Chikungunya virus nonstructural protein 1 as an antiviral target

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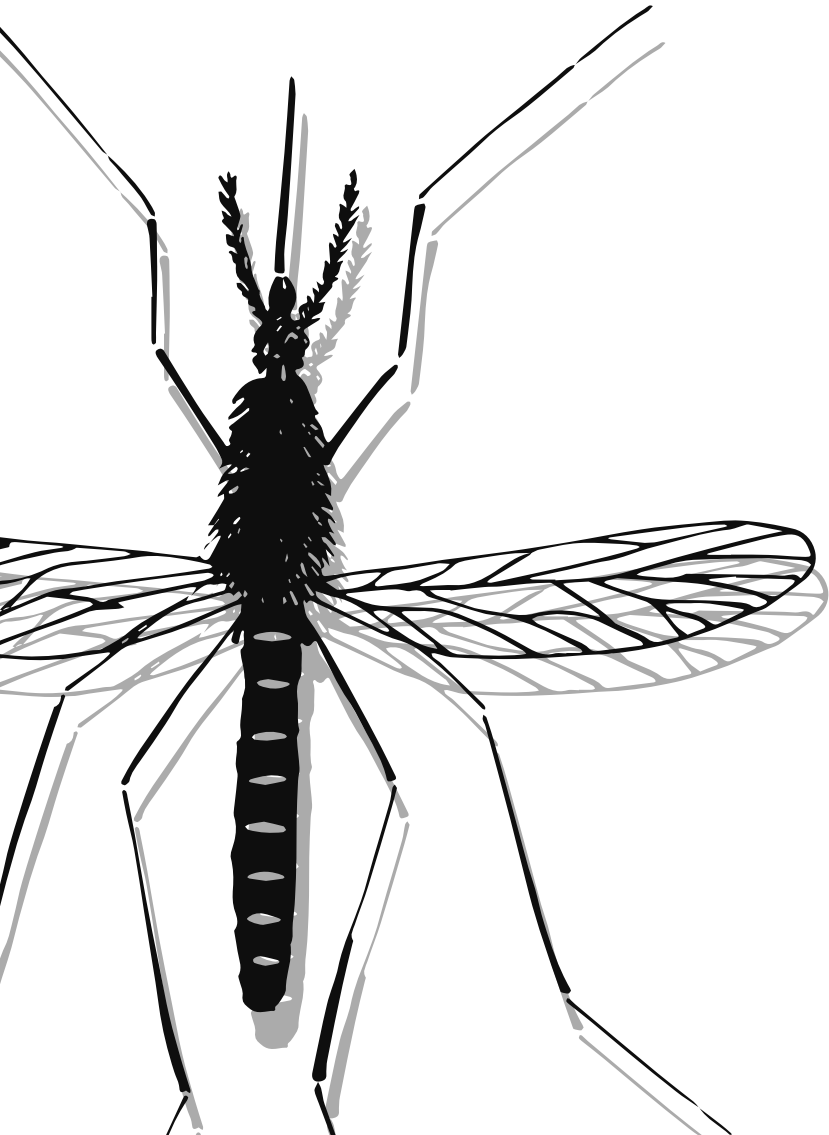**Title**: Chikungunya virus nonstructural protein 1 as an antiviral target
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CHAPTER 2

Small molecule inhibitors of Chikungunya virus: mechanisms of action and antiviral drug resistance

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Abstract

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus that has spread to more than 60 countries worldwide. CHIKV infection leads to a febrile illness known as Chikungunya fever (CHIKF), which is characterized by long-lasting and debilitating joint and muscle pain. CHIKV can cause large-scale epidemics with high attack rates, which substantiates the need for development of effective therapeutics suitable for outbreak containment. In this review, we highlight the different strategies used for developing CHIKV small-molecule inhibitors, ranging from high-throughput cell-based screening, *in silico* screens and enzymatic assays with purified viral proteins. We further discuss the current status for the most promising molecules including *in vitro* and *in vivo* findings. In particular, we focus on describing host and/or viral targets, mode of action and mechanisms of antiviral drug resistance and associated mutations. Knowledge of the key molecular determinants of drug resistance will aid selection of the most promising antiviral agent(s) for clinical use. For these reasons, we also summarize the available information about drug-resistant phenotypes in *Aedes* mosquito vectors. From this review, it is evident that more of the active molecules need to be evaluated in pre-clinical and clinical models to address the current lack of antiviral treatment for CHIKF.

Introduction

Chikungunya virus (CHIKV) is a mosquito-borne alphavirus belonging to the *Togaviridae* family that can cause explosive epidemics of acute and chronic arthritis in humans. The main vectors responsible for its transmission are the day-biting *Aedes aegypti* and *Aedes albopictus* mosquitoes. CHIKV was first isolated from a febrile patient in 1952/1953 in what is currently Tanzania (1). In the following years it caused periodic local outbreaks in Africa and Asia. In 2004, CHIKV re-emerged in coastal Kenya (2), from where it spread to immunologically naïve populations in La Reunion island, surrounding Indian Ocean islands and South Asia during 2005-2006. During this outbreak, a new CHIKV variant harbouring the A226V amino acid substitution in the E1 glycoprotein was isolated (3), which-unlike earlier isolates- was more efficiently transmitted by the *Aedes albopictus* mosquitoes that are abundant in the temperate regions of the Americas, Europe and Africa. In late 2013, CHIKV caused the first locally-transmitted outbreak on the Caribbean island of St Martin (4), resulting in more than 2.5 million cases across Central and South America in the period between 2014-2017 (https://www.paho.org/hq/index.php?option=com_topics&view=rdmore&cid=5927&Itemid=40931&lang=en). In Europe, the first autochthonous outbreak was described in Italy in 2007 (5) and since then renewed CHIKV transmission has occurred in Italy in 2017 (6) and southern France in 2010, 2014 and 2017 (7-9).

Chikungunya fever (CHIKF) typically begins with a sudden onset of fever 3 to 7 days after a bite of an infected mosquito, followed by symptoms like rash, myalgia and polyarthralgia. Polyarthralgia is mainly symmetrical and peripheral, affecting the small joints of wrists, ankles and phalanges, as well as the larger joints such as the knee and the elbow (10). The patients usually report incapacitating pain that can last for weeks to months. CHIKF treatment has been focused entirely on relieving patients' symptoms with analgesics, antipyretics and anti-inflammatory agents. Nevertheless, some of these drugs can have serious side effects upon prolonged use. The current lack of clinically approved therapeutics and adequate control measures for CHIKF warrants the development of safe and effective antiviral therapy.

In this review, a comprehensive overview of small-molecule inhibitors of CHIKV is presented in Tables 1-4 grouped by the approach by which they were identified. Table 1 and 2 contain inhibitors that were identified by cell-based screening, Table 3 contains compounds identified by *in silico* approaches and Table 4 lists compounds that were identified in enzymatic assays. It is important to note that the values presented in Tables 1-4 are not directly comparable as the experimental parameters/setup vary

between studies: e.g. the use of different virus isolates, multiplicity of infection (MOI), readouts [virus-induced cytopathic effect (CPE) vs titers vs qRT-PCR], time of harvest, type mouse models. Since this review focuses on small-molecule inhibitors, we have not included siRNA-mediated gene silencing approaches.

Strategies for anti-CHIKV drug discovery and design

Considering the global distribution of CHIKV and its mosquito vectors, the potential for additional spread and its impact on human health, the development of preventive measures is imperative. Several approaches have been used for identification of potential CHIKV inhibitors, including cell-based high-throughput screening (HTS) campaigns, rational and structure-based drug design using crystal structures or homology modelling of viral proteins. One of the conventional approaches for CHIKV drug discovery uses cell-based screening with readouts that measure CPE. The CPE reduction assay screens can provide information on antiviral activity of compounds and their cytotoxicity can be assessed in parallel on uninfected cells in the same plate. Such screens are often deployed to test known clinically approved drugs, in a process referred to as drug repurposing. The advantages of this route are decreased costs related to drug approval and an accelerated process to potential licensing of the compound. Due to increased computational power, HTS has emerged recently as an efficient process for screening thousands of compounds from large compound libraries including FDA-approved and novel molecules. The emergence of computer-aided drug design has also greatly contributed to the development of CHIKV inhibitors. These approaches are based on the structure of a viral protein to perform *in silico* virtual screens. Compounds identified by *in silico* computer-based screening can be further optimized by acquiring an understanding of the compound's structure-activity relationship (SAR) and improved derivatives can be synthesized for validation in enzymatic and cell-based assays. However, the computer-aided design for compounds targeting the CHIKV replicase is limited because, thus far, only the structures of the N-terminal and C-terminal region of nonstructural protein 2 (nsP2), representing the RNA helicase and protease domains, respectively, and the N-terminal macrodomain of nsP3 have been resolved (11-13). More opportunities for molecular docking studies arise from CHIKV structural proteins, as the structures for the envelope (E) proteins and the capsid (C) protein have been determined. Although the crystal structures for complete CHIKV nsPs are not yet available, researchers have used various purification

methods to obtain enzymatically active recombinant nsPs for use in cell-free assays. Validation of compounds originating from *in silico* virtual screens in enzymatic assays with purified proteins is especially important for confirmation of target specificity. In cell-based assays, resistance selection in the presence of (suboptimal concentrations of) compound has been widely used to identify the viral target of compounds. Tables 1 and 2 provide a comprehensive overview of all resistance mutations that have been identified so far, information which -together with cross-resistance studies- can aid in the elucidation of the mode of action of compounds that might be identified in future screening efforts. Besides the compound's mode of action, an understanding of molecular determinants of resistance can provide useful information about the virus replication cycle and pathogenesis.

Compounds targeting CHIKV entry and egress

The alphavirus virion is composed of a nucleocapsid core with T=4 icosahedral symmetry surrounded by a host-derived lipid bilayer which is decorated with 80 trimeric spikes of E1-E2 heterodimers (14-16). The E2 protein mediates viral entry by attachment to the receptors on the cell surface (17). This process is followed by clathrin-mediated endocytosis which delivers the viral particle to the early endosomes (18). The low pH in the endosomal compartment triggers conformational changes in the E1-E2 heterodimer and results in the insertion of the E1 fusion protein into the endosomal membrane. More specifically, the E1 glycoprotein is converted from a nonfusogenic form to a highly stable fusogenic E1 homotrimer (19, 20). This event will ultimately create a fusion pore and release the nucleocapsid into the cytosol. Compounds that exhibit antiviral activity against entry, fusion and egress of CHIKV are listed in Table 1.

Envelope protein E1

Obatoclax, an anti-cancer compound, was found to inhibit CHIKV infection early in the replication cycle by neutralizing the acidic endosomal environment required for fusion. The L369I mutation in domain III of the E1 fusion glycoprotein conferred at least partial resistance to obatoclax and generated resistant viruses with enhanced fusogenic potential (21).

Envelope protein E2

Arbidol/umifenovir has been licensed as an anti-influenza agent in both Russia and China and inhibits a wide-range of viruses. Arbidol inhibits early stages of the CHIKV replication cycle, which was confirmed by selection of an arbidol-resistant variant carrying a G407R mutation in the structural polyprotein (22), which corresponds to a G82R mutation in the E2 glycoprotein. This mutation also causes attenuation of the CHIKV vaccine strain 181/25, presumably by increasing interactions with glycosaminoglycans on the host cell surface (23). Arbidol derivatives with increased potency and selective inhibition of CHIKV have been developed, but the precise mechanism of action of these compounds remains unresolved (24).

Suramin is an approved drug for treatment of parasitic infections in humans and was shown to inhibit CHIKV entry in three independent studies (25-27). The compound likely influences CHIKV attachment to cells and may prevent conformational changes of the E1/E2 heterodimer that are required for viral fusion. Selection of suramin-resistant variants revealed that the mutations N5R and H18Q in the E2 glycoprotein cause some resistance to the compound. Molecular docking with the mature CHIKV spike suggested that suramin interacts with the N-terminal loop and domain A in the E2 glycoprotein, an interaction that would negatively affect virion binding to the receptor (28). Suramin treatment of C57BL/6 mice infected with different clinical isolates of CHIKV ameliorated CHIKV-induced foot swelling, inflammation and cartilage damage (29).

Capsid protein

Picolinic acid was found to bind to the hydrophobic pocket of the C protein, which might inhibit the C protein's interaction with the cytoplasmic domain of the E2 glycoprotein. The antiviral activity of picolinic acid was also confirmed in CHIKV-infected cells, although the inhibitory concentration was rather high for clinical applications (30).

6K

The 6K protein belongs to the family of viroporins or ion channel-forming proteins. It is a highly hydrophobic protein with membrane fusogenic properties. Viroporins allow for movement of small molecules and ions across membranes, which can be important during viral entry, replication and egress. The potential of 6K to serve as a therapeutic target is illustrated by the drug amantadine, a well-known influenza inhibitor that

targets the ion-channel forming M2 protein of influenza viruses (31). Amantadine inhibited ion channel activity and altered particle morphology in biophysical systems. Mechanistically, 6K likely needs to interact with E2 for its delivery to the plasma membrane where it forms an ion channel. The antiviral effect of amantadine on CHIKV was also confirmed in infected cells (32).

Drugs targeting CHIKV entry with an uncharacterized mode of action

Chloroquine (CHL) is an old anti-malarial drug with a broad range of antiviral activities against a variety of viruses. CHL had been deployed in clinical trials long before its anti-CHIKV activity was established in cell culture. The rationale for this unusual strategy was that CHL had conferred benefits in lessening joint inflammation in patients with rheumatoid arthritis during trials in the late 1950s (33). The efficacy of CHL phosphate was first investigated in a small patient cohort, which led to an alleviation of patient symptoms and justified its further use for the treatment of CHIKV-associated arthritis (34). However, the benefits of CHL for the treatment of acute CHIKV infection were disproved in the "CuraChik" trial conducted during the 2005-2006 La Reunion epidemic (https://clinicaltrials.gov/ct2/show/NCT00391313). Moreover, CHL-treated patients had more frequent complaints of arthralgia compared to placebo recipients (35). In an Indian trial during the 2006 CHIKV epidemic, CHL also did not yield benefits in relieving symptoms of musculoskeletal pain and arthritis compared to the commonly used non-steroidal anti-inflammatory drug meloxicam (36). These and other studies supported the hypothesis that CHL might enhance viral replication, which was later demonstrated *in vivo* in CHL-treated BALB/c mice infected with another arthritogenic alphavirus, Semliki Forest virus (37). In CHIKV-infected cells, CHL seems to block or delay virus internalization depending on the time of treatment. It is effective at early stages of viral infection, likely by impairing cell-virus surface interactions and blocking endosomal acidification (38).

Doxycycline is a tetracycline antibiotic used for treatment of bacterial infections that has also shown promising anti-CHIKV activity. It was postulated that the anti-CHIKV activity of doxycycline is directed towards viral entry rather than viral replication. Docking studies with the CHIKV nsP2 cysteine protease and E2 glycoprotein indicated that the compound could bind to both these viral targets. However, cell-based assays confirmed that doxycycline inhibits viral entry, likely by impairing conformational changes in the E2 glycoprotein. Treatment of adult ICR mice with doxycycline alone did not result in an improved outcome in comparison with combination treatment with ribavirin (39).

Curcumin is a turmeric plant extract that has been used for treatment of gastrointestinal disorders in Asia. The antiviral effect of curcumin on CHIKV has been demonstrated using pseudotyped viral particles (40) and subsequently a study using wild-type CHIKV showed that curcumin reduces the infectivity of CHIKV particles and their binding at the cell surface (41). A CHIKV insect cell fusion inhibition assay was used to screen for fusion inhibitors and identified two compounds, niclosamide and nitazoxanide, as prospective CHIKV inhibitors. In other assays both compounds were confirmed to inhibit CHIKV entry and suppress cell-to-cell transmission (42).

Apigenin, a natural compound with a 5,7-dihydroxyflavone structure, has shown moderate anti-CHIKV activity. Flavonoids have been previously reported to suppress the entry pathway of members of other virus families. However, the flavonoids tested against CHIKV, among which apigenin was the most potent, strongly inhibited CHIKV replicon levels and had no effect in an SFV entry assay (43), suggesting they do not target entry. Synthetic flavaglines such as FL3 were based on a class of naturally occurring plant compounds with activity in the low nanomolar range. FL3 inhibited CHIKV infection at the entry step by serving as a prohibitin ligand and disrupting the interaction between CHIKV and the prohibitin receptor (44).

Compounds targeting CHIKV replication

The incoming alphavirus genomic RNA is translated into two polyproteins: P123 and P1234. P123 is the more abundant of the two and P1234 arises as a result of the translational read-through of an opal stop codon (with about 10 % efficiency) at the end of nsP3 coding sequence (45). These polyproteins are processed by the protease domain of nsp2. Cleavage intermediates as well as fully cleaved individual viral nsPs play specific roles in CHIKV (-) and (+) strand RNA synthesis. The functions of nsPs have been largely characterized by using recombinant viruses with mutations in nsPs, in biochemical assays and by *in silico* identification of enzymatic sequence motifs. CHIKV inhibitors targeting individual nsPs are described in Tables 2, 3 and 4.

Nonstructural protein 1 (nsP1)

Alphavirus nsP1 (535 amino acids) is a viral mRNA capping enzyme with guanine-N7-methyltransferase (MTase) and guanylyltransferase (GTase) activities responsible for capping of the 5' ends of the newly synthesized genomic 42S mRNA and 26S subgenomic (sg) mRNA. The MTase catalyses the transfer of the methyl group from

S-adenosylmethionine (SAM) to the N7 position of a GTP molecule, forming m^7GTP and releasing S-adenosylhomocysteine (SAH) as by-product (46). The GTase binds the m^7GTP and forms a covalent intermediate, m^7GMP-nsP1, while releasing a pyrophosphate (PPi) (47). The m^7GMP is then transferred to the 5'-diphosphate RNA, which is generated through the RNA 5' triphosphatase activity of nsP2 (48), to create a methylated cap structure at the 5' terminus. This cap structure is essential for viral mRNA translation and prevents mRNA degradation by host 5'-exonucleases. The middle part of nsP1, spanning the amino acid residues 245 to 264, contains an amphipathic helix responsible for association of the alphavirus replication complex with membranes (49). Specifically, the presence of the amphipathic helix and palmitoylated cysteines 417-419 (50) allows nsP1 and nsP1-containing replication complexes to anchor to cholesterol-enriched membrane microdomains (51). Importantly, site-directed mutagenesis of conserved residues in alphavirus nsP1 indicated that abrogation of nsP1 enzymatic activities is detrimental for virus replication (52).

In recent years, the number of reports describing an inhibitory effect of molecules specifically targeting nsP1 functions has substantially increased (Table 2 and 4). Owing to its uniquely viral enzymatic activities, nsP1 represents an excellent target for antiviral compounds, while not affecting host cell mRNA capping that proceeds through a fundamentally different mechanism. The 3-aryl-[1,2,3]triazolo[4,5-d] pyrimidin-7(6H)-ones were first reported as a class of potent and selective inhibitors of CHIKV replication that target nsP1, with MADTP-314 as the prototype compound (53, 54). A resistance selection procedure with MADTP-314 and subsequent reverse genetics indicated that the single amino acid substitution P34S in the N-terminal part of nsP1 was responsible for resistance to MADTP-314 and several analogues of this compound (55). Recently, the 2-(4-(phenylsulfonyl)piperazine-1-yl)pyrimidine analogues known as the CHVB series, with CHVB-032 as the prototypical compound, were identified as potent and selective CHIKV inhibitors (56). Reverse genetics identified two mutations in the C-terminal region of nsP1, namely S454G and W456R, to be responsible for resistance to CHVB-032 and its analogues. Interestingly, the two families of compounds seem to target the same nsP1 functions, as the MADTP-resistant nsP1-P34S mutant is cross-resistant to CHVB-032 and its analogue CHVB-066 and, vice versa, the CHVB-resistant nsp1-S454G+W456R mutant is cross-resistant to a MADTP-314 analogue, MADTP-372 (57). Yet another phenotypic compound screen identified 6'-β-fluoro-homoaristeromycin (FHA) and 6'-fluoro-homoneplanocin A (FHNA) as potent CHIKV inhibitors with a very high therapeutic index (58, 59). The mutations G230R and K299E

in nsP1 were identified by resistance selection and reverse genetics to confer resistance to both FHA and FHNA (59). Difluoromethylornithine (DFMO), an inhibitor of ornithine decarboxylase 1, was shown to have a broad-spectrum antiviral effect on a variety of RNA viruses, including CHIKV (60). Interestingly, CHIKV can overcome polyamine depletion by acquiring mutations in nsP1. The combination of the G230R and V326M mutations in CHIKV nsP1 and *->opal524R in nsP3 was essential to confer resistance to DFMO (61). Despite its promising *in vitro* effects, there was little protection against CHIKV-induced disease in C57BL/6 mice that were fed DFMO in their drinking water prior to infection (60).

Mycophenolic acid (MPA) is a well-known immunosuppressive agent and ribavirin is a broad-spectrum guanosine analogue with immunomodulatory properties. Both compounds target the host enzyme inosine monophosphate dehydrogenase (IMPDH), which is important for the *de novo* synthesis of guanosine monophosphate (GMP) and the regulation of intracellular GTP levels. GTP is critical for at least two processes in alphavirus replication: it serves as a methyl acceptor molecule during mRNA capping and as a building block in nsP4-mediated RNA synthesis, as will be described below. The anti-CHIKV activity of MPA was shown to be associated with the depletion of the guanosine pool in cell culture (62). Similar to FHNA, treatment with MPA resulted in a release of virions with reduced specific infectivity (PFU per genome copy numbers). Earlier studies with SINV mapped the mutations responsible for the MPA-resistant phenotype to the region encoding nsP1. Viruses with the MPA-resistant phenotype were also resistant to ribavirin (63, 64). Later on, in-depth reverse genetics studies with a SINV cDNA clone demonstrated that only the mutations S23N and V302M in nsP1 were essential for MPA resistance (65). To date, CHIKV nsP1 crystal structure is not yet available, which makes it hard to appreciate the structural context of the various compound-resistant mutations and understand the molecular mechanisms underlying drug resistance. Taken together, the majority of CHIKV nsP1 inhibitors for which the molecular determinants of resistance were studied require a combination of two nsP1 mutations for compound-specific resistance. Since singular mutations did not cause resistance in most cases, the chance of the emergence of drug-resistant CHIKV variants during treatment appears to be low for these compounds.

Other CHIKV nsP1 inhibitors have been identified by using purified CHIKV nsP1 in enzymatic assays (Table 4). 5-iodotubercidin, an adenosine analogue, was recently discovered by screening with a novel capillary electrophoresis-based assay for MTase activity of CHIKV nsP1. The activity of the compound in this enzymatic assay was

validated in cell culture (66). This enzymatic assay uncouples the MTase and GTase activities and thus can be used to identify specific alphavirus nsP1 MTase inhibitors. An fluorescence polarization-based assay measuring competition for the GTP-binding site was used in a large HTS screen that identified lobaric acid as a potent CHIKV nsP1 inhibitor, which was also validated using live virus in cell-based assays (67). Since GTP binding is essential to perform the MTase step in mRNA capping, the assay identifies competitive MTase inhibitors.

Nonstructural protein 2 (nsP2)

Alphavirus nsP2 (798 aa) is a multifunctional protein which possesses several enzymatic activities, including nucleoside triphosphatase (NTPase) (68, 69), helicase (70) and RNA 5' triphosphatase (RTPase) activity (48) in the N-terminal part of the protein and protease activity (71, 72) and a SAM-dependent RNA methyltransferase-like (SAM MTase-like) domain in the C-terminal part of nsP2. The NTPase and helicase functions are important for unwinding double-stranded RNA during CHIKV replication and the RTPase activity removes the γ-phosphate from the 5' end of the RNA before the transfer of the cap-0 structure. The nsP2 protease activity is responsible for nsp123 and nsp1234 polyprotein processing (72). In the case of Old World alphaviruses, nsP2 can also induce host transcriptional shut-off and cytophatic effects (73). During host shut-off, nsP2 translocates to the nuclei of vertebrate cells to induce polyubiquitination of the catalytic subunit of the DNA-dependent RNA polymerase II, RPB1, and in this way subverts the cellular antiviral response (74, 75). The C-terminal SAM MTase-like domain plays a critical role in the nuclear function of alphavirus nsP2 (76) and inhibits the interferon response (77).

The crystal structure of the nsP2 protease domain has been solved and the protein is now used as an important target for antiviral development using computer-aided drug design (Table 3). A virtual screening campaign of a library of commercially available compounds using a homology model of CHIKV nsP2 protease identified compound 1 as an initial hit. The compound displayed anti-CHIKV activity in cell-based assays and SAR studies on 25 structural analogues yielded compound 25 which showed improved efficacy and lower cytotoxicity compared to lead compound 1 (78). Another study reported on five arylalkylidene derivates of 1,3-thiazolidin-4-one with anti-CHIKV activity in the low micromolar range, with compound 7 being the most potent. Using molecular docking, the compounds were shown to partially interact with the crystal structure of the nsP2 protease (79). Although the above-mentioned

studies identified novel nsP2-targeting molecules, they did not provide experimental evidence, e.g. using enzymatic assays, to demonstrate that nsP2 was *de facto* the target of these compounds. Computer-aided drug design was combined with cell-free assays for target validation of a set of 12 compounds designed against the CHIKV nsP2 protease using target-based modelling. The most promising compound 8 potently inhibited CHIKV replication in cell culture and was moderately active in the protease assay with recombinant CHIKV nsP2 (80). This illustrates the importance of confirming *in silico* predictions in enzymatic assays with purified protein as virtual binding does not always correlate with inhibition of enzymatic activity *in vitro*. Other inhibitors targeting nsP2 include small peptidomimetics discovered using a unique approach of quantum mechanical-based ligand descriptors. Compounds with lower molecular weight displayed greater inhibitory activity, likely due to superior access to the target pocket (81).

Nonstructural protein 3 (nsP3)

The functional role of alphavirus nsP3 (530 aa) is the least defined of all CHIKV nsPs. Three domains can be distinguished in nsP3: an N-terminal macrodomain (13), a Zn-binding alphavirus unique domain (AUD) (82) and a C-terminal hypervariable domain (HVD) (83). While the N-terminal part of nsP3 is well-conserved, the C-terminal HVD has very low sequence similarity even between closely related alphaviruses. Alphaviruses use their HVDs to recruit RNA-binding proteins typically found in stress granules, such as the G3BP proteins used by CHIKV, for the formation of pre-RCs that promote viral replication (84). Other cellular proteins from different families, specific for virus species and cell types, have been found to interact with nsP3 HVD (85). They function as the major determinants of cell specificity during viral replication. The nsP3 macrodomain affects various critical processes in the alphavirus replication cycle including nsP3 phosphorylation, (-) strand RNA synthesis, host translational shut-off and virulence (86). Importantly, ADP ribosylation of cellular proteins, a post-translational modification involved in a variety of cellular processes, is regulated by nsP3 macrodomain. The nsP3 macrodomain possesses both ADP-ribosyl-binding and ADP-ribosylhydrolase activities, by which it binds ADP-ribose and hydrolyses ADP-ribosylated residues on cellular proteins. The nsP3 macrodomain-mediated ADP-ribosyl-binding is necessary for initiating nsP synthesis and establishing RCs, while the ADP-ribosylhydrolase activity is important for amplification of RCs. Thus, the interaction of nsP3 macrodomain with ADP-ribosylated proteins is required for

efficient alphavirus replication (86). Besides that, the ADP-ribosylhydrolase activity is a determinant of neurovirulence in mice (87). nsP3 AUD appears to be a multifunctional domain that plays a role in virus genome replication (88).

To date, only a handful of small-molecule inhibitors targeting CHIKV nsP3 have been reported, perhaps due to the enigmatic role of nsP3 in viral replication. However, in-depth characterization of the nsP3 functional domains in recent years has contributed to the exploitation of nsP3 as a potential drug target. Given its highly conserved nature and the available crystal structure, the macrodomain represents an ideal site for development of specific anti-CHIKV antivirals. Baicalin is one of the very few compounds shown to interact with nsP3 using computational approach (89) with a confirmed anti-CHIKV activity in cell culture (90) (Table 3). However, the latter study revealed that baicalin inhibited early stages of CHIKV replication and has strong virucidal activity. Moreover, baicalin was shown to interact with the CHIKV E glycoprotein using molecular docking, which led to discrepancy with the previous study suggesting that nsP3 is the viral target of this compound. This again illustrates that it should become the norm that antiviral activity and mode of action of small-molecule inhibitors discovered using computer-aided drug design are validated in cell-based assays.

Nonstructural protein 4 (nsP4)

The nsP4 (611 aa) is the most conserved protein in the alphavirus family that functions as an RNA-dependent RNA polymerase (RdRp) responsible for replication of 49S genomic (+) strand RNA and transcription of the 26S sgRNA. In the early phase of the replication cycle, nsP4 together with P123 is part of an early replication complex (RC) that is responsible for the synthesis of full-length (-) strand RNA that will serve as template for synthesis of genomic and sgRNA. Following polyprotein processing, the late RC consisting of fully cleaved nsP1-4, mediates the synthesis of genomic RNA and 26S sgRNA (91). NsP4 also possesses a terminal adenylyltransferase (TATase) activity that catalyses the addition of a poly-A tail to the 3' end of the genome (92). In addition, nsP4 contains the signature GDD motif of the RdRp's catalytic core and mutation of both aspartate residues to alanine results in a complete loss of TATase activity (92).

Many RdRp inhibitors are nucleoside analogues. Because RdRp activity is absent from host cells, it represents a suitable target for development of antiviral agents (Table 2). Sofosbuvir is a uridine analogue that is clinically approved for the treatment of hepatitis C virus infection. It is administered as a uridine monophosphate

(UMP) prodrug that needs to be metabolized to yield the pharmacologically active compound sofosbuvir triphosphate (93). nsP4 is the predicted target of sofosbuvir in CHIKV-infected cells, based on molecular docking of the compound on the putative CHIKV nsP4 model. Treatment of hepatoma cells with sofosbuvir decreased CHIKV replication *in vitro* and its administration to infected adult Swiss mice resulted in reduced arthralgia-related paw inflammation (94).

β-D-N^4-hydroxycytidine (NHC) is a nucleoside analogue that inhibits CHIKV replication after it is converted to its active form NHC triphosphate. However, a direct relationship between NHC and its mode of action in CHIKV-infected cells has not yet been established. It was proposed that NHC may interfere with CHIKV replication through chain termination or mutagenesis (95). Studies with Venezuelan equine encephalitis virus, a New World alphavirus, demonstrated that resistance to NHC develops very inefficiently and is determined by a synergistic effect of multiple mutations in nsP4. Especially three nsP4-specific mutations, namely P187S, A189V and I190T, located in the index finger domain of the predicted nsP4 structure, are thought to be responsible for resistance to NHC. Interestingly, the NHC-resistant phenotype can revert back to wild-type after incorporation of the A201V mutation in nsP4, which has a negative effect on viral resistance to NHC (96).

Initially developed as an anti-influenza inhibitor, favipiravir (T-705) is a broad-spectrum nucleoside analogue that has also shown inhibitory activity against CHIKV (97). All of the favipiravir-resistant variants acquired the unique K291R mutation in nsP4, which is a highly conserved residue in the F1 motif of the RdRps of (+) strand RNA viruses (97). In addition, treatment with favipiravir reduced viral loads in the brain of infected AG129 mice and protected them from severe neurological disease. Favipiravir was also tested in immunocompetent C57BL/6J mice during the acute and chronic phase of CHIKV infection. Treatment with favipiravir during the acute phase rendered viral RNA, viral antigens and infectious particles undetectable. However, such a reduction was not observed upon favipiravir treatment during the chronic phase (98). Given that full-length CHIKV RNA could not be recovered from chronically-infected mice, it suggests that the viral RNA can be defective and unable to form infectious particles, corroborating earlier findings from studies with patient material.

As discussed above, ribavirin can block CHIKV replication by at least two different mechanisms, one of which is through RdRp inhibition. CHIKV passaging in the presence of ribavirin yielded a high-fidelity mutant containing a C483Y mutation in nsP4 (99). Interestingly, this CHIKV mutant generated populations with restricted genetic

diversity, which appears to result from a novel mechanism that differs from those typical for nucleoside analogues, such as chain termination or lethal mutagenesis. The impact of resistance mutations on the 3-D structure of nsP4 was based on homology modelling, since a crystal structure of the CHIKV RdRp is not yet available.

Despite the majority of nsP4-targeting compounds belonging to well-known classes of nucleoside analogues, compound screens have identified other potential drug candidates. For example, an HTS of advanced clinical candidates identified digoxin, a cardiac glycoside that antagonizes the sodium-potassium ATPase, as a potent CHIKV inhibitor. The V209I mutation in nsP4, situated in a well-conserved region of nsP4, was found to play a pivotal role in the development of digoxin-mediated resistance (100). Cytoplasmic proteins involved in CHIKV replication could also be targeted due to their direct interaction with CHIKV proteins. Hsp90 proteins are host proteins that serve as molecular chaperones with a wide array of functions. The cytoplasmic subunit HsP90α was shown to be the predominant interacting partner of nsP4 in co-immunoprecipitation experiments. Hsp90 proteins seem to play a role in stabilizing RCs during alphaviral infections. Hsp90 inhibitors HS-10 and SNX-2112 inhibited CHIKV replication in cell culture and they reduced CHIKV-induced joint swelling and viral load in infected mice (101). Direct evidence leading to nsP4 being the viral target of these compounds is however still lacking.

Inhibitors of CHIKV replication with an undefined target

6-azauridine is a broad-spectrum nucleoside analogue that has been widely used in patients for other indications. Its metabolite 6-azauridine 5'monophosphate was reported to inhibit the replication of several DNA and RNA viruses via targeting of the host orotidylic acid decarboxylase (102). Others have proposed a different mechanism of action, based on the interference with cellular UTP metabolism, leading to the so-called 'error' catastrophe (103). Multiple studies have demonstrated that 6-azauridine potently inhibits CHIKV replication, but its viral target has not been determined (104). A chemical library has identified a novel potent broad-spectrum small-molecule inhibitor RYL-634 with antiviral activity against many pathogenic viruses, including CHIKV. Dihydroorotate dehydrogenase (DHODH) was validated as the target enzyme of RYL-634 using activity-based protein profiling (105).

Atovaquone is a ubiquinone analogue and a well-known antimalarial and antiparasitic drug. Previous studies indicated that it can function through the inhibition of mitochondrial function or DHODH, the latter being required for the *de novo*

pyrimidine synthesis. Recently, atovaquone was found to inhibit CHIKV replication, but the mechanism underlying its precise mode of action remains unstudied. For Zika virus it was shown that atovaquone blocks DHODH and thereby leads to the depletion of intracellular nucleotide pools (106).

Another HTS campaign identified berberine, ivermectin and abamectin as strong inhibitors of CHIKV replication. Ivermectin and abamectin are broad-spectrum anti-parasitic drugs used for the treatment of humans and agricultural crops, respectively. Berberine possess antimicrobial properties and it has been tested for antiviral activity against a range of viruses, including herpes simplex virus, influenza virus and cytomegalovirus (107). Further elucidation of its anti-CHIKV effect showed that berberine impairs mitogen-activated protein kinase signalling pathways, although the specific molecular target of berberine remains unknown. Another study found that berberine affects post-replications steps in the CHIKV replication cycle by targeting interactions between genomic RNA and C protein that are required for correct nucleocapsid assembly (108). Moreover, treatment of CHIKV-infected C57BL6/J mice with berberine alleviated the symptoms of CHIKV-induced inflammatory disease (109).

Harringtonine, a cephalotaxine alkaloid, was discovered by screening a natural product compound library and found to also potently inhibit CHIKV replication. This compound acts on the post-entry stage of the CHIKV replication cycle and strongly interferes with CHIKV protein synthesis. It was postulated that harringtonine inhibits the host cell translation machinery and thereby leads to the suppression of translation of CHIKV nsPs and structural proteins (110). Silymarin is a flavonoid with anti-CHIKV activity that was also found to exert its antiviral activity at the post-entry stage (111). Imipramine is an FDA-approved antidepressant that exerts its antiviral effects at two distinct stages in CHIKV replication, the fusion/entry step and a post-fusion replication step. Because optimal fusion reactions and intracellular replication are both dependent on cholesterol, these processes are highly susceptible to imipramine, a class II cationic amphiphilic drug targeting cholesterol trafficking pathway (112). Tomatidine is a natural steroidal alkaloid that interferes with post-entry steps in the CHIKV replication cycle (113). Silvestrol is a natural compound that belongs to the flavaglines and is a specific inhibitor of the host RNA helicase eIF4A, which is part of the translation initiation complex. Silvestrol treatment of CHIKV-infected cells delayed the translation of viral proteins and prevented the host transcriptional shut-off (114). Andrographolide, a bicyclic diterpenoid lactone (115), micafungin, an antifungal agent (116), and MBZM-N-IBT (117) all inhibit CHIKV replication but their mechanism of action remains unknown.

Are drug-resistant mutants attenuated in mosquitoes?

Compared to single-host RNA viruses, the alternating use of insect and mammalian hosts restricts arbovirus adaptation to environmental pressures like treatment with antiviral compounds. Selection and fitness of new variants is influenced by replication competence in both vertebrate and invertebrate hosts. To date, only a few studies have specifically assessed the fitness of drug-resistant mutants in mosquitoes. Such information would be valuable for selecting an antiviral agent with minimal risk of inducing and spreading drug resistance in the environment. For example, a high-fidelity ribavirin-resistant variant containing the C483Y mutation in nsP4 had lower fitness in *Ae. aegypti* mosquitoes (99). Likewise, the favipiravir-resistant mutant carrying the K291R mutation in nsP4 also disseminated poorly in the bodies of *Ae. aegypti* mosquitoes and showed decreased transmission potential, while the MATDP-resistant mutant with the P34S mutation in nsP1 showed the same transmission efficiency as wild-type virus (118). Furthermore, a DFMO-resistant triple mutant carrying the G230R and V326M mutations in nsP1 and the nsP3-opal524R mutation replicated to higher titers in *Ae. albopictus* mosquitoes compared to wild-type virus (61). These studies indicate that drug-resistant mutants can have quite different phenotypes in mosquitoes and emphasize the need to determine their transmission potential for those antiviral drugs intended for clinical use.

How choice of cell line in CHIKV antiviral drug discovery can affect outcome

Vertebrate cells such as BHK-21 (baby hamster kidney) cells or Vero E6 (African green monkey kidney) cells are the most widely used cell lines for CHIKV antiviral drug screening. Fibroblast cell lines, such as MRC-5 (human lung fibroblast) or HFF-1 (human foreskin fibroblast) have also been used in multiple studies. Less frequently used cell lines include immortalized cells such as HeLa (human cervical carcinoma), Huh-7 or HepG2 (human hepatocellular carcinoma) and U2OS (human osteosarcoma). Cell lines usually vary with respect to drug uptake and intracellular metabolism. Therefore, it is anticipated that compounds that first need to be metabolized into their active form, such as nucleoside analogues, might show cell line-dependent differences in their antiviral activity profile. A study evaluating the anti-CHIKV efficacy of ribavirin and favipiravir, which need to be converted to their active triphosphate forms by host cell kinases, revealed differences in antiviral efficacy depending on the cell line used

for evaluation (119). Similarly, for immunomodulatory agents (not discussed in this review) the correct choice of cell line is especially relevant.

Concluding remarks

The lack of effective control measures, spread of new vectors, increased human travel and urbanization have largely contributed to CHIKV re-emergence between 2004-2020. The origin and the scale of a future chikungunya outbreak is hard to predict, which underscores the importance of developing effective countermeasures. Identifying and developing direct-acting and host-targeting antiviral drug options against CHIKV infection offers a promising approach for limiting viral replication and spread.

The major complaint of patients suffering from CHIKF is debilitating joint and muscle pain, which results in lost productivity and reduced quality of life. Antiviral treatment would represent a suitable measure to prevent and treat CHIKV infections and significantly lower the burden of disease in affected areas. A combination therapy for CHIKF may prove useful to reduce the likelihood of developing drug resistance, given that compounds with different viral/host targets can produce synergistic effects. In addition, chronic CHIKF patients with exacerbated response of their immune system can also be treated with immunomodulatory agents to alleviate joint arthralgia and inflammation.

Validation of CHIKV small-molecule inhibitors is currently performed in a variety of *in vitro* and *in vivo* models. Ideally, *in vivo* antiviral testing is performed in animal models that replicate the clinical course of CHIKV infection in humans. While the use of an immunocompromised acute model, such as AG129 mice, may provide more stringent conditions for antiviral evaluation, the use of an immunocompetent arthralgia model is more clinically relevant. The maximum benefit of an antiviral compound for treatment of CHIKF patients would be achieved by early administration during the acute phase of infection, in order to reduce the viral load and decrease the likelihood of developing chronic manifestations. Clinical studies with patient material have already indicated that residual viral material (RNA/protein) in joint tissue, rather than replicating virus, likely contributes to the immunopathology that is associated with CHIKV infection (120). Consequently, late antiviral treatment, i.e. during the chronic phase of CHIKV infection, targeting specific CHIKV functions and host pathways involved in viral replication would be less effective given the absence or low quantities of full-length viral RNA. This stresses the importance of fully understanding

the fundamental aspects of CHIKV-host interactions in patients with both acute and chronic disease.

In summary, the development of CHIKV small-molecule inhibitors is justified for both prophylactic and therapeutic use. Given the current absence of a vaccine, a clinically approved CHIKV small-molecule inhibitor would be especially advantageous in outbreak containment. Alternatively, it could be prescribed as a form of prophylaxis to local citizens in affected areas or to travellers at-risk.

Acknowledgements

Table 1: Compounds targeting CHIKV entry and egress[a]

| | | | | In vitro efficacy | | | In vivo efficacy | | | |
Compound[b]	Viral target	Resistance mutation(s)	CHIKV strain (genotype)[c]	EC50 (µM) or other readout[d]	CC50 (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model	Reference
obatoclax(R)	E1	L369I (SFV)	LR2006 OPY1 (ECSA)	0.03 ± 0.01	20.1 ± 4.8	BHK-21	-	-	-	(21)
arbidol	E2	G82R	LR2006 OPY1 (ECSA)	12.2 ± 2.2	376	MRC5	-	-	-	(22)
suramin(R)	E2	N5R, H18Q	CHIKV-LS3	79 ± 11.6	> 1,000	VeroE6	0611aTw, 0810bTw, 0706aTw (Asian)	reduced viral load, foot swelling and histopathologic lesions	C57BL/6	(25, 28, 29)
picolinic acid	C	-	DRDE-07 (ECSA)	60% inhibition with 2 mM dose	n.s.	Vero	-	-	-	(30)
amantadine	6k	-	S27 (ECSA)	29.5	>200	Vero	-	-	-	(32)
chloroquine(R)	-	-	DRDE-06 (ECSA)	7.0 ± 1.5	> 260	Vero	-	-	-	(38)
doxycycline(R)	-	-	n.s. (ECSA)	10.95 ± 2.12	>100	Vero	061573 (ECSA)	no significant reduction in viral titre or pathology	ICR	(39)

Compound[b]	Viral target	Resistance mutation(s)	CHIKV strain (genotype)[c]	In vitro efficacy			In vivo efficacy			Reference
				EC$_{50}$ (μM) or other readout[d]	CC$_{50}$ (μM)[a]	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model	
curcumin	-	-	LR06-049 (ECSA)	3.89	11.6	HeLa	-	-	-	(41)
niclosamide	-	-	S27 (ECSA)	0.95 ± 0.22	> 20	BHK-21	-	-	-	(42)
nitazoxanide	-	-	S27 (ECSA)	2.96 ± 0.18	> 25	BHK-21	-	-	-	(42)
apigenin	-	-	LR2006 OPY1 (ECSA)	70.8	> 200	BHK-21	-	-	-	(43)
FL3	-	-	clinical isolate (ECSA)	0.0224	0.119	HEK-293T	-	-	-	(44)

aCC$_{50}$, 50% cytotoxic concentration; EC$_{50}$, 50% effective concentration; n.s. – not specified;- not determined/not done (in vivo studies); R – repurposed compound. The numbering of mutations that provide resistance is based on the CHIKV genome sequence of the strain indicated in the table, unless indicated otherwise.
bIf the study described a family/class of compounds with antiviral activity, only the antiviral activity of the most potent and/or the most representative compound is reported.
cOnly compounds for which the antiviral activity was tested using infectious CHIKV are included; compounds identified using only replicon/surrogate systems for which confirmatory experiments with infectious CHIKV were lacking are excluded.
dWhere a compound showed activity in multiple cell lines and against multiple CHIKV strains, the best value (with corresponding cell line) is reported.

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Table 2: Compounds targeting CHIKV replication[a]

Compound[b]	Viral target	Resistance mutation(s)	CHIKV strain (genotype)[c]	EC$_{50}$ (µM) or other readout[d]	CC$_{50}$ (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model	Reference
				In vitro efficacy			**In vivo efficacy**			
MADTP-314 (N) (DA)	nsP1	P34S	IO 899 (ECSA)	26 ± 11	> 743	Vero	-	-	-	(53-55)
CHVB-032 (N) (DA)	nsP1	S454G, W456R	IO 899 (ECSA)	2.7	> 75	Vero	-	-	-	(56, 57)
6'-β-fluoro-homoaristeromycin (N, NA) (DA)	nsP1	G230R, K299E	CHIKV-LS3	0.12 ± 0.04	> 250	VeroE6	-	-	-	(58, 59)
6'-fluoro-homoneplanocin A (N, NA) (DA)	nsP1	G230R, K299E	CHIKV-LS3	0.18 ± 0.11	> 250	VeroE6	-	-	-	(59)
difluoromethylornithine (R) (HT)	nsP1	G230R, V326M (nsP1) & *524R (nsP3)	LR06-049 (ECSA)	3 log$_{10}$ reduction in titre with 500 µM dose	> 500	BHK-21	LR06-049 (ECSA)	modest reduction in CHIKV titres	C57BL/6	(60, 61, 121)
mycophenolic acid (R) (HT)	nsP1	S23N, V302M (SINV)	DRDE-06 (ECSA)	0.21 ±0.06	30 ± 3.1	Vero	-	-	-	(62, 63, 65)

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Compound[b]	Viral target	Resistance mutation(s)	CHIKV strain (genotype)[c]	In vitro efficacy			In vivo efficacy			Reference
				EC_{50} (µM) or other readout[d]	CC_{50} (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model	
ribaviri (NA) (HT/DA)	nsP1/ nsP4	(Q21K), S23N, V302M (SINV)/ C483Y (CHIKV)	Ross C347 (ECSA)	341.1	>30,000	Vero	-	-	-	(63, 99, 104)
sofosbuvir (NA) (DA)	nsP4	-	n.s. (Asian)	2.7 ± 0.5	402 ± 32	Huh-7	n.s.	reduced CHIKV-induced oedema and viral replication	Swiss Webster mice arthralgia model	(94)
β-D-N4-hydroxycytidine (NA) (DA)	nsP4	P187S, A189V, I190T (VEEV)	LR2006 OPY1 (ECSA)	0.2 ± 0.1	7.7	Vero	-	-	-	(95, 96)
favipiravir (NA) (DA)	nsP4	K291R	IO 899 (ECSA)	25 ± 3	> 636	Vero-A	S27 (ECSA)	reduced mortality by >50% and improved disease outcome	AG129 lethal model	(97)

				In vitro efficacy			In vivo efficacy			
Compound[b]	Viral target	Resistance mutation(s)	CHIKV strain (genotype)[c]	EC$_{50}$ (µM) or other readout[d]	CC$_{50}$ (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model	Reference
							IO 899 (ECSA)	reduced viral replication in joints and extremities during acute phase	C57BL/6J	(98)
digoxin (R) (HT)	nsP4	V209I	SL15649 (ECSA)	0.048	> 10	U2OS	-	-	-	(100)
HS-10 (HT)	nsP4	-	Ross (ECSA)	> 2 log$_{10}$ reduction in titre with 6.25 µM dose	> 100	HEK-293T	DMERIO9/08 (ECSA)	reduced inflammation and viremia	SvA129	(101)
SNX-2112 (HT)	nsP4	-	Ross (ECSA)	> 2 log$_{10}$ reduction in titre with 6.25 µM dose	> 100	HEK-293T	DMERIO9/08 (ECSA)	reduced inflammation and viremia	SvA129	(101)

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| | | | | In vitro efficacy | | | In vivo efficacy | | | |
Compound[b]	Viral target	Resistance mutation(s)	CHIKV strain (genotype)[c]	EC$_{50}$ (µM) or other readout[d]	CC$_{50}$ (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model	Reference
6-azauridine (R)(NA) (HT/DA)	-	-	Ross C347 (ECSA)	0.8	208	Vero	-	-	-	(104)
RYL-634	-	-	n.s.	0.26	> 2.5	Vero	-	-	-	(105)
atovaquone (R)	-	-	LR06-049 (ECSA)	< 0.75	> 11.25	Vero	-	-	-	(106)
berberine	-	-	LR2006 OPY1 (ECSA)	> 5 log$_{10}$ reduction in titre with 3 µM dose	> 100	BHK-21	LR2006 OPY1 (ECSA)	reduced inflammation and joint swelling	C57BL/6J	(107, 109)
ivermectin (R)	-	-	LR2006 OPY1 (ECSA)	> 4 log$_{10}$ reduction in titre with 3 µM dose	37.9 ± 7.6	BHK-21	-	-	-	(107)
abamectin (R)	-	-	LR2006 OPY1 (ECSA)	> 4 log$_{10}$ reduction in titre with 3 µM dose	28.2 ± 1.1	BHK-21	-	-	-	(107)
harringtonine	-	-	0708 (ECSA)	0.24	> 100	BHK-21	-	-	-	(110)
silymarin	-	-	MY/065/08/ FN295485 (ECSA)	35	881	Vero	-	-	-	(111)

Compound[b]	Viral target	Resistance mutation(s)	CHIKV strain (genotype)[c]	In vitro efficacy				In vivo efficacy			Reference
				EC$_{50}$ (µM) or other readout[d]	CC$_{50}$ (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model		
andrographolide	-	-	0708 (ECSA)	77.39	1,098	HepG2	-	-	-		(115)
micafungin (R)	-	-	S27 (ECSA)	20.6 ± 1.7	> 100	U2OS	-	-	-		(116)
MBZM-N-IBT	-	-	S27 (ECSA)	38.7	> 800	Vero	-	-	-		(117)
imipramine (R)	-	-	LR2006 OPY1 (ECSA)	3 log$_{10}$ reduction in titre with 75 µM dose	> 100	HFF1	-	-	-		(112)
tomatidine	-	-	LR2006 OPY1 (ECSA)	1.3	156	Huh-7	-	-	-		(113)
silvestrol (HT)	-	-	IO 899 (ECSA)	0.00189	> 0.03	HEK-293T	-	-	-		(114)

[a]n.s. – not specified;- not determined/not done (in vivo studies); N, novel; NA, nucleoside analogue; R, repurposed compound; VEEV, Venezuelan equine encephalitis virus. The numbering of mutations that provide resistance is based on the CHIKV genome of the strain indicated in the table, unless indicated otherwise. DA, direct-acting compounds; HT, host-targeting compounds; HT/DA, both host-targeting and direct-acting compounds.

[b]If the study described a family/class of compounds with antiviral activity, only the antiviral activity of the most potent and or the most representative compound is reported.

[c]Only compounds for which the antiviral activity was tested using infectious CHIKV are included; compounds identified using only replicon/ surrogate systems for which confirmatory experiments with infectious CHIKV were lacking were excluded.

[d]Where a compound showed activity in multiple cell lines and against multiple CHIKV strains, the best value (with corresponding cell line) is reported.

Table 3: CHIKV inhibitors identified by in silico approaches (molecular docking, homology modelling, pharmacophore modelling)

Compound	Viral target	Resistance mutation(s)	Confirmed in enzymatic assays?	Confirmed in infected cells?	In vitro efficacy				In vivo efficacy			Reference
					CHIKV strain (genotype)	EC50 (µM) or other readout[b]	CC50 (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model	
25	nsP2	-[a]	no	yes	IO 899 (ECSA)	3.2 ± 1.8	101 ± 50	Vero	-	-	-	(78)
7	nsP2	-	no	yes	LR2006 OPY1 (ECSA)	0.42	>100	Vero	-	-	-	(79)
8	nsP2	-	yes	yes	LR2006 OPY1 (ECSA)	~1.5	>200	BHK-21	-	-	-	(80)
3a	nsP2	-	no	yes	n.s.	8.76 µg/ml	n.s.	Vero	-	-	-	(81)
4b	nsP2	-	no	yes	n.s.	8.94 µg/ml	n.s.	Vero	-	-	-	(81)
baicalin	nsP3	-	no	yes	MY/065/08/ FN295485 (ECSA)	5	>600	Vero	-	-	-	(90)

[a]-, not determined/not done (*in vivo* studies)
[b]Data represent µM unless otherwise indicated.

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Table 4: CHIKV inhibitors identified in in vitro biochemical assay/ assays with purified protein[a]

Compound	Viral target	Resistance mutation(s)	Confirmed in infected cells?	CHIKV strain (genotype)	*In vitro* efficacy				*In vivo* efficacy			Reference
					EC$_{50}$ (µM)	CC$_{50}$ (µM)	Cell line	CHIKV strain (genotype)	Efficacy	Mouse model		
5-iodotubercidin (NA)	nsP1	-	Yes	clinical isolate 119067	0.409	> 50	Vero	-	-	-	-	(66)
lobaric acid	nsP1	-	Yes	LR2006 OPY1 (ECSA)	9.9 ± 2.6	76.3 ± 2.1	BHK	-	-	-	-	(67)
sinefungin	nsP1	-	Yes	CHIKV LS3	184.9 ± 38.4	> 1,000	VeroE6	-	-	-	-	(66, 122) Kovacikova et al., unpublished

[a]For compounds identified using *in silico* approaches, only those compounds for which the antiviral activity was demonstrated in cell culture using infectious CHIKV are reported; all other compounds for which activity was claimed against CHIKV from in silico screens, but for which no activity in cell-based assays was reported, are excluded from this table.-, not determined/not done (*in vivo* studies)

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