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Towards antivirals against chikungunya virus

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ABSTRACT

Chikungunya virus (CHIKV) has re-emerged in recent decades, causing major outbreaks of chikungunya fever in many parts of Africa and Asia, and since the end of 2013 also in Central and South America. Infections are usually associated with a low mortality rate, but can proceed into a painful chronic stage, during which patients may suffer from polyarthralgia and joint stiffness for weeks and even several years. There are no vaccines or antiviral drugs available for the prevention or treatment of CHIKV infections. Current therapy therefore consists solely of the administration of analgesics, antipyretics and anti-inflammatory agents to relieve symptoms. We here review molecules that have been reported to inhibit CHIKV replication, either as direct-acting antivirals, host-targeting drugs or those that act via a yet unknown mechanism. This article forms part of a symposium in *Antiviral Research* on "Chikungunya discovers the New World."

Contents

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1. Introduction

Chikungunya virus (CHIKV) is an alphavirus transmitted mainly by female mosquitoes of the species *Aedes aegypti* and *Aedes albopictus*. It causes an acute disease characterized by fever, arthralgia and in some cases a maculopapular rash (Thiberville et al., 2013). Infection is rarely fatal, but in many cases it evolves into a chronic stage of persistent disabling polyarthritis that can severely incapacitate the patient for weeks and even up to several years (Simon et al., 2011).

There are no vaccines or antivirals available for the prevention or treatment of CHIKV infection. Current therapy consists of the use of analgesics, antipyretics and anti-inflammatory agents, such as paracetamol and non-steroidal anti-inflammatory drugs

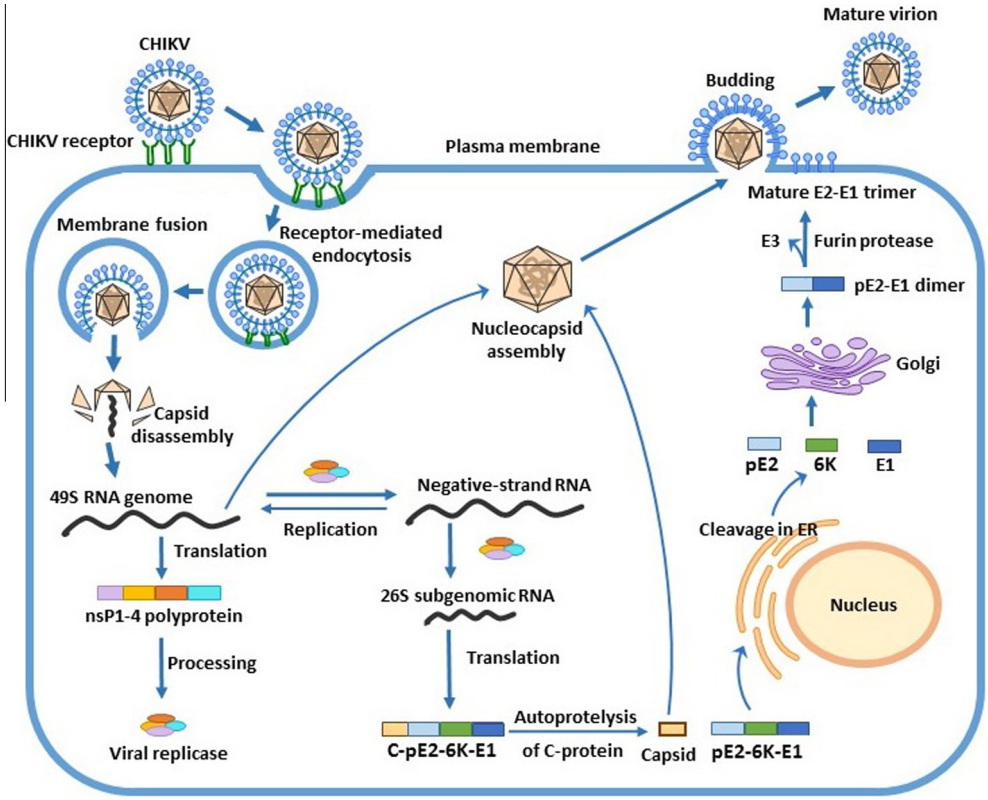

Fig. 1. Schematic representation of the replication cycle of chikungunya virus. CHIKV enters the cell by endocytosis following the binding of the E2 protein to specific receptor(s) on the cell surface. Within the endosome, the low pH triggers the fusion of the viral envelope with the endosomal membrane, leading to the release of the nucleocapsid into the cytoplasm. The nucleocapsid disassembles to liberate the viral genome, which is translated to produce the viral nonstructural proteins (nsP1–4). After processing, the nonstructural proteins complex to form the viral replicase, which catalyzes the synthesis of a negative-sense RNA strand to serve as a template for synthesis of both the full-length positive-sense genome and the subgenomic (26S) RNA. The subgenomic (26S) RNA is translated to produce the structural polyprotein (C-E3-E2-6K-E1), which is then cleaved to produce the individual structural proteins, followed by assembly of the viral components. The assembled virus particle is released by budding through the plasma membrane, where it acquires the envelope with embedded viral glycoproteins.

(NSAIDs) (Thiberville et al., 2013). Aspirin should be avoided because of the risk for bleeding or the potential risk of the development of Reye's syndrome (Kucharz and Cebula-Byrska, 2012). In addition, disease-modifying antirheumatic drugs (DMARDs) such as methotrexate and sulphasalazine could be used in severe cases when NSAIDs are not effective (Ali Ou Alla and Combe, 2011; Kucharz and Cebula-Byrska, 2012). Because of the worldwide re-emergence of CHIKV (Weaver and Forrester, 2015), the development of potent antiviral drugs is urgently needed.

2. Replication cycle of CHIKV

Similar to other alphaviruses, the entry of CHIKV into the host cell is facilitated by the interaction of the E2 envelope glycoprotein with receptors on the surface of the target cells (Fig. 1). Upon receptor binding, the virus is rapidly internalized through endocytosis. Within the endosome, conformational changes take place in the viral envelope glycoproteins due to the low pH environment, allowing the fusion between the E1 envelope glycoprotein and the endosomal membrane (Gould et al., 2010). This results in the release of the viral nucleocapsid into the cytoplasm where it is disassembled to release the viral RNA genome. Consequently, the viral genome is translated by the host cell machinery to generate the non-structural polyprotein which is cleaved to yield the nsP123 precursor and free nsP4 protein. The nsP123 precursor interacts with nsP4 and host proteins to form an initial replication complex (viral replicase) which synthesizes the negative-strand RNA (Solignat et al., 2009). The negative-strand RNA is then used as a template to synthesize positive-strand genomic RNA and subgenomic RNA (26S RNA). The 26S RNA serves as the mRNA for the synthesis of the polypeptide containing the structural viral proteins C-pE2-6K-E1 (Gould et al., 2010).

The capsid protein (C) is then released from this polypeptide by its autoprotease activity, whereas the remaining pE2-6K-E1 polypeptide is processed in the endoplasmic reticulum (ER). The pE2 and E1 glycoproteins form heterodimer complexes that migrate towards the cell membrane through the Golgi complex. During this migration towards the cell surface, pE2 is cleaved by a cellular furin or furin-like proteinases to form mature E2 and E3. Finally, the nucleocapsid complexes assemble in the cytoplasm and bud through the cell membrane acquiring a lipid bilayer envelope that contains the virus-encoded glycoprotein E1-E2 (Gould et al., 2010). Several steps of the viral life cycle can be targeted to inhibit CHIKV replication.

3. Methods for the *in vitro* and *in vivo* evaluation of antiviral compounds

3.1. Viruses

3.1.1. Bio-safe viruses

Bio-safe surrogates for CHIKV can be used to identify antiviral molecules in order to avoid the need for BSL-3 facilities. These include for example BHK replicon cell lines containing a persistently replicating CHIKV replicon (Pohjala et al., 2011) and a Semliki Forest virus (SFV) strain with *Renilla* luciferase (Pohjala et al., 2011). However, using a replicon model in which only viral replication occurs, involves the risk of not identifying molecules that inhibit other steps of the viral life cycle, such as virion entry and release. CHIKV pseudoparticles carrying the envelope proteins and tagged with a luciferase reporter (Selvarajah et al., 2013; Weber et al., 2015) and a heat-sensitive SFV strain (SFVts9-Rluc), characterized by severe defects in RNA replication at an elevated temperature (Pohjala et al., 2011), could be used to evaluate the effect of antiviral agents on CHIKV entry.

3.1.2. Infectious viruses

To identify molecules with anti-CHIKV activity, the best option is to use infectious viruses that have a complete life cycle. Clinical isolates that have been used for antiviral screening include CHIKV-0708 (GenBank: FJ513654) (Kaur and Chu, 2013; Lam et al., 2012), the DRDE-06 strain (GenBank: EF210157) (Khan et al., 2011) and DMERI09/08 strain (Rathore et al., 2014). Also laboratory CHIKV strains such as ROSS (Briolant et al., 2004; Lam et al., 2012; Rathore et al., 2014), LR2006_OPY1 (GenBank: DQ443544.2) (Jadav et al., 2015) and the Indian Ocean strain 899 strain (GenBank: FJ959103.1) (Delang et al., 2014) were used when identifying inhibitors of CHIKV replication. The CHIKV-122508 isolate (GenBank: FJ445502.2) which contains the A226V mutation in the E1 protein has also been used to evaluate the efficacy of antiviral compounds (Gupta et al., 2014; Kaur and Chu, 2013). Recombinant CHIKV with the green fluorescence protein (CHIKV-118-GFP) (Cruz et al., 2013) or a luciferase gene (Pohjala et al., 2011) could provide an easy read-out for the evaluation of antiviral activity.

3.2. Methods to evaluate in vitro antiviral activity

Cell viability/cytopathogenic effect (CPE) reduction assays are usually employed for the initial identification of molecules with activity against CHIKV (Bassetto et al., 2013; Bourjot et al., 2014; Cruz et al., 2013; Delang et al., 2014; Jadav et al., 2015). The advantage of this type of cell-based assay is the possibility of discovering/identifying new antiviral targets. It also allows evaluation of the cytotoxic effect of putative antiviral molecules.

African green monkey kidney (Vero) cells are the most commonly used cells in these assays. Other cell types used for screening for CHIKV antivirals include baby hamster kidney (BHK) cells, human fetal lung fibroblast (MRC-5) cells, human embryonic kidney 293 (HEK-239T) cells, bronchial epithelial cells, and human hepatocarcinoma (HuH-7) cells. These commonly used cell lines have however no clinical relevance in CHIKV disease. Human muscle satellite cells (Ozden et al., 2007) or macrophages may be more relevant cell lines, but they are not suitable for high-throughput screening campaigns. Virus-induced CPE can be scored microscopically and/or quantitatively measured by colorimetric assays such as resazurin fluorescence reduction assay (Cruz et al., 2013; Jadav et al., 2015), the MTS/PMS method (Delang et al., 2014) or neutral red dye uptake (Khan et al., 2011).

3.3. Animal models

Several animal models have been developed to study the pathogenesis of CHIKV infection. Lethal infection models using adult immunodeficient mice including AG129 (Delang et al., 2014; Fric et al., 2013) and *Ifnar*$^{-/-}$ mice (Pal et al., 2013) have been used to assess the antiviral efficacy of small molecules and monoclonal antibodies against CHIKV-induced death. On the other hand, immunocompetent mice such as C57BL/6 (Goh et al., 2013; Lam et al., 2012; Parashar et al., 2013; Selvarajah et al., 2013) and Swiss albino mice (Parashar et al., 2013) are non-lethal infection models that can be used to assess the efficacy of drug therapy against CHIKV-induced arthritis and inflammation.

4. Direct-acting antivirals

4.1. Inhibitors of CHIKV entry

4.1.1. Chloroquine

Chloroquine, an antimalarial drug, has *in vitro* antiviral activity against a number of viruses, including HIV, severe acute

Table 1
Direct-acting antivirals against chikungunya virus.

Antiviral agent	Mechanism of action	*In vitro* efficacy	*In vivo* efficacy	References
Chloroquine	Inhibition of fusion of the viral E1 protein with the endosomal membrane by raising the endosomal pH	Inhibition of CHIKV infection in Vero A cells	No significant efficacy in a macaque model or clinical trials in CHIKV infected patients	Chopra et al. (2014), Khan et al. (2010) and Roques et al. (2007)
Arbidol	Interference with the binding of CHIKV to host receptors and alteration of cellular membranes	Inhibition of CHIKV infection in MRC-5 cells (EC50 = 12 μM)	Not determined	Delogu et al. (2011)
Flavaglines	Interference with the binding of CHIKV Prohibitin-1	Moderate antiviral effect on CHIKV replication in HEK293T/17 cells (EC50 of FL3 = 22.4 nM)	Not determined	Wintachai et al. (2015)
SiRNAs targeting nsP1, E2	Inhibition of protein synthesis	Inhibition of CHIKV replication in Vero-E6 cells (>90%)	Complete inhibition of CHIKV replication in infected Swiss albino and C57 BL/6 mice when administered 3 days post-infection	Parashar et al. (2013)
Harringtonine and homoharringtonine	Inhibition of protein synthesis	Inhibition of CHIKV replication in BHK21 cells (EC50 = 0.24 μM)	Not determined	Kaur et al. (2013)
Arylalkylidene derivatives of 1,3-thiazolidin-4-one	Inhibition of CHIKV nsP2 protease activity	Inhibition of CHIKV replication in Vero A cells (EC50 of the best compound = 0.42 μM)	Not determined	Jadav et al. (2015)
Ribavirin	Inhibition of viral genome replication, mostly via GTP pools depletion	Inhibition of CHIKV replication in Vero cells (EC50 = 341 μM). Synergistic inhibitory effect in combination with IFN-α2b and doxycycline	Reduced the viral load and inflammation in infected ICR mice when combined with doxycycline	Briolant et al. (2004) and Rothan et al. (2015)
6-Azauridine	Inhibition of orotidine monophosphate decarboxylase enzyme (depletion of UTP pools)	Inhibition of CHIKV replication in Vero cells (EC50 = 0.82 μM)	Not determined	Briolant et al. (2004)
Favipiravir (T-705)	Inhibition of viral genome replication	Inhibition of CHIKV-induced CPE in Vero A cells (EC50 = 25 μM)	Reduction of the mortality rate in infected AG129 mice with >50% and protection from neurological disease	Delang et al. (2014)
Monoclonal antibody C9	Interaction with CHIKV E2 glycoprotein	Neutralization of CHIKV pseudovirions in HEK293T cells and replication-competent CHIKV in Vero cells	As prophylaxis: complete protection of infected C57BL/6 mice from arthritis and viremia As therapy: 100% survival of CHIKV infected mice when given at 8 or 18 h post infection	Selvarajah et al. (2013)

respiratory syndrome (SARS) coronavirus and alphaviruses (Khan et al., 2010). Chloroquine inhibits CHIKV replication in Vero A cells in a dose-dependent manner (Khan et al., 2010) and is believed to interfere with the endosome-mediated CHIKV internalization by raising the endosomal pH, thereby preventing the E1 fusion step (Bernard et al., 2010). However, clinical trials of chloroquine in CHIKV-infected patients could not prove its efficacy for treatment of CHIKV infection. Experiments in a macaque model also failed to demonstrate an antiviral effect of chloroquine (Roques et al., 2007). Furthermore, in a more recent study performed in India no benefit of chloroquine treatment over meloxicam (an NSAID) was observed in the treatment of early musculoskeletal pain and arthritis following acute CHIKV infection (Chopra et al., 2014). This divergence between the *in vitro* and *in vivo* antiviral efficacy of chloroquine was also reported for other viruses such as influenza (Paton et al., 2011) and Ebola virus (Falzarano et al., 2015). Similarly, clinical trials of chloroquine against influenza virus and Ebola virus failed to prove its effectiveness, despite significant *in vitro* activity against these viruses.

4.1.2. Arbidol

Arbidol is a broad-spectrum antiviral that was approved in Russia and China for the treatment and prophylaxis of influenza and other respiratory infections (Blaising et al., 2014). Arbidol was reported to inhibit CHIKV infection in MRC-5 cells with an EC_{50} of 12 μM (Delogu et al., 2011). Two analogues of arbidol were recently identified that had a somewhat higher selectivity index for inhibition of *in vitro* replication of CHIKV (Di Mola et al., 2014). An arbidol-resistant mutant strain was identified with a mutation of a glycine to an arginine (G407R) in the CHIKV E2 glycoprotein (Delogu et al., 2011). This amino acid position may be involved in binding to host receptors. The compound has been shown to inhibit CHIKV hemagglutination, suggesting that arbidol interferes with the replication cycle at the cell adsorption step (Delogu et al., 2011). Arbidol could also be incorporated into cellular membranes leading to alterations of the membrane structure and thereby interfering with stages of the virus life cycle that are membrane-dependent such as fusion with the endosomal membrane (Blaising et al., 2014) (Table 1).

4.1.3. Phenothiazines

Using a novel virus entry assay for the identification of alphavirus entry inhibitors, six compounds with a 10H-phenothiazine core, including chlorpromazine, perphenazine, ethopropazine, thiethylperazine, thioridazine and methdilazine were identified as possible alphavirus entry inhibitors. A heat-sensitive mutant of Semliki Forest virus (SFVts9-Rluc) was used as a bio-safe surrogate for CHIKV. The anti-CHIKV activity of the identified molecules was then confirmed using a recombinant strain carrying a luciferase reporter gene (CHIKV-Rluc). The precise molecular mechanism by which these compounds inhibit viral entry has yet to be elucidated (Pohjala et al., 2011).

4.1.4. Epigallocatechin gallate (green tea component)

Epigallocatechin gallate (EGCG) is the major constituent of green tea extract. EGCG exerts *in vitro* antiviral activity against several viruses, including HIV, influenza and hepatitis C virus. Recently, EGCG was reported to inhibit *in vitro* CHIKV replication (Weber et al., 2015). The compound was shown to inhibit the entry

of CHIKV pseudoparticles (carrying the CHIKV envelope proteins) into the target cell.

4.1.5. Flavaglines

Flavaglines are a family of natural products that were shown to have anticancer and cardio- and neuroprotective properties (Ribeiro et al., 2012). Moreover, flavaglines also target prohibitins (Polier et al., 2012). Prohibitin-1 was previously identified as a receptor for CHIKV entry into mammalian cells (Wintachai et al., 2012). The synthetic flavaglines sulfonyl amidine 1 m, FL3 and FL23 exert a moderate antiviral effect on CHIKV replication in HEK293T/17 cells (Wintachai et al., 2015). Two compounds (1 m and FL23) were suggested to inhibit an entry step, whereas FL3 could act at a post-entry stage. The co-localization of prohibitin-1 and the CHIKV E2 glycoprotein was markedly reduced in the presence of flavaglines, which may suggest an effect on receptor binding of the virus (Wintachai et al., 2015) (Table 1).

4.2. Inhibitors of viral protein synthesis

4.2.1. RNA interference targeting CHIKV genes

Small interfering RNA (siRNA) sequences targeting the CHIKV nsP3 and E1 genes were designed and evaluated against CHIKV in Vero cells (Dash et al., 2008). These siRNAs were shown to reduce CHIKV titers by 99.6% in siRNA-transfected cells at 24 hours postinfection, but this reduction was not sustained at 72 hours (Dash et al., 2008). This is possibly due to susceptibility of siRNAs to intracellular degradation and the rapid replication nature of CHIKV. SiRNAs targeting nsP1, E2 and the combination of both resulted in more than 90% inhibition of CHIKV replication in Vero-E6 cells. Treatment of CHIKV-infected Swiss albino and C57 BL/6 mice with these siRNAs completely inhibited CHIKV replication when administered 72 h post-infection (Parashar et al., 2013) (Table 1).

Short hairpin RNAs (shRNAs) against CHIKV E1 and nsP1 shRNAs resulted in significant and sustained inhibition of infection, whereas shRNA targeting the capsid resulted in a modest inhibitory effect (Lam et al., 2012). Pretreatment of C57BL/6 suckling mice with 60 μg of shRNA E1 completely protected against CHIKV-induced disease. Survival was 100% at 15 days post-infection, compared to 0% survival at 10 days p.i. in controls (Lam et al., 2012). No resistant variants emerged after 50 passages of CHIKV in cells stably expressing the E1 shRNA.

4.2.2. Harringtonine and homoharringtonine

Harringtonine, a cephalotaxine alkaloid derived from *Cephalotaxus harringtonia*, was reported as an *in vitro* inhibitor of CHIKV replication (EC_{50} of 0.24 μM) (Kaur et al., 2013). In addition, homoharringtonine, a more stable analogue of harringtonine that was recently approved by the FDA for the treatment of chronic myeloid leukemia, also inhibited CHIKV replication. The anti-CHIKV activity of harringtonine and homoharringtonine was suggested to be the result of the inhibition of the host cell protein translation machinery (Kaur et al., 2013). Interestingly, harringtonine and homoharringtonine were more potent against a CHIKV strain carrying the E1 A226V mutation than a strain carrying the wild type E1. The mechanism behind this difference has not yet been resolved (Table 1).

4.3. Inhibitors of CHIKV non-structural protein 2

The non-structural protein 2 (nsP2) of CHIKV is multifunctional, with RNA helicase, RNA triphosphatase and nucleoside triphosphatase activity within the N-terminal half and auto-protease activity for processing of the nonstructural viral polyprotein in its C-terminal half (Fros et al., 2013; Solignat et al., 2009). CHIKV nsP2 is also involved in shutting off host cell mRNA transcription and translation. Moreover, CHIKV nsP2 was found to suppress type I/II interferon-stimulated JAK/STAT signaling leading to inhibition of the host antiviral response (Fros et al., 2013). Because of these diverse functions, nsP2 could be a target for CHIKV inhibitors. Especially the protease function of CHIKV nsP2 is of interest, as proteases of other viruses (such as HIV and HCV) were shown before to be excellent targets for the development of antiviral drugs.

A structure-based virtual screening strategy was applied to select for CHIKV nsP2 inhibitors, based on the nsP2 protease binding site. Among the selected hits, one lead compound (compound 1) resulted in inhibition of CHIKV-induced CPE (EC_{50} of 5 μM) in Vero cells (Bassetto et al., 2013). The compound was predicted to bind to the central part of the nsP2 protease active site. In another study, a series of arylalkylidene derivatives of 1,3-thiazolidin-4-one were evaluated for their *in vitro* antiviral activity. Five compounds were shown to inhibit CHIKV in a CPE reduction assay (EC_{50} of the best compound was 0.42 μM) (Table 1). The authors proposed, based on molecular docking studies, that the compounds may target the CHIKV nsP2 protease (Jadav et al., 2015).

To identify inhibitors that target the nsP2-mediated shutoff of the host-cell transcription machinery, a phenotypic assay was employed. In this assay, the expression of a luciferase reporter gene is regulated by the human transcription factor Fos that is fused to the DNA-binding domain of Gal4, a yeast transcription factor (Lucas-Hourani et al., 2012). When CHIKV nsP2 is co-expressed, the Fos-induced expression of luciferase is inhibited. One compound, ID1452-2, was identified which partially blocked the nsP2 activity and significantly inhibited *in vitro* replication (EC_{50} of 31 μM) (Lucas-Hourani et al., 2012).

4.4. Inhibitors of viral genome replication

4.4.1. Ribavirin

Ribavirin is a synthetic guanosine analogue with broad-spectrum antiviral activity. Ribavirin was approved for treatment of infections with the respiratory syncytial virus in infants (Turner et al., 2014) and chronic hepatitis C virus infections in combination with pegylated IFN-α and/or direct-acting antivirals (Pawlotsky, 2014). Ribavirin has also been used off-label for treatment of other viral infections such as Lassa virus infection (Ölschläger et al., 2011). Ribavirin was shown to exert *in vitro* antiviral activity against CHIKV (EC_{50} of 341 μM) and resulted in a synergistic inhibitory effect when combined with IFN-α2b (Briolant et al., 2004). The combination of ribavirin and doxycycline also resulted in a good antiviral effect against CHIKV replication in Vero cells and reduced the viral load and inflammation in infected ICR mice (Rothan et al., 2015) (Table 1).

Several mechanisms of antiviral action have been assigned to ribavirin (Paeshuyse et al., 2011). The major mechanism by which ribavirin inhibits the replication of RNA viruses such as flavi- and paramyxoviruses is the inhibition of inosine monophosphate dehydrogenase enzyme (IMPDH) resulting in the depletion of GTP pools (Leyssen et al., 2006). The other suggested mechanisms of the antiviral activity of ribavirin include the inhibition of viral RNA capping and the induction of an error catastrophe (an increased mutation rate as a result of the incorporation of ribavirin-5′-mono-phosphate by the viral polymerase) (Paeshuyse et al., 2011).

4.4.2. Mycophenolic acid

Mycophenolic acid (MPA) is a non-competitive inhibitor of IMPDH which is widely used as an immunosuppressant to prevent the rejection of transplant organs. MPA was reported to effectively

inhibit CHIKV replication (in Vero cells). MPA is even more potent in depleting intracellular GTP-pools than ribavirin. The anti-CHIKV activity of the compound can be ascribed to this mechanism (Khan et al., 2011).

4.4.3. 6-Azauridine

6-Azauridine is a uridine analogue with broad-spectrum antiviral activity against both DNA and RNA viruses (Rada and Dragún, 1977) and also inhibits *in vitro* CHIKV replication (in Vero cells, EC_{50} of 0.82 μM) (Briolant et al., 2004). The compound, which is a competitive inhibitor of orotidine monophosphate decarboxylase enzyme, depletes intracellular UTP-pools, explaining its activity on rapidly replicating viruses such as CHIKV (Rada and Dragún, 1977) (Table 1).

4.4.4. Favipiravir (T-705)

Favipiravir (T-705) is a broad-spectrum antiviral agent that was recently approved in Japan for the treatment of influenza virus infections. Interestingly, T-705 exerts antiviral activity against several RNA viruses, including Rift valley fever virus (Caroline et al., 2014; Scharton et al., 2014), arenaviruses (Gowen et al., 2013; Mendenhall et al., 2011) and hantaviruses (Safronetz et al., 2013), both in cell culture and in animal models. T-705 has also been shown to be endowed with *in vitro* and *in vivo* antiviral activity against the Ebola virus (Oestereich et al., 2014; Smither et al., 2014). Clinical trials are currently ongoing in Western Africa to evaluate its efficacy in patients (Mentré et al., 2015).

T-705 is converted intracellularly to its ribofuranosyl 5′-triphosphate form, which competitively inhibited the incorporation of ATP and GTP by the viral RNA-dependent RNA polymerase (RdRp) (Furuta et al., 2013). The precise molecular mechanism of the antiviral activity of T-705 has not been totally elucidated yet. The proposed mechanisms of actions of T-705 are (i) chain termination of the nascent viral RNA strand and/or (ii) the induction of lethal mutagenesis in the viral genome (Furuta et al., 2013).

T-705 and its defluorinated analogue, T-1105, inhibited the replication of different laboratory strains and clinical isolates of CHIKV in Vero cells, including a strain isolated from the recent outbreak in the Caribbean region (Delang et al., 2014). In addition, treatment of infected AG129 mice with T-705 (oral dose, 300 mg/kg/day) protected these mice from severe neurological disease and reduced the mortality rate with more than 50%. No effect was observed on the specific infectivity of CHIKV (calculated as the ratio of infectious virus yield $\times 10^{-3}$ to the genome copy number), suggesting that error catastrophe is not underlying the inhibitory effect. Low-level T-705-resistant CHIKV variants were selected that carry a K291R mutation in the F1 motif of the RNA-dependent RNA polymerase. This mutation was shown to be responsible for the phenotypic resistance against T-705. Interestingly, this lysine residue is highly conserved in the polymerase of other +ssRNA viruses, which may provide an explanation for the broad-spectrum antiviral activity of T-705 (Delang et al., 2014) (Table 1).

4.5. Monoclonal antibodies

4.5.1. Human MAbs

Two MAbs, 5F10 and 8B10, were isolated from a patient with a CHIKV infection history and were found to target the viral envelope glycoprotein (Warter et al., 2011). Both 5F10 and 8B10 showed specific neutralization of different strains of CHIKV in a plaque reduction assay (Warter et al., 2011). MAbs 5F10 and 8B10 also protected 100% of infected AG129 mice when given prophylactically (Fric et al., 2013). Post-exposure administration of 5F10 and 8B10 did not protect the infected mice, but resulted in delayed mortality by ten days (Fric et al., 2013). Another monoclonal antibody, MAb C9, was also isolated from a patient that recovered from CHIKV infection. The epitope of MAb C9 was identified in the acid-sensitive region of the CHIKV E2 glycoprotein (Selvarajah et al., 2013). MAb C9 neutralized both CHIKV pseudovirions and replication-competent viruses *in vitro* and completely protected CHIKV infected C57BL/6 mice from arthritis and viremia when given one day before infection. Moreover, treatment with Mab C9 resulted in 100% survival of CHIKV infected mice when given at 8 or 18 h post infection (Selvarajah et al., 2013) (Table 1).

4.5.2. Murine MAbs

In a screening assay of a panel of murine MAbs against CHIKV, four MAbs (CHK-102, CHK-152, CHK-166, and CHK-263) resulted in complete protection of infected *Ifnar*$^{-/-}$ mice from CHIKV-induced mortality when administered prophylactically (Pal et al., 2013). Moreover, therapeutic administration of a single dose of a combination of two of these MAbs protected the infected mice from mortality when administered 60 hours after infection (Pal et al., 2013). The epitopes of these MAbs were localized in the E1 and E2 glycoproteins. The most effective MAb, CHK-152, was humanized and showed similar neutralizing efficacy compared to the murine MAb (Pal et al., 2013).

In another study, a number of MAbs targeting CHIKV E2 glycoprotein were generated in mice and evaluated for their CHIKV-neutralizing activity (Goh et al., 2013). Three of these

Table 2
Host-targeting antivirals against chikungunya virus.

Antiviral agent	Mechanism of action	*In vitro* efficacy	*In vivo* efficacy	References
dec-RVKR-cmk	Inhibition of virus maturation via inhibition of cellular furins	Inhibition of CHIKV infection in human muscle satellite cells	Not determined	Ozden et al. (2008)
Prostratin and TPA	Not determined. Prostratin and TPA are protein kinase C activators	Inhibition of CHIKV-induced CPE in Vero A cells (EC50 = 2.6 μM, EC50 PMA = 0.0029 μM)	Not determined	Bourjot et al. (2012)
Kinase inhibitors	Inhibition of kinases involved in apoptosis	Reduction of the number of apoptotic blebs in infected HuH-7 cells with moderate reduction of infectious virus yield	Not determined	Cruz et al. (2013)
HSP-90 inhibitors (HS-10 and SNX-2112)	Not determined. HSP-90 was found to interact with CHIKV nsP3 and nsP4	Reduction of CHIKV infection in a dose-dependent manner in HEK-293T cells	Reduction of viral titers in the serum of infected SvA129 mice at 48 h post-infection and protection against inflammation	Rathore et al. (2014)
Polyinosinic acid: polycytidylic acid	Stimulation of IFN-α, IFN-β and antiviral genes such as OAS and MxA	Inhibition of CHIKV-induced CPE in human bronchial epithelial cells	Not determined	Li et al. (2012)
RIG-I agonists	Stimulation of immune response	Inhibition of CHIKV-induced CPE in MRC-5 cells and reduction of virus titers	Not determined	Olagnier et al. (2014)

MAbs (91.3A2, 4.6F5 and 4.10C12) showed efficient neutralization of CHIKV in Vero cells. In addition, passive immunization of C57BL/6 mice with MAbs 1.3A2 and 4.6F5 protected from CHIKV-induced arthritis and viremia (Goh et al., 2013). Another murine MAb (CK47) specific for the CHIKV E1 glycoprotein was isolated from a mouse immunized with CHIKV. In cell-based assays, MAb CK47 inhibited the budding and release of CHIKV from infected cells with no effect on virus entry or intracellular replication (Masrinoul et al., 2014).

5. Host-targeting antivirals

5.1. Furin inhibitors

Cellular furins are involved in the processing of the E3E2 viral glycoprotein to produce mature virions. Decanoyl-RVKR -chloromethyl ketone (dec-RVKR-cmk), an irreversible furin inhibitor, inhibited CHIKV infection in human muscle satellite cells (Ozden et al., 2008). The inhibition of furins by dec-RVKR-cmk resulted in the formation of immature viral particles and reduced the viral spreading. Furthermore, the combination of dec-RVKR-cmk and chloroquine resulted in an additive antiviral effect against CHIKV with almost total suppression of virus spread and yield. Pretreatment of cells with dec-RVKR-cmk inhibited viral entry (Ozden et al., 2008).

5.2. Modulators of cellular kinases

5.2.1. Protein kinases C (PKC) activators

The protein kinases C (PKC) are a family of serine-threonine kinases that regulate many cellular functions such as cell cycle control, proliferation, differentiation, metastasis and apoptosis. Prostratin and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) are tigliane diterpenoids with a basic phorbol carbon skeleton (Bourjot et al., 2012). Because of their structural similarity to diacylglycerol, TPA and prostratin act as potent activators of PKC. Prostratin and TPA were previously reported to exert antiviral activity against HIV (McKernan et al., 2012) and to inhibit CHIKV replication in Vero cells (Bourjot et al., 2012) (Table 2). The role of PKC in the CHIKV life cycle is not clear yet.

5.2.2. Kinase inhibitors

Screening of a kinase inhibitor library against *in vitro* CHIKV infection resulted in the identification of six compounds (of which one with a benzofuran core scaffold, one with a pyrrolopyridine scaffold and one with a thiazol-carboxamide scaffold) that reduced the number of apoptotic blebs in infected cells and resulted in a moderate reduction of infectious virus yield. It was proposed that these compounds interfere with CHIKV-induced CPE formation through the inhibition of kinases involved in apoptosis (Cruz et al., 2013) (Table 2).

5.3. HSP-90 inhibitors

Heat shock protein 90 (HSP-90) is a highly abundant molecular chaperone which is involved in many cellular processes and signaling pathways through interaction with its client proteins. There are two main isoforms: stress-induced HSP-90α and constitutively expressed HSP-90β. HSP-90 has been reported to play a key role in the replication of many viruses such as the hepatitis C virus, human cytomegalovirus and influenza virus. The HSP-90 inhibitors HS-10 and SNX-2112 inhibited *in vitro* CHIKV replication in a dose-dependent manner in infected HEK-239T cells (Rathore et al., 2014). Treatment of infected mice (SvA129) with HS-10 and SNX-2112 (dose: 10 mg/kg twice a day) resulted in a significant reduction in the serum viral titers at 48 h post-infection and the treated mice showed no swelling or inflammation during the course of infection.

Using co-immunoprecipitation and mass spectrometry, it has been proposed that HSP-90 interacts with CHIKV nsP3 and nsP4. SiRNA knockdown of the HSP-90α subunit resulted in a greater inhibition of CHIKV replication than knockdown of the HSP-90β subunit. It was also suggested that HSP-90α may play an important role in the stabilization of CHIKV nsP4 and the formation of the CHIKV replication complex (Rathore et al., 2014). Further studies are needed to understand the role of HSP-90 in the life cycle of CHIKV\ (Table 2).

5.4. Modulators of the host immune response

5.4.1. Interferon-α

Treatment with IFN-α resulted in significant inhibition of the *in vitro* replication of CHIKV (Briolant et al., 2004). A strain carrying the E1 A226V mutation proved to be more sensitive to the antiviral activity of recombinant IFN-α than a strain carrying the wild-type genotype (Bordi et al., 2011).

5.4.2. Polyinosinic acid:polycytidylic acid

Polyinosinic acid:polycytidylic acid [poly(I:C)] is a synthetic analogue of dsRNA which acts as a potent inducer of IFN. Poly(I:C) was reported to inhibit CHIKV-induced CPE in human bronchial epithelial cells and to significantly reduce virus titers in infected cells. The anti-CHIKV activity of poly(I:C) may be attributed to the upregulation of TLR3 resulting in the stimulation of IFN-α, IFN-β and antiviral genes such as OAS and MxA (Li et al., 2012) (Table 2).

5.4.3. RIG-I agonists

RIG-I (retinoic acid-inducible gene I) is a member of the RIG-I like receptor family which recognizes viral dsRNA resulting in

Table 3
Antivirals against chikungunya virus with unidentified targets and mechanism of action.

Antiviral agent	*In vitro* efficacy	*In vivo* efficacy	References
Trigocherrierin A	Inhibition of CHIKV-induced CPE in Vero A cells (EC50 = 0.6 μM)	Not determined	Bourjot et al. (2014)
Aplysiatoxin	Inhibition of CHIKV replication SJCRH30 cells (EC50 of the best compound = 1.3 μM)	Not determined	Gupta et al. (2014)
5,7-Dihydroxyflavones	Inhibition of CHIKV replication in both a BHK replicon cell line (*Rluc* EC50 = 28–60 μM) and infectious virus-based assays	Not determined	Pohjala et al. (2011)
[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones	Inhibition of CHIKV-induced CPE in Vero A cells (EC50 of the best compound <1 μM)	Not determined	Gigante et al. (2014)
Benzouracil–coumarin–arene conjugates	Inhibition of CHIKV-induced CPE in Vero A cells (EC50 = 10.2–19.1 μM)	Not determined	Hwu et al. (2015)

the activation of multiple antiviral factors that inhibit viral infection. RIG-I can also be induced by synthesized dsRNA molecules bearing an exposed 5′-triphosphate end (5′ppp) (Hornung et al., 2006; Pichlmair et al., 2006). Interestingly, stimulation of RIG-I in MRC-5 cells by an optimized 5′triphosphorylated RNA molecule protected these cells against CHIKV infection and reduced the virus titers (Olagnier et al., 2014). The antiviral response triggered by this 5′pppRNA was independent of the type I IFN response. Major advantages of boosting the innate immune response by RIG-I agonists are the low possibility of resistance development and the broad antiviral spectrum (Table 2).

6. Inhibitors with an unidentified target

6.1. Trigocherrierin A

Trigocherrierin A, a new daphnane diterpenoid orthoester, has been isolated from the ethanol extract of *Trigonostemon cherrieri* leaves (Bourjot et al., 2014). Trigocherrierin A was reported to inhibit the *in vitro* replication of CHIKV (EC_{50} of 0.6 μM) by a yet unknown mechanism.

6.2. Aplysiatoxin

Debromoaplysiatoxin and 3-methoxydebromoaplysiatoxin (isolated for the first time from the marine cyanobacterium, *Trichodesmium erythraeum*) were reported to inhibit the *in vitro* replication of CHIKV (Gupta et al., 2014). It was suggested that these compounds might block a post-entry step in the CHIKV replication cycle (Table 3).

6.3. 5,7-Dihydroxyflavones

Four natural 5,7-dihydroxyflavones (apigenin, chrysin, naringenin and silybin) were reported as inhibitors of CHIKV using both a replicon cell line and infectious virus-based assays (Pohjala et al., 2011) (Table 3).

6.4. [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones

The molecular target is still unknown. A new class of compounds, [1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-ones, has been reported to inhibit the *in vitro* CHIKV replication with EC50 values in the low micromolar range and selectivity indices up to 600 (Gigante et al., 2014). Interestingly, these compounds showed specific anti-CHIKV activity with little or no antiviral activity against other members of the Togaviridae family (Gigante et al., 2014). Mechanistic studies are currently ongoing (Table 3).

6.5. Benzouracil–coumarin–arene conjugates

A number of uracil–coumarin–arene conjugates were synthesized and evaluated for their antiviral activity against CHIKV replication in Vero cells (Hwu et al., 2015). Five of these conjugates elicited significant inhibition of the *in vitro* CHIKV replication with low toxicity (Hwu et al., 2015). Structure-activity relationship studies revealed that the compounds with a benzouracil–SCH2–coumarin–OSO2–arene scaffold were the most potent in this series. (Hwu et al., 2015) (Table 3).

7. Conclusion

The re-emergence of CHIKV, together with the sometimes severe complications that can be associated with acute chikungunya fever, underline the need for potent antivirals. Various classes of active compounds have been reported, which target a viral or a host factor. However, *in vivo* efficacy has not yet been evaluated in animal models for most of these molecules. The mechanism by which viral replication is inhibited has only been demonstrated for a handful of compounds. Although most of the reported inhibitors have relatively weak to modest *in vitro* anti-CHIKV activity, they may be used as a starting point to develop more potent and specific inhibitors of CHIKV/alphavirus replication.

Some of the reported CHIKV inhibitors such as favipiravir, ribavirin, IFN-α and arbidol are approved to treat patients with other viral infections. Because these molecules have already been intensively studied in patients, the evaluation in CHIKV-infected patients may possibly be fast-tracked. The performance of controlled clinical trials could probably be facilitated by the current epidemic in South America, where millions of people are being infected in urban areas. Once the virus has become endemic, it will become more difficult to perform clinical trials in sporadic infections. Because there is no approved antiviral for CHIKV, a promising compound could be evaluated in patients in comparison to a placebo, and in the setting of this first, explosive year of the epidemic, it should thus be possible to detect antiviral efficacy, or its absence, relatively quickly.

In this context, the anti-CHIKV activity of the broad-spectrum antiviral favipiravir may be particularly interesting. Approved to treat influenza virus infections in Japan, this drug is now being evaluated for the treatment of Ebola virus infections in Western Africa. However, given the growing number of patients suffering from CHIKV infections, it may be justified to develop specific CHIKV drugs. Ideally such inhibitors should exert pan-alphavirus activity, so that they can for example be used for the treatment of infections with the Ross River virus or the equine encephalitis viruses. Nowadays, highly potent drugs are only available for the treatment of a limited number of viruses: HIV, herpesviruses, hepatitis B and C virus. When investing sufficient time and effort, it should also be possible to develop safe and potent drugs for the treatment and/or prophylaxis of alphavirus infections.

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