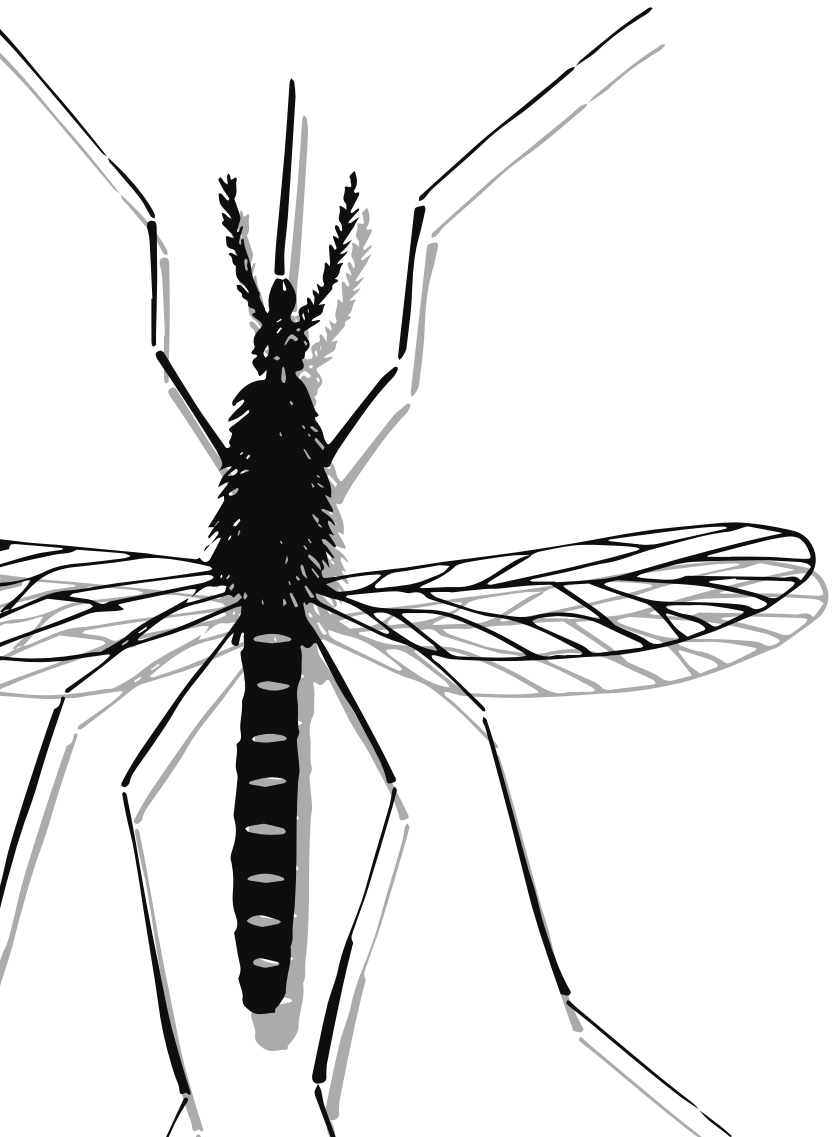
Chikungunya virus nonstructural protein 1 as an antiviral target

Kovacikova, K.



CHAPTER 7

General Discussion

The highly epidemic potential of CHIKV was only truly appreciated after the large epidemics occurred at the beginning of the 21st century. CHIKV remains a serious health problem, mostly in developing countries with poor healthcare infrastructure and high incidence of *Aedes* mosquito vectors. So far, registered antiviral therapies and vaccines are not available and CHIKF treatment is only symptomatic. The main objective of this thesis was to develop novel antiviral strategies against CHIKV. The research described in this thesis focused on identifying prospective CHIKV inhibitors and characterizing their modes of action using cell-based assays, enzymatic assays with purified protein and molecular docking. More specifically, **Chapters 3-6** describe studies ranging from the identification of small-molecule inhibitors in cell-based screens to in-depth elucidation of the mechanism of action of selected potent inhibitors, which were found to inhibit CHIKV nsP1. CHIKV nsP1 was first identified as an antiviral target in 2016, with a report describing the MADTP compound series as potent inhibitors of this protein (1). NsP1 performs unique virus-specific enzymatic functions responsible for viral RNA capping, thus representing a good target for antiviral therapy. This thesis has contributed to the CHIKV antiviral field by the identification of novel RNA capping inhibitors with specific yet distinct inhibitory effects on the nsP1 enzymatic functions. Furthermore, this thesis for the first time describes the use of a CHIKV nsP1 cryo-EM structure for molecular docking studies with small-molecule inhibitors. This final chapter places the results of this thesis in the context of the current literature and discusses some unexpected findings. The highlights and limitations of the antiviral studies with CHIKV nsP1 are summarized and discussed below.

Identification of CHIKV inhibitors and characterization of their mechanisms of action

Compounds identified as prospective CHIKV nsP1 inhibitors originate from screens of repurposed or natural compounds as well as newly synthesized molecules. The CHIKV nsP1 inhibitors described in this thesis have been chemically synthesized and contain novel chemical moieties, as described in **Chapters 2-3.** 6'-fluorinated-aristeromycin and 6'-fluorinated-homoaristeromycin analogues were designed as dual-target compounds against both a viral and a host target. Therefore, their inhibitory activity was first validated in an enzymatic assay with a recombinant S-adenosyl-L-homocysteine (SAH) hydrolase and subsequently in CHIKV CPE-reduction assays (**Chapter 3**). Two of the prototype compounds, namely 6'-β-fluoro-

homoaristeromycin (FHA) and 6′-fluoro-homoneplanocin A (FHNA), showed stronger inhibition in CHIKV CPE-reduction assays (EC_{50} 0.12 ± 0.04 μM and 0.18 ± 0.11 μM, respectively) compared to the enzymatic assays with SAH hydrolase, suggesting their antiviral effect could be direct rather than indirect via inhibition of the host SAH hydrolase (**Chapters 3-4**). The inhibition of the latter host enzyme indirectly interferes with the nsP1-mediated methylation reactions via a negative feedback mechanism due to SAH build-up. Despite the inhibition of SAH hydrolase having been studied with various SAH hydrolase inhibitors in cell culture in the past, a direct link between the inhibition of SAH hydrolase and FHA/FHNA has not been established in our study using cell-based assays. The opportunities for measuring metabolites such as SAM and SAH in infected treated and untreated cells have been explored but have proved to be complex, as many players feed into the cellular methylation pathway. It was also not possible to perform the SAH hydrolase knockdown by siRNA mediated gene silencing, for example, as the SAH hydrolase is considered an essential host factor in the cellular methylation pathway. Despite lack of direct evidence that FHA/ FHNA act via inhibition of the host SAH hydrolase, the viral target of these compounds was determined by resistance passaging and generation of resistant mutants by reverse genetics. The resistance passaging protocol used and described in **Chapters 4-5** differs from the conventional passaging approach performed in the presence of suboptimal compound concentrations. Instead, this method relies on using a high viral dose in the presence of compound concentrations that confer full protection in CPE-reduction assays. The wells containing 'viral breakthrough', i.e. viruses with mutations that increase fitness, are then further passaged, again in the presence of a compound dose conferring full protection against virus-induced CPE, as in the previous step. The objective is to obtain a viral population where the mutants conferring resistance to the compound represent the majority. While this selection procedure allows rapid identification of resistant mutants, it does not discriminate between mutations that originated due to resistance to the compound and those that arose as a consequence of cell culture adaptation. Mutations responsible for cell culture adaptation in VeroE6 cells, such as R171Q and T301K in CHIKV nsP1 and opal524R in CHIKV nsP3, have been detected previously (2). Therefore, the mutation of opal524R in CHIKV nsP3 identified in our study was considered as non-specific. Because the resistance passaging method described here does not allow for parallel passaging of wt virus to reveal such non-specific mutations, all identified mutations have to be reverse-engineered into an infectious cDNA clone, either singularly or in combinations. The inability to

exclude non-specific mutations is the major downside of this method, as opposed to conventional passaging where wt virus is passaged in parallel in untreated cells. Our mechanism of action studies described in **Chapters 4-5** identified CHIKV nsP1, the enzyme responsible for viral RNA capping, as the target of both FHA and FHNA, and the CHVB compounds. Reverse genetics confirmed that resistance against FHA/FHNA and CHVB compounds required two mutations in CHIKV nsP1. The equivalents of the mutations that confer resistance to FHNA in CHIKV, namely G230R and K299E, were introduced into an SFV infectious cDNA clone. The SFV nsP1 mutants with a single mutation K231R or K300E and the K231R/K300E double mutant were tested for resistance in SFV CPE-reduction assays. Unlike for CHIKV nsP1 mutants, none of these SFV nsP1 mutants proved to be resistant to FHNA (data not shown). However, it is important to mention that these mutations were not selected by resistance passaging, therefore the mutations responsible for SFV resistance to FHNA were either located elsewhere in the nsP1 sequence and/or more than two mutations were responsible for the resistance phenotype. Importantly, the enzymatic assays with wt SFV nsP1 were able to demonstrate at least a partial direct inhibitory effect of the metabolized form of FHNA, the 3'-keto form. Unfortunately, despite several efforts, the 3'-keto form of FHNA could not be chemically synthesized (unpublished). Assays with the purified SFV nsP1 carrying the K231R and K300E mutations showed that this protein was enzymatically less active and also partially resistant to the inhibitory effect of the 3'-keto form of FHNA (**Chapter 4**), in contrast to the same mutant in SFV CPE-reduction assays. This clearly demonstrates differences between the conformation of the protein in infected cells and its accessibility to inhibitors compared to *in vitro* assays. This topic is revisited in more detail below. Although the direct link between FHA/FHA and the inhibition of SAH hydrolase could not be established, the effect of both SAM and SAH on the activity of purified wt SFV nsP1 and mutant K231R/K300E nsP1 was tested in enzymatic assays. The results of these assays disproved an (inhibitory) effect of both SAH and SAM on the protein, indicating that the mechanism of action of FHA/FHNA is independent of the inhibition of SAH hydrolase.

Is RNA capping a good target for antiviral drug development?

The 5' cap structure is unique to eukaryotic cellular and viral mRNAs, as it is absent from other domains of life. Although the capping pathway is well conserved in all eukaryotes, the structure and genetic organization of the capping enzymes differ

between species (3). In the conventional RNA capping pathway of eukaryotic cells, the cap is formed by three enzymatic reactions at the 5' end of the nascent mRNA (Fig. 1). First, the 5' triphosphate end of pre-mRNA is hydrolysed to a diphosphate by an RNA 5' triphosphatase (RTPase). Second, a guanylyltransferase (GTase) adds guanosine 5' monophosphate (GMP) to the diphosphate RNA via a covalent enzyme-GMP intermediate. Finally, the GpppN cap is methylated at the N7 position by a guanine-N7 methyltransferase (MTase). This process generates the minimal cap-0 structure (m^7GpppN) found mainly in lower eukaryotes. In higher eukaryotes, further methylation by ribose 2'-O-methyltransferases (2'-O MTases) occurs at the 2' position of the first nucleotides in the RNA transcript, to generate cap-1 (m^7GpppNmN) and cap-2 (m^7GpppNmNmN) structures (4). S-adenosyl-L-methionine (SAM) serves as a methyl donor for both the N-7 and 2'-O methylation reactions. S-adenosyl-L-homocysteine (SAH) is a demethylated by-product that is released during the reaction and can inhibit SAM-dependent MTases.

In contrast to some viruses that require two methylation steps, alphaviruses need only one methylation event for production of a functional 5' cap. An alphavirus 5' cap is identical to the cellular 5' RNA cap, albeit its synthesis proceeds via a non-conventional mechanism. The alphavirus nsP1 MTase first methylates a GTP molecule to produce 7-methylguanosine (m^7GTP) (5). The nsP1 GTase is then responsible for the covalent attachment of m^7GTP onto nsP1, resulting in the formation of the covalent m^7GMP-nsP1 intermediate and releasing a pyrophosphate (PPi) (6, 7). The nsP2 RTPase removes the 5' γ-phosphate from nascent RNAs to produce 5' diphosphate-containing RNAs ready to receive the methylated m^7GMP cap (8). Finally, the nsP1 GTase transfers m^7GMP onto the diphosphate RNAs. Thus, both alphavirus nsP1 and nsP2 are involved in RNA capping. The cap structure protects the mRNA from degradation by host 5'-3' exoribonucleases as well as against a number of innate immune sensors that detect the presence of uncapped viral RNAs as 'non-self'. Lastly, the cap is required for translation by host ribosomes that recognize the N7-methylated cap structure

Alphavirus RNA capping is an interesting target for CHIKV antiviral drug development owing to the virus-specific enzymatic functions and mechanistic differences between cellular and viral 5' cap formation. While cellular MTase and GTase enzymes often contain canonical catalytic motifs (KDKE and KxDG, respectively), alphavirus nsP1 does not contain these signature sequences, opening the possibility of selective inhibition (9). Studies with an SINV infectious cDNA clone have also shown that point mutations of H39, D91, R94 and Y249 that abolish nsP1 enzymatic activity

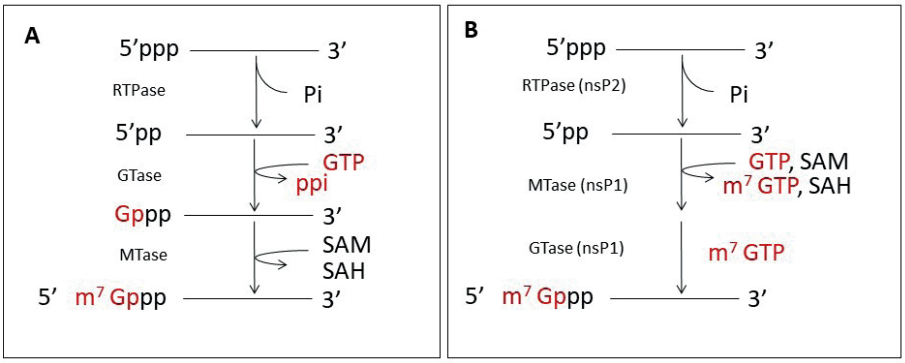


Figure 1: Comparison of the host (A) and alphavirus (B) RNA capping pathway.

render the virus non-viable (10). It is possible to achieve a complete block of replication by targeting the 5' cap, as illustrated by the CHVB compounds that fully blocked CHIKV replication at specific doses in the low micromolar range in a $TCID_{50}$-based assay (**Chapter 5**). As alphavirus RNA capping occurs exclusively in the cytoplasm drugs designed to specifically target this process are not expected to trigger adverse reactions. In the alphavirus replication cycle, two RNA molecules are co-transcriptionally capped – the genome and the sgRNA. Inhibition of this process should have a profound negative effect on alphavirus replication. Furthermore, the activation of innate immune pathways, including the type I IFN response, by the presence of uncapped RNA molecules, is expected to enhance the impact of drugs targeting RNA capping. Under such circumstances, RNAs with immature or incomplete cap structures would be targeted for degradation by the RNA decay machinery.

Nevertheless, some considerations need to be made when developing CHIKV nsP1 inhibitors. Antiviral drug resistance to CHIKV nsP1 inhibitors is known to develop, typically requiring the presence of two mutations, as discussed in **Chapter 2**. A virus can also develop alternative pathways to resist the effect of antiviral compounds. For example, DENV was reported to alternate between cap-dependent and cap-independent translation mechanisms (11), which could be problematic for the development of specific DENV capping inhibitors. Another potential downside of using nsP1 as a specific antiviral target is the limited level of conservation between different members of the *Togaviridae* family. It is known that alphavirus nsP1 displays approximately 60-65% sequence similarity despite the enzymatically active part being well-conserved. The unique structural features that increase the selectivity of this

antiviral target could come at the cost of a broad-spectrum antiviral effect. Despite the latest findings by Jones *et al.* that alphavirus nsP1 forms a conserved structure composed of 12 nsP1 protomers arranged into a ring-shaped membrane-associated complex (12), it is still not understood why some inhibitors are specific towards CHIKV nsP1 while others are not. This is exemplified by the CHVB compound series described in **Chapter 5**, which inhibited various CHIKV strains but was not active against related alphaviruses. Perhaps unsurprisingly, the MADTP compound series that is predicted to bind in the same pocket as the CHVB series based on docking studies described in **Chapter 6**, also had a limited antiviral spectrum. The MADTP-314 compound and its analogues potently inhibited several CHIKV strains, but were less active against VEEV and not active against closely related alphaviruses like ONNV (1).

Detection and role of uncapped RNAs produced during the alphavirus replication cycle

The absence of a 5' cap structure was identified as a primary molecular determinant of SINV particle infectivity (13). The viral RNAs produced during an infection are not universally 5' capped as previously thought. In fact, it has been shown that a significant amount of uncapped genomic RNAs, composed primarily of the 5' monophosphate RNAs, are packaged into particles during the course of a SINV infection (13). There appears to be no preferential packaging of either 5' capped or uncapped genomic RNA in mammalian or mosquito cells during a SINV infection. A more detailed mechanistic study in tissue culture models revealed that nsP1 mutations can modulate the production of uncapped RNAs. Specifically, alanine substitutions at positions Y286 and N376 of SINV nsP1 led to decreased RNA capping efficiencies and an alanine substitution at position D355 increased RNA capping efficiencies (14). Importantly, SINV appears to be much more sensitive to mutations that increase RNA capping efficiency compared to those that decrease RNA capping efficiency. In addition, increasing the viral capped-to-uncapped RNA ratio had a detrimental effect on overall viral particle production (14). These observations strongly indicate that there is a role for uncapped RNAs during the replication cycle, whereby decreased amount of uncapped RNAs negatively affects viral particle assembly. However, the precise underlying molecular mechanisms are unknown. It would be interesting to know whether uncapped RNAs play a similar role during CHIKV replication and whether any of the mutations responsible for resistance to CHIKV nsP1 inhibitors, such as FHA/

FHNA or CHVB compounds, are able to modulate capping efficiencies. Our data from **Chapter 4** have shown that treatment with the capping inhibitors FHA and FHNA led to an increased genomes-to-PFU ratio. The balance between the capped and uncapped RNAs might have been altered in favour of uncapped RNAs early during the replication cycle. To measure the ratio of capped-to-uncapped RNAs, an XRN1-based qRT-PCR assay was set up to discriminate the amounts of these molecules in a sample (data not shown). This assay was originally designed for SFV, a model alphavirus that has been extensively studied in our laboratory, based on the principle described by LaPointe et al. (14) and on the assumption that SFV and SINV have similar capping efficiencies. The assay uses the 5'-3' exoribonuclease XRN1 that digests 5' monophosphate RNAs, but leaves 5'di/triphosphate RNAs and 5' caps intact. After an enzymatic digestion by XRN1, only capped RNAs are expected to be left in the sample preparation. In addition, the samples are internally spiked with a yellow fever virus (YFV) *in vitro*-transcribed RNA fragment. Control reactions contain YFV RNA that is first treated with RNA 5' pyrophosphodydrolase (RppH) that removes 5' di/triphosphate RNAs and 5' caps, leaving only 5' monophosphate RNAs for degradation by XRN1. The net result of the combined RppH and XRN1 treatment is the absence of YFV RNA. The ratio of capped-to-uncapped RNAs is then compared between XRN1-treated and untreated samples by detection with qRT-PCR using specific SFV and YFV probes. Experimentally, we have determined that there is approximately 3% YFV RNA remaining after digestion with both RppH and XRN1, demonstrating the assay is sufficiently sensitive to show differences between samples. However, in our assay with FHNA-treated and untreated intracellular and extracellular SFV RNA, there was large variation between the sample replicates in the assay. Therefore, it was not possible to draw a conclusion about the effect of FHNA treatment on SFV RNA capping. This could be attributed to the instability of uncapped viral transcripts, especially outside of the cellular environment. Thus, RNA degradation could be the main reason for replicate variability. It must be stated that the effect of FHNA on CHIKV RNA capping is expected to be greater, because FHNA was a more potent inhibitor of CHIKV compared to SFV, as described in **Chapter 4**. Furthermore, the SFV nsP1 K231R/K300E double mutant was not resistant to FHNA treatment in cell culture, suggesting that these mutations unlikely play a role in altering the capped-to-uncapped ratio during the SFV replication cycle. Other systems for detecting capped and uncapped RNAs include immunoaffinity purification of m^7G-capped transcripts using the anti-m^3G/m^7G antibody (Ab)-coupled beads. This method was used for the characterization of

the 5' termini of the genomic and four sg RNAs of Berne torovirus (15), a member of the nidovirus order. Our protocol differed in using qRT-PCR for detection of CHIKV RNA instead of ^{32}P-labelled oligonucleotide probes for Northern blot hybridization analysis. This assay was used to determine the amount of capped CHIKV RNA bound to the Ab-coupled beads. However, despite our best efforts, we failed to ascertain the specificity of the anti-m^{3}G/m^{7}G Ab when using an uncapped CHIKV transcript, which impacted the overall reliability of this assay set-up. For these reasons and unresolved contamination problems, we decided not to quantify capped CHIKV RNA using this assay (data not shown). Another method uses qRT-PCR for detection of RNA-adapter-containing RNAs obtained by enzymatic treatment of intracellular or extracellular SINV RNAs. In this approach, 5' monophosphate-containing RNAs are generated by an initial treatment with a phosphatase followed by treatment with either a decapping enzyme or a kinase. The resulting capped and uncapped reaction mixtures serve as substrates for a ligation with an RNA adapter. In this way, the RNA-adapter-containing molecules can be compared to the total amount of viral genomic RNA in order to determine the ratio of capped-to-uncapped RNAs (13). However, the major disadvantages of the immunoprecipitation method with the anti-m^{3}G/m^{7}G Ab and the adapter ligation method are that both are labour-intensive and require multiple (enzymatic) steps to obtain capped and uncapped RNAs for quantification by qRT-PCR. The XRN1-based qRT-PCR assay is simpler, faster and less error-prone compared to the other methods, which is why it remains the best method for determining the ratio of capped-to-uncapped RNAs from virally-infected samples.

Deciphering the enzymatic functions of alphavirus nsP1: what we have learned from the past

In vitro assays with SFV-infected cell lysates were developed already in the early 1980s to understand the SFV-specific enzyme functions in more detail. The first *in vitro* assays used ^{3}H-labelled SAM to produce methylated caps at the 5' termini of genomic and sg RNAs by the so called 'methylating enzyme', found in the mitochondrial pellet fractions (P15) of SFV-infected cells (16). The discovery that the enzyme responsible for the 'mehylating' activity, termed MTase, originated from a virus-specific protein came soon after (17). MTase activity was associated with the SFV replication complex based on evidence demonstrating that MTase from SFV-infected P15 extracts differed in substrate specificity from the cellular enzyme because, unlike the host enzyme, it

could catalyse the methylation of GTP to m^7GTP. Furthermore, the MTase reaction could take place in the presence of EDTA, although the activity was reduced if divalent cations (Mg^{2+}) were absent from the reaction. Preparations of SFV-infected P15 fractions at different time points also revealed that the MTase activity appeared during the first 2 h post-infection, coinciding with the synthesis of a viral protein during the early stages of the SFV replication cycle (17). The direct link between MTase activity and nsP1 was revealed by expressing a SINV nsP1 mutant resistant to low methionine levels in *E. coli.* This mutant expressed an MTase with much higher affinity for SAM compared to wt nsP1 (18). Later on, SFV nsP1 was also successfully expressed in *E. coli* and recombinant baculovirus (AcNPV-nsP1)-infected cells, although finding a suitable purification method was not successful at the time due to heavy protein aggregation in these expression systems (5, 7). The MTase assays with the *E. coli* lysates confirmed that the reaction is specific for GTP and dGTP while the natural cap analogue of SFV RNAs, GpppA, could not serve as a methyl acceptor molecule. Furthermore, the enzyme could not methylate capped SFV transcripts, a feature in which alphavirus nsP1 MTase differs from other virus families (5). The ability of SFV nsP1 to form a covalently bound enzyme-guanylate complex typical for GTases was discovered using P15 fractions from recombinant baculovirus (AcNPV-nsP1)-infected cells. The GTase assays, which included α^{32}P-GTP and SAM in the reaction mixture of nsP1-containing cell lysates, used the formation of a 68 kDa ^{32}P-labelled m^7GMP-nsP1 covalent intermediate as a readout. As opposed to the MTase activity, the GTase activity was dependent on the presence of divalent cations (Mg^{2+} or Mn^{2+}) for the reaction to proceed. The covalent linkage between nsP1 and m^7GMP was found to occur via a phosphamide bond (6). Using crosslinking assays with *E. coli* lysate containing recombinant SFV nsP1, residues D64 and D90 were found to be critical for SAM binding whereas residue H38 was linked to covalent binding of m^7GMP (7). These residues are conserved across the alphavirus-like superfamily, which suggested that other members of this family use similar mechanisms for 5' cap formation. Altogether, these studies indicated that alphavirus nsP1 is a source of at least two unique virus-specific enzymatic functions, the MTase and the GTase, and that the GTase is dependent on MTase. Later studies focused on deciphering the functional details of these enzymatic activities using radiolabel- and fluorescence-based assays with purified wt and mutant proteins.

SINV nsP1 expressed in *E. coli* and purified to homogeneity was the first purified alphavirus nsP1 capping enzyme. The protein retained its MTase and GTase activities, which indicated that membrane association is not required for

the enzymatic activity (19). In a more recent study with VEEV nsP1 purified from *E. coli*, a non-radioactive Western blot assay using the anti-m^3G/m^7G Ab was set up to study the GTase independently from the MTase (20). This reaction used m^7GTP and SAH as substrates while, in the past, a chemically synthesized [^{14}C]m^7GTP was used. This non-radioactive GTase assay confirmed the results of the previous study, which showed that ^{14}C-labelled nsP1 could only be synthesized in the presence of both [^{14}C]m^7GTP and SAH and that addition of SAM had no effect (7). The Western blot assay relies on the detection of the m^7GMP-nsP1 covalent complex using immunoblotting with anti-m^3G/m^7G Ab. Mutational analyses of the MTase and GTase activities revealed that substitutions of residues H37 and D63 completely abolished the formation of the m^7GMP covalent complex, as discovered for SFV nsP1. It also revealed that replacement of other residues, namely D354 and R365, increased the complex formation and that substitutions of Y285, N369 and N375 decreased complex formation. Some of these residues were found to be involved in modulating RNA capping efficiencies, as discussed before. Lastly, the guanylyltransfer of m^7GMP onto a 5' diphosphate RNA oligonucleotide was demonstrated using an assay with purified VEEV nsP1 (20). Therefore, it is now possible to uncouple all steps of the alphavirus RNA capping pathway using enzymatic assays reflecting each of the successive steps.

On the basis of the above-mentioned literature, we have established a purification protocol for SFV nsP1. It should be noted that the SFV nsP1 that was used for the activity assays in **Chapters 4-6** was purified to about 70-80% homogeneity, based on the SDS-PAGE analysis and Coomassie Blue staining. An assay detecting the formation of the m^7GMP-nsP1 covalent intermediate, which studies both MTase and GTase activities, and uses $\alpha^{32}P$-GTP and SAM as substrates, was developed to assess the antiviral effect of compounds described in **Chapters 4-6** and as discussed below. The MTase assays for SFV nsP1 were developed based on enzymatic assays with SINV and VEEV nsP1, as described in (19, 20). In our experimental conditions, the MTase reaction was stopped with excess SAH and loaded directly on the DEAE membrane. The detection of radioactive signal in this assay relies on the fact that the positively charged membrane binds to the negatively-charged nucleic acids such as [^3H]GIDP while the rest is washed away. The radioactivity that is retained on the membrane is then absorbed by the scintillation liquid and measured in the scintillation counter. However, this approach failed to detect SFV nsP1 MTase activity despite several attempts (counts per minute at or below background level; data not shown), while the MTase activity was detected for the vaccinia virus (VV) capping enzyme used as

a positive control. To avoid detection of background due to [^{3}H]SAM and to avoid clogging on the membrane, [^{3}H]GIDP was extracted 3x with phenol buffered in 100 mM Tris-HCl pH 8.0 to yield a pure preparation, similar to a previously published protocol (19). The extraction is based on the separation of [^{3}H]SAM into the phenol phase and the methylated acceptor [^{3}H]GIDP into the aqueous phase. The radioactivity in the extracted aqueous phase was then mixed with the scintillation liquid and measured by scintillation counting. Nevertheless, this method also did not lead to detection of the MTase activity of SFV nsp1 (data not shown). It could be that the SFV nsP1 preparation that was used for these assays was contaminated with traces of SAH due to incomplete purification, which would have distorted the assay equilibrium and would have had a negative effect on the enzymatic activity. It is also very likely that this assay is not sensitive enough to detect the MTase activity compared to the radioactive assay with α^{32}P-GTP and SAM measuring both MTase and GTase activities.

Inhibitors of alphavirus nsP1 in enzymatic assays

The main purpose of developing the covalent complex formation assay with SFV nsP1 was to assess the antiviral effect of RNA capping inhibitors on this antiviral target, as described in **Chapters 4-6**. Obtaining purified CHIKV nsP1 has been notoriously difficult, which is why our enzymatic assays were developed using a close relative of CHIKV nsP1, SFV nsP1. The quest for alphavirus RNA capping inhibitors has started more than 20 years ago with the development of functional enzymatic assays that uncoupled the individual steps of the RNA capping pathway. One of the first antiviral enzymatic assays with SFV nsP1 used partially purified bacterial supernatant (S15) fractions to study the effect of GTP analogues. Although some of the cap analogues could effectively inhibit the MTase and GTase of SFV nsP1, they could also interfere with translation by competing for ribosomal binding (21). More recently, sinefungin and aurintricarboxylic acid (ATA), a known inhibitor of flavivirus RNA capping, were shown to inhibit MTase and GTase activities in antiviral assays with purified VEEV nsP1. In contrast, ribavirin triphosphate (RTP) was very poorly active in both assays (20). This demonstrates that alphavirus nsP1 behaves differently from other enzymes such as the VV capping enzyme, as RTP can act directly as a substrate for the VV capping enzyme and form a covalent RMP-enzyme intermediate (22). The above-mentioned assays were based on the use of substrates containing radioactive isotopes, the use of which has diminished over the last decade. Despite their sensitivity, radioactive assays

are often discontinued due to potential health risks, reduced production of radioactive isotopes by commercial suppliers, limited half-life and costly waste disposal. More recently, non-radioactive methods have been developed that obviate the need for radiolabelled substrates. For example, a high throughput (HT) ELISA-based GTase assay was used for screening of 1220 compounds and identified 18 compounds inhibiting guanylylated VEEV nsP1. The best compounds showed improved IC_{50} values compared to the reference compound sinefungin (IC_{50} 29.1 ± 2.6 μM) (23). A screen using a similar ELISA-based assay with CHIKV nsP1 confirmed the results with VEEV nsP1, with sinefungin and ATA having low IC_{50} values of 2.7 μM and 5.7 μM, respectively, while RTP was a poor inhibitor of CHIKV nsP1 with an IC_{50} in the millimolar range (24). Another method, a fluorescence polarization (FP)-based assay with CHIKV nsP1, was developed to monitor the displacement of fluorescently-labelled GTP analogues in real time using a pure monomeric CHIKV nsP1. This was the first report describing structural features of GTP that are important for GTP binding within CHIKV nsP1 (25). The same HT FP-based assay was used to screen 3051 compounds and identified lobaric acid as a potent competitive inhibitor of GTP binding (K_i 7.0 ± 0.6 μM) and anti-CHIKV activity (EC_{50} 5.9 ± 1.4 μM) (26). Our studies in Chapters **4-6** describing the *in vitro* antiviral effect of CHVB and MADTP compounds, sinefungin, FHNA and its modified 3'-keto form were performed with SFV nsP1. CHVB compounds completely blocked the formation of the ^{32}P-labelled m^7GMP-nsP1 covalent intermediate while MADTP-372, sinefungin and the 3'-keto form of FHNA reduced the signal to a lesser extent. Overall, these assays demonstrated the antiviral effect of CHIKV nsP1 inhibitors in an assay system with a purified alphavirus nsP1 protein.

Discovery of alphavirus nsP1 capping complex and implications for future antiviral research

Towards the end of 2020, a new discovery describing alphavirus nsP1 capping rings has challenged our interpretations of the biochemical data on alphavirus nsP1 that accumulated over the last decades. For the first time, it was demonstrated that CHIKV nsP1 is active in ring-like oligomeric complexes and that these complexes are conserved among alphaviruses and beyond (12). The CHIKV nsP1 complex assembles into a dodecameric ring with a central pore, that is composed of a crown, a waist and a membrane-binding skirt. The active sites of the capping MTase-GTase domain form positively-charged pockets at the top of the crown while the membrane-binding

spikes protrude from the bottom of the skirt. The oligomerization of the nsP1 complex is needed to allosterically activate the enzyme and stabilize the conformation of the capping domain. Therefore, the enzymatic activity of monomeric nsP1 seems to be only residual compared to the enzymatic activity of oligomeric nsP1, which is expected to several times exceed that of the monomeric nsP1. Furthermore, these enzymatically active rings are associated with the membrane within the spherule necks, controlling access to these cellular invaginations harbouring newly synthesized viral RNA, as suggested in the past (27).

In **Chapter 6**, we performed molecular docking studies with the CHIKV nsP1 cryo-EM structure. Our results revealed that CHVB and MADTP series dock at the SAM-binding site of the oligomeric nsP1 complex. Interestingly, they tend to dock at the GTP-binding site of the monomeric nsP1. Therefore, it's important to further elucidate whether the nsP1 complex is the predominant structural form in infected cells as well as the speed of nsP1 complex assembly. Besides the capping domain, CHIKV nsP1 also contains the RAMBO domain that makes up the waist and skirt regions of the complex. The FHNA series docks at a binding pocket in this domain, leading to disruption of membrane binding and oligomerization of nsP1. The identification of the predicted compound-docking sites in these functionally distinct regions of CHIKV nsP1 is supported by the resistance-inducing mutations that localize in the vicinity of these sites. The residues mutated in the MADTP- and CHVB-resistant viruses gate the active site and the residues involved in FHNA resistance line a pocket in the RAMBO domain, as further described in **Chapter 6**. Altogether, our molecular docking studies classified the CHIKV nsP1 inhibitors into functionally distinct groups. However, some of our earlier findings, including the specific antiviral activity of MADTP and CHVB series against CHIKV but not against related alphaviruses, could not be addressed with the available structural information. Therefore, the structural context for inhibition of MADTP or CHVB series still needs to be further investigated, given that their docking positions can change depending on the oligomeric state of nsP1. This is counterintuitive, as one would expect a defined binding pose for inhibitors specifically targeting CHIKV nsP1. Nevertheless, it must be re-emphasized that the current CHIKV nsP1 structure did not contain ligands in the active site, and that CHIKV nsP1 is predicted to undergo conformational reorganization affecting the active site. The characterization of the precise location of SAM- and GTP-binding sites within the alphavirus nsp1 structure, including the thus far elusive residues involved in MTase and GTase activities, would represent the first step towards identification of broad-spectrum small molecule

inhibitors of alphavirus nsP1. This information could also be harnessed for *in silico* screening and rational design of selective alphavirus nsP1 inhibitors. Another potential avenue for drug design would be the development of allosteric inhibitors of alphavirus nsp1, by targeting the druggable pocket in the RAMBO domain of nsP1.

Do arboviruses pose a global threat for the future?

Mosquitoes, like all other organisms, respond to selective pressures imposed by their changing environments. Climate change and urbanisation have been the major drivers of arbovirus spread to new territories with previously unexposed populations. The changing epidemiology in these new regions could be disruptive to healthcare systems and economies of the affected countries that have not had a history of arboviral diseases. For example, predictive models indicate that arboviruses will pose an increased threat to Africa due to their year-around circulation and the abundance of *Aedes* mosquitos on this continent. This shift can be explained by differences in thermal optima between the mosquito vectors for malaria and arboviruses, such as dengue and chikungunya. While malaria transmission by *Anopheles gambiae* peaks at 25°C, arbovirus transmission by *Ae. aegypti* peaks at 29°C (28, 29), which can lead to geographical shifts in *Ae. aegypti*-transmitted viruses with warmer temperatures, for example in sub-Saharan Africa. The shift from malaria to arboviral diseases has already occurred in much of Latin America, where malaria transmission has substantially declined in the past three decades and, at the same time, dengue, chikungunya and Zika incidence have increased (30). While malaria control programs have undoubtly played an indispensable role in controlling malaria transmission, the influence of temperature and human movement on vector-borne disease transmission has been largely unrecognized. In addition, CHIKV has expanded to cooler regions of the world due to adaptation to a new vector - *Ae. albopictus* -, which thrives at lower environmental temperatures compared to *Ae. aegypti*. This indicates that, besides ecological changes, alteration of the genetic make-up of viruses such as CHIKV can affect vector competence and their distribution in the environment. The latest statistics show that the most CHIKV-affected countries lie in Central and South America, with Brazil having the largest number of reported cases, and in South-East Asia, dominated by Malaysia and Thailand. There have been no autochthonous cases of CHIKV reported in continental Europe in 2019 or 2020 (https://www.ecdc.europa.eu/en/chikungunya-monthly).

Concluding remarks

In this thesis, several important topics related to inhibition of CHIKV nsP1 by small-molecule inhibitors have been discussed. Here, a few key points of importance to the field of (alpha)virology and antiviral drug development are highlighted. First, it was shown that potent and specific inhibitors of the CHIKV RNA capping can be developed by using novel synthetic approaches to fill the CHIKV antiviral drug discovery pipeline. Moreover, it was demonstrated that the resistance to CHIKV nsP1 inhibitors can develop, and that the barrier to resistance typically consists of two amino acid substitutions in the non-conserved regions of nsP1. Besides identifying the viral drug target in cell-based assays, it is important to validate the viral target of direct-acting CHIKV nsP1 inhibitors in different experimental systems, for example, in enzymatic assays with purified protein, to confirm target specificity. As shown with the use of the recently solved CHIKV nsP1 cryo-EM structure, which is assembled into a dodecameric ring, the oligomerization state of nsP1 can have an impact on the molecular docking of CHIKV nsP1 inhibitors and can influence the interpretation of our findings from cell-based and enzymatic assays. More importantly, our studies showed that the use of a CHIKV nsP1 structure is instrumental for identifying and validating druggable pockets or binding sites. In addition, such knowledge can contribute to rational design and improvement of CHIKV active molecules for development into a much-needed antiviral therapy. Given that antiviral drug resistance is known to develop for CHIKV inhibitors, it appears that combination therapy consisting of two or more direct-acting inhibitors or a direct-acting inhibitor together with a host-directed inhibitor or an immunomodulatory agent is the best approach for tackling CHIKF and that this strategy can increase the barrier to resistance. To conclude, this thesis stresses the importance of further research in the arbovirus field as arboviruses will undoubtly represent a global health threat in the future due to their unpredictable epidemiology.

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