays have been developed. Most of these assays, such as the MTT or WST assays, measure the metabolic activity of cells at the endpoint of the analysis. However, HCV replication is also strictly dependent on host cell growth; the highest levels of HCV replication (proteins, RNA) are observed in actively growing cells, and a sharp decline is observed in resting cells. Most likely, this phenomenon occurs because cellular factors that are required for HCV RNA replication and/or translation vary in abundance and are limited in non-dividing cells (123, 351). Therefore, compounds that cause cytostatic effects may also supress HCV replication. Thus, assays that also monitor the effects of the tested compounds on cell growth are clearly more adequate. One such assay is based on the use of the xCELLigence System RTCA MP Instrument (ACEA Biosciences). This system measures the growth of the cells in a real-time setting, and cytostatic effects of compounds can be detected easily. For these reasons, all cytotoxicity measurements were performed using this system and verified using the MTT assay and visual microscopy observations.

All selected compounds were diluted in DMSO and applied to Huh-luc/neo-ET cells as described in section 3, at final concentrations of 1, 10 and 20 μM. Higher concentrations were excluded, as at concentrations of 50 μM or higher cell damage was observed visually using microscopy. Using the xCELLigence System, it was found that at the 20 μM concentration, only compounds 23330 and 23332 lacked cytotoxic/cytostatic effects, compounds 32787, 24757 and 31356 had moderate effects on cell growth, and substances 19145 and 32385 profoundly inhibited cell growth. At the 10 μM concentration, the cytotoxic effects were substantially lower, and at the 1 μM concentration, no cytotoxicity was observed (**I**, Supplementary Material 3 Fig. S1). When the same compounds were analysed using the MTT assay, only relatively mild cytotoxic effects were observed, even at the highest (20 μM) concentrations. At this concentration, only compounds 24757 and 32385 reduced cell viability by ≈50%. Interestingly, no cytotoxicity was detected for compound 19145 (Fig. 5). This observation contrasted with the findings obtained using the xCELLigence System, where this compound exerted clear effects on the cells at both the 10 μM and 20 μM concentrations (**I**, Supplementary Material 3, Fig. S1A). One possible explanation for this discrepancy is that compound 19145 is not directly toxic to the cells but rather has a cytostatic effect. Thus, this analysis confirmed that the MTT assay is less sensitive and less informative than the use of the xCELLigence System.

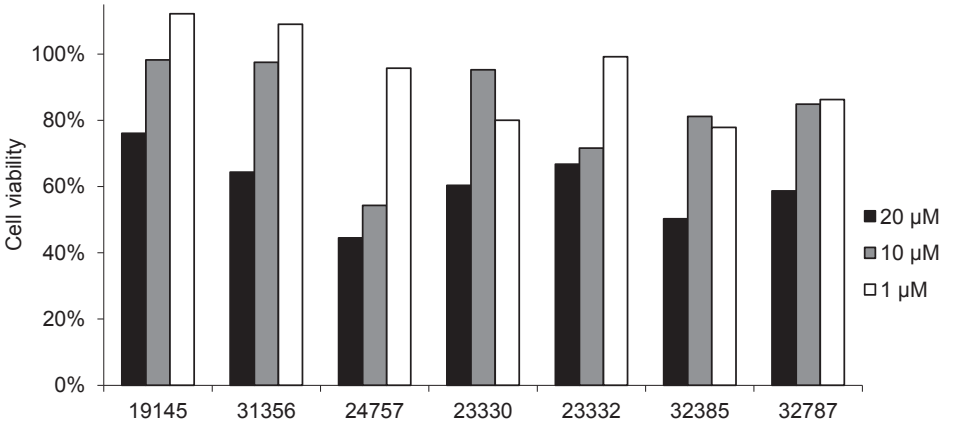

Figure 5. Effects of compounds 19145, 31356, 24757, 23330, 23332, 32385, and 32787 on the viability of Huh7-luc/neo-ET cells. Cell viability was measured using the MTT assay. The viability of cells treated with the vehicle control (0.2% DMSO) is taken as 100%. The results of one (of two) reproducible experiment are shown.

4.1.1.3. *In silico* predicted compounds inhibit HCV replication in cell culture

The quality of *in silico* prediction depends on the methods used and on the amount and quality of the input data. Furthermore, the properties of compounds used in biological assays may not correspond to the properties of *in silico* predicted hits. The inhibitory properties of compounds may also be reduced due to their poor bioavailability. For these reasons, compounds selected using *in silico* approaches often lack the expected properties.

Compounds were applied to sub-confluent Huh-luc/neo-ET cell cultures at a final concentration of 10 μM. The cells were lysed at 24, 48, 96, 120, and 144 h post-treatment, and luciferase activity, which is proportional to the copy number of HCV RNA in the cells (352), was measured. The obtained luciferase activities were first normalized to the amount of total protein (measured using the Bradford assay), and the obtained values were normalized to the vehicle control (taken as 100%). No inhibition of HCV replication was observed at 24 h post-treatment in any case (Fig. 6). The exception was compound 32385, for which the observed inhibition originated from its cytotoxic/cytostatic effects (**I**, Supplementary Material 3 Fig. S1). For most of the compounds, the lack of inhibition probably occurred because considerable time is required for the compounds to cause a reduction of HCV mRNA levels and/or because of Fluc is relatively stable protein with half life ≈ 3 h. In line with these assumptions, the inhibition of HCV replication became apparent at 48 h post-treatment (except for compounds 31356 and 23332); at 72 h post-treatment, inhibition was observed for all compounds. At the later time points, inhibition either increased slightly (23330; 23332, 32787) or remained at the same level as that observed at 72 h post-treatment (19145, 31356, 24757) (Fig. 6). Thus, at the 10 μM concentration, all of the selected compounds were able to inhibit HCV replication, although for compounds 19145 and 32385, this effect may be indirect (caused by cytotoxic/cytostatic side effects of the compound). This emphasizes the need for use of the most sensitive method(s) for detection of side effects of compounds. Compounds 19145 and 32385 would have been considered to be direct HCV inhibitors if only MTT assay (which did not reveal cytotoxicity at 10 μM concentration (Fig. 5)) had been used. It should also be noted that the inhibition of HCV replication (with the exception of cytotoxic compound 32385) was moderate, being most pronounced for compounds 23330 and 32787 (Fig. 6).

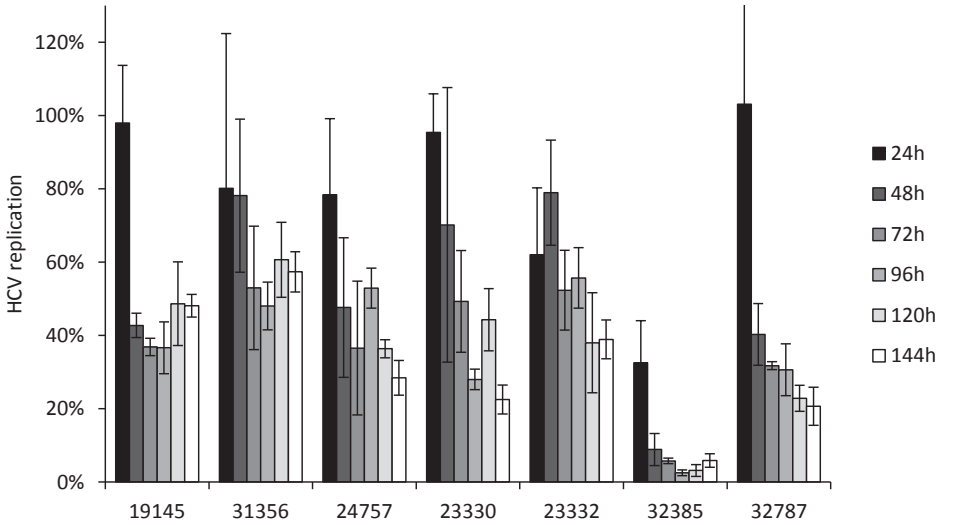

Figure 6. Time dependence of the inhibition of HCV replication in Huh-luc/neo-ET cells treated with the compounds at a final concentration of 10 μM. The cells were lysed at the indicated time points; the luciferase activities and total protein contents of the lysates were measured. The luciferase activities, which were normalized to the protein content, in cells treated with the vehicle control were taken as 100%. The means of triplicate experiments are shown with the standard error.

Finally, the concentration dependence of HCV replication inhibition was analysed. Due to the limited amount of available compounds, this experiment was performed only once; hence, it is not known if the data presented below are reproducible. The cells were lysed at 48 h post-treatment, and the analysis was carried out as described above. Compound 23330 inhibited HCV replication at the 0.1 μM concentration but not at the 1 μM concentration (Fig. 7). Thus, the inhibition observed at the lower concentration was an experimental error. No other compound was able to inhibit HCV replication at the 0.1 μM concentration. Instead, some compounds (such as 24757) slightly activated HCV replication (Fig. 7). Overall, only two compounds demonstrated concentration-dependent inhibition. Compound 32385 inhibited HCV replication by 30% at the 1 μM concentration. At the 10 μM concentration, replication was reduced by nearly 80% (Fig. 7); however, the effect was overshadowed by the observed cytotoxicity (**I**; Supplementary Material 3 Fig. S1). Compound 23332 also showed concentration-dependent inhibition and suppressed HCV replication by approximately 50% at the 1 μM and 10 μM concentrations. As compound 23332 was not toxic at these concentrations (**I**; Supplementary Material 3 Fig. S1), this effect likely results from direct anti-HCV activity. It is also possible that in this experiment, the full potency of compound 23332 was not revealed,

as the antiviral effects of this compound are more prominent at late time points (Fig. 6). Compounds 23330, 31356, 24275 and 19145 also inhibited HCV replication at 10 μM, but no inhibition was observed at lower concentrations.

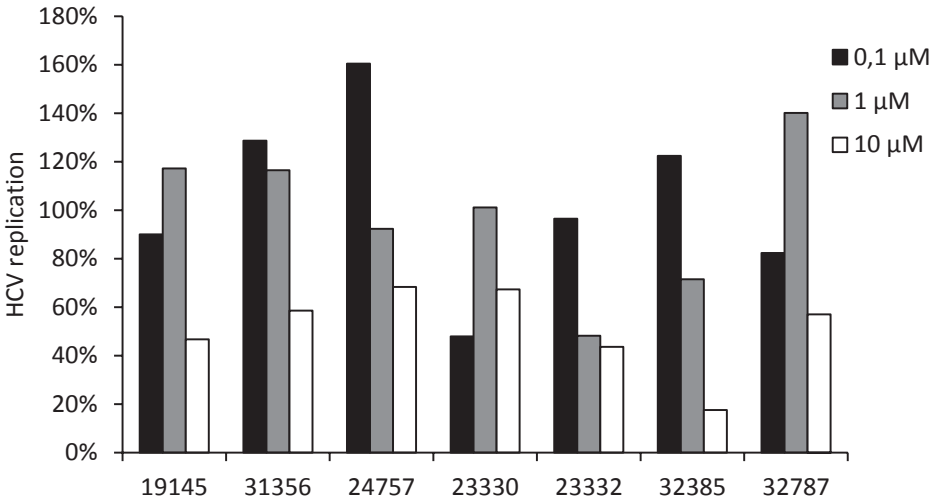

Figure 7. Concentration dependence of HCV replication inhibition. Huh-luc/neo-ET cells were treated with the compounds at final concentrations of 0.1 μM, 1 μM or 10 μM. The cells were lysed at 48 h post-treatment. The luciferase activities and total protein contents of the lysates were measured. The luciferase activities, which were normalized to the protein content, in cells treated with the vehicle control were taken as 100%. The experiment was performed only once; hence, no error bars can be shown.

Based on the obtained data, we can conclude that compound 23332 (and possibly some other compounds) can directly inhibit HCV replication. However, we lack actual proof that this effect occurs due to the inhibition of NS3/4A protease activity. It became clear that data from a cell-free protease assay would be very important for supporting the data acquired from cell culture experiments. Interestingly, most of recent studies dedicated to the development of CHIKV protease inhibitors suffer from similar shortcomings (320, 321). It should also be concluded that all analysed substances, including compound 23332, were not very potent inhibitors. Too few concentrations were tested to enable the reliable calculation of an inhibitory concentration 50 (IC50), but it could be estimated that the IC50 of compound 23332 is in the low micromolar range. The low potency of the hit compounds found in the initial screens is not surprising and serves as indication that *in silico* design should be an iterative process that involves multiple rounds of hit compound optimization. However, even if only a single compound (of seven) had specific anti-HCV activity, it could be concluded that the discovery rate of hit compounds with some anti-HCV activity using the applied approach was relatively high (at least when compared to the

screening of chemical libraries that are not pre-selected *in silico*). It would have been interesting to analyse the anti-HCV activities of the structures directly generated by the FQSAR process. This information would have been important for estimating how much potency was lost (or gained, which is rather unlikely) due to the replacement of the predicted inhibitors with structurally similar commercially available compounds.

Finally, this study was the first experience of our research group collaborating with a theoretical chemistry research team. The lessons learned from this work were indeed helpful for subsequent joint projects dedicated to the development of non-nucleoside inhibitors of HIV-1 reverse transcriptase (Viira et al., submitted), for the screening of inhibitors of CHIKV replication (Pohjala et al., 2011), and for the generation of a unique library of rationally designed inhibitors of the CHIKV nsP2 protease (Das et al., unpublished data).

4.1.2. Design and analysis of modified ASOs targeting a highly structured coding region of the HCV genome (II)

ASOs are the most straightforward type of inhibitors, and their design appears to be extremely simple. However, in practice, the development of efficient and specific ASO inhibitors is much more complicated (see 1.3.3). In the next study, numerous aspects of ASO inhibitor development were analysed using the HCV genome as a target.

4.1.2.1. Selection of target sites for highly efficient ASOs

The potency of an ASO is largely dependent on the accessibility of its target site. Additionally, an ASO must bind to its target with sufficient specificity and efficiency, and it must also affect the functions of the targeted RNA.

The genomic RNA of HCV is highly structured (353) and serves as example of RNA that is difficult to target. The true high-order structure of the coding region of the HCV genome is not known. In contrast, the HCV 5' UTR structure is well characterized, and different ASOs that bind to the IRES or other parts of the 5' UTR have been designed (86, 354). However, the 5' UTR is only a small part (less than 5%) of the whole HCV genome, and this strategy therefore omits many potential ASO target sites.

One obvious approach includes targeting sequences that are conserved between different HCV GTs. Another possibility is to target regions of genomes that have a certain nucleotide composition (for example, a high C/G content). Regions lacking extensive secondary structures could be predicted using minimum free energy calculations. Here, all of these approaches were attempted but failed to produce significant results. Therefore, an empirical method was developed and used for ASO target site selection.

We used an RNA interference (RNAi)-guided approach to select ASO target sites in the HCV coding region. It was assumed that efficient inhibition of HCV replication by certain siRNAs serves as indication of the accessibility of the corresponding target site. To ensure the detection of useful target sites, an in-house algorithm (developed by Andrei Nikonov) was used to design 28 different siRNAs targeting sequences located in the NS3-NS5B coding region of HCV GT1b (**II**, Table 3). These siRNAs (and relevant positive and negative control siRNAs) were used to target the replication of the HCV replicon in Huh-luc/neo-ET cells. As expected, not all siRNAs could inhibit HCV replication (**II**, Fig. 2B), either because their target sites were inaccessible (true negative results) or because the siRNAs were not efficient for other reasons (false negative results). Twelve siRNAs had minimal or no effects on HCV replication (as in **I**, HCV replication was estimated by measuring the luciferase activity produced by the HCV replicon). The inhibition achieved by fourteen siRNAs was considered moderate. From the panel of 28 siRNAs, only siRNA3570 and siRNA4676 were capable of supressing HCV replication to a greater extent than positive control siRNAs targeting the largely unstructured region encoding the luciferase reporter. The target sites of these siRNAs, which were designated as sites 3570 and 4676, were subsequently used as binding sites for a set of ASOs.

4.1.2.2. 8-oxo-2′-deoxyguanosine (8-oxo-dG) residues affect the binding of ASOs to their targets

Most of the currently used modifications in ASO structures affect the oligonucleotide backbone; alternatively bulky and non-natural nucleobase analogues are used. In contrast, 8-oxo-2'-deoxyguanosine (8-oxo-dG) and 5-hydroxy-2'-deoxycytidine (5-OH-dC) residues contain minimally modified nucleobases that do occur naturally (these residues represent products of oxidative DNA damage). Quantum-chemical calculations revealed that both of these nucleobases possess two tautomeric forms: major and minor (**II**, Fig. 1A). The properties of these forms are different; the minor tautomeric form mediates abnormally strong binding towards the complementary nucleotide (355), while the major form of 8-oxo-dG has been shown to bind the complementary cytidine more weakly than the non-modified guanosine (356). It was not known which tautomeric form of the modified nucleobase (minor or major) has a bigger impact on the properties of ASOs containing such modified nucleobases.

An effective ASO must form a stable duplex with its target sequence. Therefore, the effect of 8-oxo-dG and 5-OH-dC modifications in ASOs on the thermal stability (melting temperature, Tm) of their duplexes with DNA and RNA targets was estimated. The analysis revealed that the incorporation of 8-oxo-dG into all-DNA ASOs reduced the Tm of the ASO:DNA and ASO:RNA duplexes. Each inserted 8-oxo-dG residue reduced the Tm by 1.6–2.5°C (**II**, Fig. 1B, 1C). Based on these data, one can speculate that under the employed conditions, 8-

oxo-dG was present in its weakly binding major tautomeric (6,8-diketo) form. Interestingly, the incorporation of 5-OH-dC residues did not affect the Tm of the ASO:target duplexes (**II**, Fig. 1B, 1C).

To analyse the effect of 8-oxo-dG residues on the ASO:RNA duplexes in a more detailed manner, the stability of duplexes of ASOs containing LNA nucleobases at their termini (LNA/DNA gapmers) with their targets was also measured. As expected, the presence of LNA bases in an ASO greatly increased the Tm of the ASO:target duplexes. Similar to the case of all-DNA ASOs, the 8-oxo-dG residues reduced the Tm of the LNA/DNA ASO:target duplexes; the reduction was approximately 2–3°C per modified residue (**II**, Fig. 5A, 5B). It is possible that the exact extent of the 8-oxo-dG modification on the Tm of an ASO may depend on multiple additional factors, including the overall nucleotide composition, the presence of additional modifications (including modifications of the oligonucleotide backbone) and the environmental conditions (pH, ionic strength of the solution, etc.). However, based on our current findings, it could be concluded that weaker binding to their targets is most likely a general property of ASOs containing 8-oxo-dG residues. The effect (if any) of the minor tautomeric form of 8-oxo-dG residues could not be detected using the applied methods.

Next, the effect of 8-oxo-dG residues on the efficiency of ASO:target duplex formation was evaluated. Depending on the conditions, such as temperature, the target RNA can have different conformations. As the normal body temperature is most relevant for practical use of ASOs, the assay was carried out at 37°C. It was found that the incorporation of 8-oxo-dG into all-DNA ASOs clearly reduces the efficiency of ASO:target duplex formation. In contrast, no effect of such modification on the formation of LNA/DNA gapmer ASO:target duplexes was observed (**II**, Fig. 5C). Thus, the negative impact of 8-oxo-dG residues was likely overshadowed by the presence of LNA bases, which ensured strong binding of an LNA/DNA gapmer ASO to its target. Hence, to obtain inhibitory compounds, 8-oxo-dG modifications should be used in combination with additional modification(s) that increase the binding efficiency of the ASO.

4.1.2.3. Design of efficient ASO inhibitors

Due to inefficient binding to their targets and short half-lives in biological environments, all-DNA ASOs are poor antisense inhibitors. More potent ASOs can be obtained by including different modifications (briefly reviewed in 1.3.3). Several such modifications were tested in the early stages of the current study (including PTO ASOs). Due to quality problems (briefly discussed in the Material and Methods section of **II**), the obtained results were inconclusive.

When combining different modifications into a single ASO, several aspects should be considered. As 8-oxo-dG residues reduce the Tm of the compound, the additional modification(s) should have an opposite effect. The inclusion of additional types of nucleobase modifications would likely complicate the inter-

pretation of the data, and such combinations would complicate the synthesis of ASOs. For example, we were never able to obtain ASOs containing both 8-oxo-dG and 5-OH-dC residues; such compounds were ordered from severeal service providers, but ultimately, no company was able to produce them. Thus, the modification(s) of the oligonucleotide backbone represents the best choice. It should also be mentioned that we do not have a facility that can synthesize ASOs or substrates for their synthesis; hence, we were dependent on what was available on the market. To our knowledge, the only substrate available (at least at the time when the study was initiated) contained 8-oxo-guanosine (or 5-hydroxy-cytidine) bound to the deoxyribose sugar. Thus, all-LNA (or all-2′-OMe RNA, all-morpholino or all-PNA) ASOs with 8-oxo-G (or 5-OH-C) modifications were very difficult to obtain, if not completely impossible. Consequently, the included modification must be compatible with standard deoxyribose sugars in the ASO backbone.

ASOs can inhibit mRNA (virus genome) translation in two different ways: via steric blockade of the translation machinery or by activating cellular RNase H, which leads to the degradation of target RNA. To act as a steric inhibitor, an ASO must bind an element required for the initiation of translation. Both selected target sites (3570 and 4676) are located in the coding region of the HCV genome. Hence, in our case, the modified ASO, in order to be efficient, should be able to trigger the degradation of the targeted RNA.

LNA bases, when incorporated into an ASO, enhance its stability and increase ASO:target duplex formation (86, 357). However, all-LNA ASOs and LNA/DNA mixomer ASOs (oligonucleotides in which DNA bases are interspaced by LNA bases) cannot activate RNase H (**II**, Fig. 6B, 6C) because RNase H requires the presence of an uninterrupted RNA:DNA duplex no less than 6 bp in length (86). Thus, LNA/DNA gapmers, which fulfil this requirement, represent the best choice. Given the availability of substrates for oligonucleotide synthesis, all 8-oxo-G or 5-OH-C modifications were located in the central (DNA) parts of these ASOs (**II**, Table 1).

4.1.2.4. 8-oxo-dG residues have a small effect on the antiviral potency of LNA/DNA gapmer ASOs

To analyse the effect of 8-oxo-dG residues on the inhibitory potency of ASOs, such modifications were incorporated into compounds targeting site 4676 in the HCV replicon RNA. As the central part of this site contains three cytosine residues, the ASOs were designed to contain three 8-oxo-dG residues. All obtained ASOs (**II**, Table 1) were evaluated for the ability to supress HCV replication in Huh-luc/neo-ET cells.

Notably, these experiments were considerably more complicated than the experiments described in publication **I**. This was because unlike low molecular mass compounds, ASOs were unable to enter Huh-luc/neo-ET cells on their

own and required a transfection procedure. Many different transfection reagents are available, but they are all designed to facilitate the delivery of commonly used types of nucleic acids: plasmid DNAs, RNA transcripts or siRNAs. Similarly, there are also many different transfection protocols, but none of them was specifically developed for the types of compounds used in this study. To overcome these issues, seven different transfection reagents and a number of protocols were tested. We concluded that with the existing reagents, it was impossible to obtain good transfection efficiencies without causing substantial cell damage. As a compromise, a method (Lipofectamine 2000 and reverse transfection protocol) that resulted in transfection of approximately 75% of the cells and caused minimal (though in some cases, still detectable) damage to the cells was selected. Thus, although we did our best to take into account the cytotoxic effects of the ASO-transfection reagent combinations, it is likely that cytotoxity, not necessarily caused by ASO itself but originating from combination of multiple factors, still had some impact on the results of some experiments.

First, an all-DNA ASO (D4676) was confirmed to be a very poor inhibitor of HCV replication (**II**, Fig. 4A). In contrast, the corresponding siRNA (4676) was a very potent inhibitor, with an effective concentration 50 (EC50) of 0.13 nM (**II**, Table 4). Second, an LNA gapmer lacking 8-oxo-dG residues (LD4676) was also an efficient inhibitor of HCV replication and had an EC50 of 5.5 nM (**II**, Table 4). Interestingly, even when applied at a 100 nM concentration (>18-fold higher than the EC50), the compound still failed to cause complete inhibition of HCV replication. Inhibition did not exceed 60% and never reached the same level observed in siRNA-treated cells (**II**, Fig. 4A). Third, the introduction of 8-oxo-dG residues into the LNA gapmer (LDM4676) enabled the ASO to supress HCV replication nearly completely in transfected cells. Thus, at the 100 nM concentration, LDM4676 inhibited HCV replication by approximately 73%, which was similar to the rate achieved with siRNA (**II**, Fig. 4A). Note that ~75% inhibition corresponds to complete inhibition, as in these experiments ~25% of the cells remained un-transfected, and HCV replication in these cells was presumably unaffected. Regardless of the more pronounced inhibition, the EC50 for LDM4676 was 7.1 nM, which was slightly higher than the EC50 of LD4674 (**II**, Table 4). Fourth, an interesting phenomenon was observed for the inverted control ASOs. The LNA/DNA gapmer LD4676inv powerfully activated HCV replication, and the presence of 8-oxo-dG residues (LDM4676inv) reduced this effect (**II**, Fig. 4C). The origin of the unexpected activation of HCV replication remains unknown, but it could be speculated that LD4676inv interacted with some non-target cellular RNAs and that this interaction somehow boosted HCV replication. If this theory is correct, the reduced off-target effects of LDM4676inv likely result from its reduced Tm.

Taken together, these data indicate that the presence of 8-oxo-dG residues in the LNA/DNA gapmer ASO led to changes in its properties. Generally, LDM4676 performed better than its counterpart LD4676. This finding led us to conclude that ASOs with 8-oxo-dG residues were better inhibitors than could be

expected based on their Tm values (**II**; Fig. 5B), leading to the hypothesis that Tm is likely not the only property of an ASO that is affected by the presence of 8-oxo-dG residues.

4.1.2.5. 8-oxo-dG modifications impact multiple properties of ASOs that can affect their antiviral potency

In addition to Tm (the binding efficiency to the target and the stability of the formed ASO:target duplex), the antiviral effect of an ASO also depends on its ability to activate RNase H-mediated target RNA degradation and on the stability of the ASO in biological environments.

To analyse the effect of 8-oxo-dG residues on RNase H-mediated target degradation, two different experiments were performed. In the first experiment, ASO:RNA duplexes were pre-made, purified and treated with bacterial RNase H (human RNase H enzymes are not commercially available). Consistent with previous studies (86), we observed that duplexes with an LNA mixomer ASO were completely resistant to RNase H (**II**, Fig. 6B, 6C). In contrast, the RNA strands of duplexes made using D4676, DM4676, LD4676 and LDM4676 were degraded by RNase H. The presence of 8-oxo-dG residues had no detectable effect on the speed or efficiency of duplex degradation (**II**, Fig. 6C). However, differences in the reaction products were observed. For duplexes containing D4676 (or LD4676), only one main labelled cleavage product was observed. At the same time, the degradation of duplexes containing DM4676 (or LDM4676) resulted in two cleavage products (**II**, Fig. 6B). Thus, the incorporation of 8-oxo-dG residues into an ASO affected the positions of the bonds in the target RNA that were cleaved by RNase H. Another interesting observation was that duplexes that were prepared using LNA/DNA gapmer ASOs were degraded more efficiently than duplexes containing all-DNA ASOs. The initial speed of degradation was similar, but for all-DNA ASO duplexes, the reaction plateaued after 0.5 min, and 20–30% of duplexes were left uncleaved. In the case of LNA/DNA gapmer ASO duplexes, the reaction continued for up to 5 min, and only 10% of the substrate was left uncleaved (**II**, Fig. 6B, 6C).

RNase H-mediated cleavage in living cells requires both duplex formation and subsequent duplex cleavage. Duplex formation can be affected by the sequences surrounding the target site and/or by the high-order structure of the targeted RNA. To model these conditions, the experiment was also performed using a 3131 nt-long fragment of HCV RNA as a target. We found that the presence of LD4676 and LDM4676 in the reaction mixture triggered rapid RNase H-mediated degradation of the targeted RNA. The differences between the compounds were very small; in both cases, after 60 min, no substrate was left (**II**, Fig. 6D). Compared to the LNA/DNA gapmers, D4676 and DM4676 triggered slower RNase H-mediated target RNA degradation. Furthermore, in this regard, DM4676 was clearly less efficient than D4676 (**II**, Fig. 6D). Com-

bined with the data obtained from previous experiments, it can be concluded that the degradation of the targeted RNA correlates with the speed and efficiency of ASO:target RNA duplex formation (**II**, Fig. 5C) and may be further affected by the more efficient degradation of duplexes containing LNA/DNA gapmers (**II**, Fig. 6B, 6C). Duplex formation itself depends on the presence of 8-oxo-dG residues, as these residues reduce the Tm of the ASO:RNA duplexes (**II,** Fig. 5C). The observed effect of nucleobase modification is more prominent in the context of all-DNA ASOs, as their Tm values are lower than those of LNA/DNA gapmers of the same sequence (**II,** Fig. 5B).

Another property of an ASO that affects its antiviral potency is its stability in biological environments. The classical approach to increasing the stability of an ASO is the introduction of PTO modifications into ASO backbone, but the introduction of LNA bases is also known to increase ASO stability. Miravirsen, which is an inhibitor of mir-122, contains a combination of these modifications and has a serum half-life of more than one week (354). As ASOs are injectable drugs, the long half-life is especially important. As it was not known how 8-oxo-dG residues affect the stability of ASOs, the D4676, DM4676, LD4676 and LDM4676 were labelled and incubated in human serum. Consistent with previous studies, we observed that LNA/DNA gapmers have a much longer (>10-fold) serum half-life than all-DNA ASOs (**II,** Fig. 7). Importantly, it was found that three 8-oxo-dG residues significantly increased the half-lives of both all-DNA (from ~8 min to ~15 min) and LNA/DNA gapmer (from ~90 min to ~130 min) ASOs (**II,** Fig. 7). Thus, the observed high antiviral activity of LDM4676 (**II**, Fig. 4A) could be partly explained by the increased stability of the compound. However, it is also clear that the increased stability of a compound in an *in vitro* assay does not necessarily indicate its superior stability under much more complex *in vivo* conditions. It is therefore important to further assess the effect of 8-oxo-dG modification on ASO stability *in vivo*.

4.1.2.6. Inhibitory properties of ASOs are affected by mutations in the target site

The efficacy of nucleic acid based inhibitors (siRNAs, ASOs) depends on the match between their sequence and the target site. In this regard, siRNAs are especially sensitive; a single nucleotide mismatch can completely destroy the efficacy of the inhibitor. In the case of ASOs, there is usually no demand for a perfect match; however, ASOs also lose potency if they cannot bind efficiently to the target. This aspect is especially important to take into account when targeting viral genomes. First, viral genomes have a natural variation of sequences. HCV, which has multiple GTs, is a prime example of such a virus. The target site 4676 (selected on the basis of the GT1b Con1 sequence) is conserved (although it may still contain a single mismatch) in GT1b. However, in GT1a viruses, up to three nucleotide differences could be present in this region, and in

other GTs, up to seven nucleotide differences could be present. Second, RNA viruses respond to antiviral treatment by developing resistance. Thus, it was important to verify the sensitivity of the modified LNA/DNA gapmer ASOs to changes in their target sites.

Mutations introduced into the coding region may hamper HCV replication and/or be reverted. To eliminate the possibility of such unwanted effects, we searched the literature for mutations located in the 4676 or 3570 target sites that were reported to have no (or minimal) effect on virus replication. No such mutation was described for site 4676. However, site 3570 contains a codon that is changed from Thr to Ala in HCV mutants that are resistant to first-generation protease inhibitors. In the Con1 sequence, this mutation (3579 A→G) is located conveniently in the middle of the 3570 target site (**II**, Fig. 3A). To test the mutation's effect on the efficiency of the modified ASO, a new compound (LDM3570) that contained three 5-OH-dC residues and a cell line designated as Huh-luc/neo-ET-3570mut were created. Using these tools, we found that this mismatch reduced the inhibitory effect of LDM3570 by ~20% but had no effect on the efficacy of LDM4676 targeting a different site (**II**, Fig. 3B). As expected, the impact of the same mutation on the efficacy of an siRNA targeting the 3570 site was much more prominent (nearly 10-fold) (**II**, Fig. 3B). There is no doubt that 5-OH-dC residues are not the perfect control for 8-oxo-dG residues, as the effects of 5-OH-dC residues on the Tm of the compound are different from the effects of 8-oxo-dG residues (**II**, Fig. 1C). Nevertheless, it can be concluded that the efficacy of a modified ASO with naturally occurring nucleobases is affected by mutations in its target site but to a lesser extent than the efficacy of the corresponding siRNAs.

4.1.2.7. Can 8-oxo-dG modifications be used for the development of improved ASO drugs?

The ASO project was large and very resource- and time-consuming (only a fraction of the total data is presented here and in publication **II**). At its end, many questions remained. One of those questions concerns the effects of 8-oxo-dG modifications in the *in vivo* system. *In vitro* analysed ASOs are active (**II**, Fig. 4A) and possess enhanced stability (**II**, Fig. 7); these properties are attractive. The intrinsic weakness of all ASOs is delivery. In *in vivo* systems, this issue becomes even more critical than in cell culture assays. The liver is actually the only easy *in vivo* target, as ONs typically end up in this organ (354, 358–360). For all other targets, specific vehicles are needed for specific delivery and cell membrane penetration. Despite some progress, the targeting of different organs remains largely unsolved. Therefore, different ASOs are primarily attractive for the treatment of liver diseases.

The progress made concerning therapies for HCV infection has been remarkable (see 1.4.5; 1.4.6). Thus, a new drug (or treatment) must not only be effi-

cient but also have some competitive advantages over the current treatments. ASOs are likely to remain injectable drugs; thus, ASOs will have some serious disadvantages compared to oral drugs. Therefore, the issue of stability (as the stability of drug correlates with the frequency with which the drug should be injected) could be the most important issue. In this regard, compounds with 8-oxo-dG residues are quite attractive (**II**, Fig. 7). Another key feature for the successful treatment of viruses (including HCV) is the resistance barrier. Theoretically, pre-existing sequence variation should not be a problem, as the sequence of an ASO can be easily adjusted to match any target sequence. From a practical point of view, this issue highlights the need to develop a family of compounds rather than a single compound. That strategy will almost certainly be associated with greatly increased costs and will thus be unlikely to be practical. Rapidly acquired resistance is another problem; modified ASOs are sensitive (although to a much lesser extent than siRNAs) to mutations in their target sites (**II**, Fig. 3B). Thus, a combination of drugs with different targets and/or with different mechanisms of action is clearly needed. ASOs could be combined with low molecular mass drugs, such as inhibitors of the NS3/4A protease. This combination could result in the inhibition of HCV replication (effect of the NS3/4A inhibitor) and the destruction of the targeted RNA genome (effect of the ASO). As one possibility, a novel ASO inhibitor targeting the HCV genome can be combined with another ASO-based drug candidate: the mir-122 inhibitor miravirsen. These compounds are chemically similar and could be delivered together; however, they have completely different mechanisms of action and can thus act in a synergistic manner. Taking into account all of the problems associated with targeting HCV RNA, the novel modified ASOs could be applied to other targets with better prospects. For instance, the sequences of cellular targets (such as mRNAs that are over-expressed in cancer cells) are more conserved and do not change nearly as quickly as viral genomes. Clearly, a number of issues must be addressed (delivery among them), but the modifications themselves (at least the use of 8-oxo-dG nucleobases) seem to hold promise.

4.2. Development of an attenuated CHIKV vaccine and analysis of the molecular basis of attenuation

A general description of antiviral vaccines and recent progress in the development of a vaccine for the prevention of CHIKV infection are presented above (1.2 and 1.5.5). Our research group has been actively involved in multinational efforts to develop a novel and efficacious vaccine for CHIKV. For this purpose, over 20 different vaccine candidates were constructed; of these candidates, 10 were tested in a mouse model (334, 336, 342, 361) and three were tested in a non-human primate model (Roques et al., submitted manuscript). The construction and analysis of such a large panel of vaccine candidates served the

simple and clear aim of producing vaccine candidates that have a realistic chance of being tested clinically and becoming licensed for clinical use. To have such a chance, we proposed that a vaccine candidate should be genetically stable, safe to use (with no pathology), effective after a single immunization, able to provide long-lasting protective immunity and easy to produce. It is relatively easy to meet some or even most of these requirements, but it is not easy to meet all of them at once. To our knowledge, we succeeded, and our vaccine candidate (based on data obtained from a 1.5-year long non-human primate study) meets all of these criteria. Our candidate also entered clinical trials in 2015 (due to the agreement between the University of Tartu and the company carrying out trials, the name of the company cannot be disclosed at this stage). To our knowledge, our vaccine is the fifth candidate CHIKV vaccine that has reached clinical trials.

The unravelling of the real effects responsible for the attenuation of this vaccine virus represents a topic of ongoing studies. As all of these studies involved a number of researchers from different laboratories, I will focus only on the parts of the corresponding studies in which I actively participated.

4.2.1. Deletions in the nsP3 HVD or the 6k region attenuate CHIKV replication and generate vaccine candidates that elicit protective immune responses in mice (III)

The main approach used for the development of our set of CHIKV vaccine candidates was rational design. We attempted to introduce mutations that presumably cause serious defects in virus infection *in vivo* and are also difficult for the virus to revert. Large deletions introduced into the reginons encoding viral proteins were considered to be the most suitable mutations for this purpose. However, for CHIKV, no deletion of this type has been studied. Therefore, we obtained an advantage from previous studies of SFV. For SFV, two deletions of a similar size (approximately 150 nucleotides) have been shown to have relatively mild effects on the general replication of the virus in cell culture but in animal models these deletions restrict virus-induced pathology. The first of these deletions removed the N-terminal part of the HVD of nsP3, which contains phosphorylation sites (362). The second deletion removed the entire region encoding the 6K protein (363) and also prevented the expression of the TF protein (although the existence of TF protein was not known at the time when the original study was performed).

These deletions were introduced into the CHIKV genome (ECSA genotype, isolate LR2006-OPY1). A mutant harbouring a deletion of the 6K region (designated as CHIKVΔ6K; **III**, Fig. 1A) was easy to design. In contrast, the deletion at the beginning of the nsP3 HVD was harder to design, as the corresponding regions of SFV and CHIKV have no sequence similarity. To overcome this obstacle, a set of 5 viruses, which harboured deletions of different sizes, was con-

structed and analysed in cell culture. All of these viruses were viable, and no notable differences were observed between them. The largest deletion (named deletion 5) substituted 62 aa residues from nsP3 (residues 1656 to 1717 of the P1234 polyprotein) with a short linker (sequence AYRAAAG). This virus, which was designated as CHIKVΔ5nsP3 (**III**, Fig. 1A), was selected for subsequent experiments. CHIKV carrying both deletions (CHIKVΔ5nsP3+Δ6K) was also constructed and tested in cell culture. This virus was strongly attenuated and was therefore not used as a vaccine candidate (our unpublished data). In contrast, both CHIKVΔ5nsP3 and CHIKVΔ6K exhibited only somewhat reduced titres (1.2×10^7 and 1.7×10^7 pfu/ml, respectively; 4.4×10^8 pfu/ml for wild-type CHIKV) and produced slightly smaller plaques than wild-type CHIKV (**III**, Fig. 1B).

With all attenuated vaccine candidates, it is essential to demonstrate that the attenuated phenotype is maintained (i.e., the attenuated virus is genetically stable). As the ultimate aim of the study was to produce a vaccine candidate suitable for use in humans, experimental proof of its genetic stability over number of passages was mandatory. For this purpose, both CHIKVΔ5nsP3 and CHIKVΔ6K were passaged in Vero cells at a low multiplicity of infection (0.01 pfu/cell); five passages were made for CHIKVΔ6K, and CHIKVΔ5nsP3 was passaged ten times (five passages were reported in **III**, five more passages were made later). For each passage, the titres of CHIKVΔ5nsP3 and CHIKVΔ6K were determined, and the plaque morphology was observed. No changes in plaque sizes (which stayed small) were observed, and the end titres of different stocks were similar to these of the P_0 stocks. Thus, no change in phenotype was observed. In addition, genomic RNA was isolated from the P_5 and P_{10} virus stocks, and the regions harbouring the deletions were RT-PCR-amplified and sequenced. This analysis confirmed that the originally introduced deletions were maintained in all analysed stocks. No reversions of deletions or changes in the aa sequences of the regions flanking the deletion were detected.

As the vaccine candidates were found to be genetically stable, they were next assayed for the ability to elicit an immune response. Both CHIKVΔ5nsP3 and CHIKVΔ6K produced some foot swelling in immunized mice; low-titre viremia was also observed for CHIKVΔ5nsP3 but not for the CHIKVΔ6K vaccine candidate (**III**, Fig. 3). Both vaccine candidates elicited high levels of humoral and cellular immune responses, with CHIKVΔ5nsP3 being more efficient (**III**, Fig. 4). That difference was also maintained (although the difference was not statistically significant) in the case of homologous prime-boost immunization (**III**, Fig. 7B). Upon challenge with wild-type CHIKV, all mice that were immunized with CHIKVΔ5nsP3 were protected against viremia and had minimal foot swelling. Viremia was detected in one mouse immunized with DNA-launched CHIKVΔ6K, and animals who received that vaccine candidate also had somewhat more pronounced foot swelling (**III**, Fig. 5). These data clearly demonstrate that both vaccine candidates were attenuated and could provide excellent protection against CHIKV infection.

Subsequent studies ((336); Roques et al., submitted manuscript) confirmed that CHIKVΔ5nsP3 was the most efficient vaccine candidate, even when compared to other tested candidates (including recombinant MVA, different DNA vaccines and recombinant proteins with adjuvants). Experiments using cynomolgus macaques, which represent a much more relevant model of CHIKV infection than mice (364), ultimately demonstrated that CHIKVΔ5nsP3 is attenuated in non-human primates and does not produce detectable symptoms. Most importantly, single immunization was sufficient to induce a high titre of neutralizing antibodies against CHIKV of the ECSA or Asian genotypes, and this response lasted for the entire observation period (~1 year). All immunized monkeys were completely protected against CHIKV challenge. Based on these data, a commercial company took over the vaccine development and initiated clinical trials of the CHIKVΔ5nsP3 vaccine candidate.

4.2.2. Functional differences in the N-terminal part of the HVD of nsP3 in SFV and CHIKV (IV and unpublished data)

Although new information about the functions of the nsP3 of alphaviruses is gradually emerging, the protein remains largely mysterious. Its N-terminal two-thirds is well conserved among alphaviruses and is folded into two structured domains (365), suggesting the existence of yet unknown enzymatic function(s). In contrast, the C-terminal HVD is intrinsically unstructured and mediates different virus-host interactions. The first known property of this region is the phosphorylation of Ser/Thr residues (366, 367). In the case of SFV, the phosphorylated region has been mapped to the N-terminal part of the HVD (368). It has been assumed, although never experimentally demonstrated, that the analogously located region of the nsP3 of CHIKV is similarly modified. The HVD is also tolerant to different deletions and marker protein insertions (268, 278, 369–371). Among other modifications, the deletion of the entire phosphorylation region (50 aa residues) is well tolerated in SFV. Viruses with such a deletion (designated SFVdel50) replicate to a high titre but are avirulent in mice (278). As described in 4.2.1, a similarly located region in the nsP3 of CHIKV also tolerates various deletions; even the removal of the whole 62 aa residue region does not prevent the virus (CHIKVΔ5nsP3) from growing to high titres. These similarities led to the logical, but erroneous, assumption that these analogously positioned regions of CHIKV and SFV nsP3 are functionally similar.

Many researchers, including members of our group, have observed consistent differences in the location of replication complexes in SFV and CHIKV in infected cells. In both cases, the replicase complexes are initially formed on the plasma membrane and subsequently internalized via the phosphatidylinositol-3-kinase (PI3K)-Akt signalling pathway using microtubules and the actin cytoskeleton (305). During the late stage of infection, SFV replication complexes are localized in large perinuclear vesicles (CPV-I), while vesicles

containing CHIKV replicase complexes are generally smaller and stay in close proximity to the plasma membrane (**IV**, compare Fig. 6A and 8E). Our colleagues observed that in contrast to wild-type SFV, SFVdel50 does not internalize its replication complexes (**IV**, Fig. 4A) and is unable to cause the hyper-activation of the cellular PI3K-Akt-mTOR pathway (**IV**, Fig. 4B, 4C). Moreover, there was a perfect correlation between the ability of SFV and its mutant forms to cause hyper-activation of the PI3K-Akt-mTOR pathway and to internalize replication complexes (**IV**, Fig. 7). This correlation was emphasized by the finding that CHIKV replication complexes are located in the cell periphery and that similar to SFVdel50, CHIKV is also unable to hyper-activate the PI3K-Akt-mTOR pathway (**IV**, Fig. 8). Swapping the HDV regions of SFV and CHIKV clearly indicated that this region was solely responsible for these phenotypic differences between SFV and CHIKV (**IV**, Fig. 10). These findings also confirmed that virus-cell interactions, which are mediated by different alphaviral nsP3 HVDs, result in different phenotypes.

During this study, we hypothesized that the determinant(s) needed for the hyper-activation of the PI3K-Akt-mTOR pathway are located inside the region of SFV nsP3 that is covered by the del50 deletion. If so, the swapping of this region with the corresponding region from CHIKV should result in chimeric nsP3 proteins with switched properties. The replacement of 50 aa residues of SFV nsP3 with 62 aa residues from nsP3 of CHIKV (thus, swapping the regions affected by del50 in SFV and by Δ5nsP3 in CHIKV) indeed resulted in a chimeric protein (SFV/CHIKV5-nsP3) that was unable to hyper-activate the PI3K-Akt-mTOR pathway (**IV**; Fig. 9D). The reciprocal swap resulted in the CHIKV/SFV50-nsP3 chimeric protein, which was also unable to hyper-activate the PI3K-Ak-mTOR pathway (**IV**; Fig. 9E). Thus, the swapping of these regions was not sufficient (unlike the swapping of the full HVDs) to transfer the ability to hyper-activate the PI3K-Akt-mTOR pathway from SFV nsP3 to the nsP3 of CHIKV. This finding indicates that the determinants required for the hyper-activation of the PI3K-Akt-mTOR pathway are not fully localized within the 50 aa region of SFV nsP3 HVD and probably include also some sequences localized between aa residues 368 and 408 of the nsP3 of SFV (e.g., between the regions affected by the del50 and delP deletions; **IV**; Fig. 4A).

The most surprising results were obtained when such swapping was performed in the context of infectious cDNA clones of SFV and CHIKV. First, the replacement of the 50 aa region of the nsP3 of SFV with 62 aa residues from the nsP3 of CHIKV was poorly tolerated. The infectivity of the obtained construct (designated as SFV/CHIKV50) was reduced (compared to the infectivity of the wild-type SFV construct) by approximately 100-fold. The reciprocal swap had an even more drastic effect: the infectivity of the obtained CHIKV/SFV50 construct was reduced (compared to the infectivity of the wild-type CHIKV construct) by approximately 10,000-fold. These data unequivocally demonstrated that the functions of similarly located regions of the nsP3 proteins from SFV and CHIKV are drastically different. Furthermore, these functions are clearly

incompatible; the deletions of these regions were well tolerated by both SFV and CHIKV, but the swapping of these regions was not. The simple conclusion is that the molecular basis of the attenuation of CHIKVΔ5nsP3 is most likely different from that of SFVdel50.

The effect caused by this swapping cannot originate from some defect in virus-host interactions. Previous studies in our laboratory showed that a 10,000-fold reduction in the infectivity of recombinant alphaviruses typically originates from a severe defect in genome replication (269, 290, 371–373). Effects of this magnitude may be caused by a defect in some essential enzymatic activity of the ns protein or by a severe defect in interactions between ns proteins, preventing correct replication complex formation. As the swapped region is structurally disordered, the second possibility is far more likely. If a construct harbouring such a mutation is capable of producing viable progeny, this scenario is always associated either with the reversion of the introduced mutation or with the selection of compensatory (second-site) mutations. As the swapping cannot be reverted (too many changes are required), a search for potential adaptive mutations in viruses rescued from the CHIKV/SFV50 construct was performed. In total, the genomes of ten progeny viruses were analysed by sequencing. In seven cases, a methionine-to-isoleucine mutation was detected in the ZBD of nsP3 (position 1552 in P1234). In the rest of the viruses, an asparagine-to-isoleucine mutation located in the C-terminal region of nsP2 (position 1318 in P1234) was detected. We found that the introduction of either of these mutations back into the CHIKV/SFV50 construct increased its infectivity nearly 10,000-fold (to a level similar to that of the wild-type CHIKV construct). This result clearly demonstrates that these mutations are true compensatory changes and not random mutations. The ability of viruses harbouring compensatory changes to hyper-activate the PI3K-Akt-mTOR pathway was not analysed, as we had already shown that viral progeny rescued from CHIKV/SFV50 lack this ability (**IV;** Fig. 9B). The rescue of the infectivity of CHIKV/SFV50 by a mutation located in the ZBD of nsP3 indicates that for some crucial viral function(s), the synchronized action of ZBD and the HVD of nsP3 is required. This finding also indicates that there is likely a physical interaction between these two domains, although it is unclear whether this interaction occurs within one molecule or as an intermolecular interaction. The compensatory effect of a mutation in the C-terminal domain of nsP2 serves as a strong indication that at some point of infection, this region acts cooperatively with the nsP3 HVD. It was previously shown that the interaction of nsP2 with the C-terminus of the macrodomain is crucial for the cleavage of the 2/3 site in P1234 (371). Combined, these findings emphasize that several functions that are essential for alphavirus replication are jointly performed by these two proteins. Clearly, to provide proof for any of these (or other) hypotheses, specific studies are needed. Furthermore, it appears that such studies may also reveal the true molecular basis of the attenuation of the CHIKVΔ5nsP3 vaccine candidate.

CONCLUSIONS

The common aim of the studies presented in this thesis was to develop new approaches for targeting the medically important viruses HCV and CHIKV. HCV is primarily associated with chronic disease; hence, we focused on the development of potential therapeutics - antiviral compounds. CHIKV is associated with acute disease (which could result in chronic symptoms); hence, we focused on the development and testing of rationally designed candidates for a preventive CHIKV vaccine. All of these studies were carried out as collaborative projects between different laboratories and/or experts in different areas (quantum-chemistry, oligonucleotide chemistry, immunology, and animal studies, among others). The main conclusions from these studies are as follows:

- The new FQSAR method-based approach allowed for the rapid prediction of hit compounds targeting the NS3/4A protease of HCV. This approach can, at least theoretically, also be applied to other targets. The main obstacle associated with this approach is the difficulty of obtaining hit compounds predicted by FQSAR. The replacement of these compounds with structurally similar and commercially available compounds increases the possibility of false negative results. In our case, of seven compounds obtained using these approaches, only two were non-cytotoxic. Therefore, although all seven compounds analysed in this project displayed some anti-HCV properties, only the effect caused by the non-cytotoxic compound 23332 can be considered direct.
- RNAi-guided selection was successfully used to reveal two potent ASO target sequences in the highly structured HCV coding region. A novel technology – the incorporation of naturally occurring minimally modified nucleobases into ASOs – was evaluated using ASOs that bind to these targets. Modified compounds containing 8-oxo-dG residues were capable of triggering the RNase H-mediated degradation of their RNA targets and had enhanced stability in biological environments. At the same time, these compounds had reduced melting temperatures and an impaired ability to form duplexes with their target RNA. The latter effect was largely compensated by the introduction of LNA bases into the ASOs. Combined, these approaches led to the development of ASO compounds with high antiviral activity. However, these inhibitors were sensitive to mutations located in their target sites and also had cytotoxic side effects. It could be concluded that technology based on the use of novel modified ONs is promising but is likely to be more suitable for targeting the unwanted expression of cellular genes (such as oncogenes) than for targeting rapidly mutating virus genomes.
- Rational design was used to develop a number of promising anti-CHIKV vaccine candidates. Two of those candidates were viruses that were attenuated by deletions of large (≥150 nucleotides) parts of coding regions. These viruses (CHIKVΔ5nsP3 and CHIKVΔ6K) were found to have a stable

attenuated phenotype, and the introduced changes were maintained during serial passages. Of all of the studied vaccine candidates, CHIKVΔ5nsP3 was the most potent; a single immunization provided full and long-lasting protection of all vaccinated animals. The design of this vaccine candidate was based on that of a previously studied SFV mutant. Surprisingly, it was found that the functions of the region affected by the deletion are different in SFV and CHIKV. In the case of SFV, this region, which is located in the N-terminal part of the HVD of nsP3, acts as part of a sequence element responsible for the hyper-activation of the PI3K-Akt-mTOR pathway. CHIKV, in contrast, lacks this property, and the corresponding region in nsP3 apparently has other function(s). It was found that although these regions of SFV and CHIKV are dispensable for virus replication, they are not interchangeable. The analysis of virus progeny rescued from constructs harbouring such swaps in the nsP3 region revealed that the region removed from the CHIKVΔ5nsP3 vaccine candidate is apparently involved in interactions with another domain of nsP3 and with the C-terminal region of nsP2. These findings provide a platform for further analysis of the biological causes of the attenuation of the CHIKVΔ5nsP3 vaccine candidate.

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SUMMARY IN ESTONIAN

Hepatiit C viiruse ja Chikungunya viiruse vastased lähenemised

Tänapäeval on võimalik ennast erinevate viiruste vastu vaktsineerida ning ka viirushaiguste ravi on muutunud oluliselt tõhusamaks. Samas leidub endiselt meditsiiniliselt tähtsaid viiruseid, mille vastu puudub vaktsiin ja/või mille põhjustatud haigustele pole siiani adekvaatset ravi. Viiruste mitmekesisus, nende keerukas elutsükkel ning sellised omadused nagu kiire muteerumine, efektiivne replikatsioon, rekombinatsioon ning varjumine immuunsüsteemi eest on vaid mõned aspektid, mis raskendavad antiviraalsete ühendite ja vaktsiinide väljatöötamist.

Hepatiit C viirus (HCV) on maksapatoloogiate (fibroos, tsirroos, maksakasvajad) üheks levinumaks tekkepõhjuseks. Hinnanguliselt on selle viirusega krooniliselt nakatunud ~3% inimkonnast. Kroonilise HCV infektsiooni raviks kasutatakse uudseid HCV vastaseid inhibiitoreid, mis võimaldavad kasutada interferoonivaba raviskeemi. Selline ravi omab kuni 99%-st efektiivsust kuid on kallis, selle efektiivsus on sõltuv viiruse genotüübist ning juba väljaarenenud patoloogiast; lisaks võib ravi põhjustada tõsiseid kõrvalmõjusid. Seetõttu on uute HCV vastaste ühendite arendamine endiselt oluline.

Antud töö esimeseks eesmärgiks oli analüüsida FQSAR meetodil leitud potentsiaalsete HCV NS3/4A proteaasi inhibiitorite omadusi. FQSAR on meetod, mis võimaldab soovitud omadustega keemiliste ühendite struktuuride genereerimist *in silico*. Sellisel viisil valiti välja seitse HCV NS3/4A proteaasi inhibiitori-kandidaati. Kõik analüüsitud ühendid omasid võimet suruda maha viiruse replikatsiooni kultiveeritavates rakkudes. Paraku võis see efekt enamikel juhtudel tuleneda ühendite tsütotoksilistest kõrvalmõjudest. Vaid üks ühend (23332) inhibeeris viiruse replikatsiooni kontsentratsioonidel, mis ei omanud kahjulikku toimet viiruse peremeesrakkudele. Samuti näitasid saadud tulemused, et rakupõhistest katsesüsteemidest saadud andmed vajavad ülekontrollimist kasutades selleks ensümaatilise aktiivsuse inhibeerimise mõõtmist.

Töö teiseks eesmärgiks oli analüüsida uudsete 8-oxo-G lämmastikaluseid sisaldavate antisense oligonukloetiidide (ASO) omadusi ning võimet inhibeerida HCV replikatsiooni. ASO sihtmärkjärjestused HCV kodeerivas regioonis valiti välja RNAi-l põhineval meetodil. ASO, mis sisaldasid LNA aluseid mõlemas oligonukleotiidi otsas inhibeerisid HCV replikatsiooni. See efekt säilis ka 8-oxo-dG modifikatsioonide lisamisel ASO kesksesse ossa. 8-oxo-dG modifikatsoon vähendas ASO sulamistemperatuuri, kuid ei mõjutanud RNaas H vahendatud sihtmärk RNA lagundamist. Lisaks suurendasid sellised modifikatsioonid ASOde stabiilsust bioloogilises keskkonnas. Seega võimaldas LNA aluste ja 8-oxo-dG modifikatsioonide kombineerimine saada kõrge HCV-vastase aktiivsusega ühendid. Samas olid saadud inhibiitorid tundlikud punktmutatsioonidele ASO sihtmärkjärjestuses ning omasid kõrgematel kontsentratsioonidel tsütotoksilist efekti. Sellest võib järeldada, et 8-oxo-G modifikat-

sioone sisaldavad ühendid omavad perspektiivi eelkõige rakuliste sihtmärkide (nagu kasvajates üle-ekspresseeritavad onkogeenid) mahasurumisel.

Chikungunya viirus (CHIKV, perekond Alfaviirus) on troopiline arboviirus mis on viimasel aastakümnel korduvalt väljunud oma tavalisest levialast ja põhjustanud epideemiaid erinevates maailmajagudes. Antud töö kolmandaks eesmärgiks oli analüüsida uudsete CHIKV-vastaste vaktsiinikandidaatide geneetilist stabiilsust ning uurida nendes sisalduvate viirust nõrgestavate mutatsioonide mõju CHIKV elutsüklile. Selleks analüüsiti kahte potentsiaalset CHIKV vaktsiinitüve, mis sisaldavad suuri (≥150 alust) deletsioone viiruse valke kodeerivates alades. Leiti, et need viirused (CHIKVΔ5nsP3 ja CHIKVΔ6K) omavad nõrgestatud fenotüüpi ka peale mitmekordset passeerimist koekultuuri rakkudes. Mitmetest analüüsitud CHIKV-vastastest vaktsiini kandidaatidest osutus kõige efektiivsemaks CHIKVΔ5nsP3. Täiendavad analüüsid näitasid, et regioon, mis on selles vaktsiinikandidaadis eemaldatud, täidab erinevate alfaviiruste infektsioonis erinevaid funktsioone. Erinevalt CHIKV'st on see region Semliki Forest viiruse (SFV) puhul vastutavaks raku PI3K-Akt-mTOR signaalraja aktiveerimise eest. Lisaks selgus, et nii CHIKV kui ka SFV taluvad vastava regiooni eemaldamist kuid mitte selle väljavahetamist teisest viirusest pärineva järjestuse vastu. Selline vahetus vähendas drastiliselt viiruste infektsioonilisust ja põhjustas adaptiivsete mutatsioonide tekkimist. Selliste mutatsioonide paiknemine viitab sellele, et CHIKV nsP3 C-terminaalne regioon interakteerub sama valgu keskmise domeeni ning nsP2 valgu C-terminaalse osaga ja et need interaktsioonid on olulised viiruse replikaasi moodustamisel. Need avastused võimaldavad edaspidi välja selgitada CHIKVΔ5nsP3 mitte-patogeense fenotüübi põhjused.

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PUBLICATIONS

CURRICULUM VITAE

Name: Margit Mutso
Date of birth: 08.10.1986
Citizenship: Estonian
E-mail: mmutso@ut.ee

Education and professional employment

1991–2005	Tartu Kivilinna Secondary School
2005 –2008	University of Tartu, BSc (Gene Technology)
2008 –2010	University of Tartu, MSc (Gene Technology)
2010 –	Doctoral studies, University of Tartu (Engineering and Technology)
2014 –	University of Tartu, Institute of Technology, specialist

List of publications

1. Karelson, M., Dobchev, D.A., Karelson, G., Tamm, T., Tämm, K., Nikonov, A., **Mutso, M.** and Merits, A. (2012) Fragment-based development of HCV protease inhibitors for the treatment of hepatitis C. *Current Computue Aided Drug Design*, **8**, 55–61.
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ELULOOKIRJELDUS

Nimi: Margit Mutso
Sünniaeg: 08.10.1986
Kodakondsus: Eesti
E-post: mmutso@ut.ee

Haridus ja erialane teenistuskäik

1991–2005	Tartu Kivilinna gümnaasium
2005–2008	Tartu Ülikool, BSc (Geenitehnoloogia)
2008–2010	Tartu Ülikool, MSc (Geenitehnoloogia)
2010–	Doktorantuur, Tartu Ülikool, Tehnoloogiainstituut, (Tehnika ja tehnoloogia)
2014–	Tartu Ülikool, Tehnoloogiainstituut, spetsialist

Publikatsioonid

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