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Daniel Oliveira Silva Martins

Atividade antiviral de Tiossemicarbazona conjugada com complexo metálico de Cobalto (III)
contra o vírus Chikungunya *in vitro*.

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Microbiologia, junto ao programa de Pós-Graduação em Microbiologia do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista “Júlio Mesquita Filho”, Campus São José do Rio Preto.

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Comissão Examinadora

Prof.^a. Dr.^a. Ana Carolina Gomes Jardim

Instituição: Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia
Orientadora

Prof.^a. Dr.^a. Carolina Colombelli Pacca

Instituição: Instituto de Educação Superior Ceres (UNICERES)

Prof.^a. Dr.^a. Fernanda de Freitas Anibal

Instituição: Universidade Federal de São Carlos (UFSCAR).

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Resumo

O vírus Chikungunya (CHIKV) é um vírus de RNA de fita simples e polaridade positiva da família Togoviridae que é transmitido pela picada da fêmea do mosquito *Aedes sp.* Os pacientes infectados com CHIKV são afetados pela doença febril Chikungunya, que apresenta febre e dores nas articulações como sintomas clínicos. Além disso, os pacientes podem desenvolver uma doença crônica que pode levar a incapacidade. Atualmente, não há tratamento antiviral contra o CHIKV, demonstrando a necessidade de desenvolvimento e pesquisa de novos agentes antivirais. Nesse contexto, alguns estudos têm demonstrado que as tiossemicarbazonas e seus complexos metálicos possuem propriedades farmacológicas como atividade antitumoral, antiparasitária, antibacteriana e antiviral. A síntese desses compostos é simples, limpa, versátil e tem alto rendimento. Apesar disso não há atividade anti-CHIKV descrita, na literatura disponível, destes compostos contra CHIKV. Portanto, este trabalho teve como objetivo avaliar a atividade antiviral de tiossemicarbazona conjugada com Cobalto (III) (CoAtc-Ph) e seus precursores metálicos contra o CHIKV *in vitro*. Para isso, células BHK 21 foram infectadas com CHIKV *nanoluciferase* (M.O.I 0,01) na presença ou ausência de cada composto em concentração não citotóxica por 16 horas. Através deste ensaio, CoAtc-Ph a 50 μ M inibiu 94% da infecção por CHIKV. Usando uma faixa mais ampla de concentrações, foi determinado que o composto tem um EC₅₀ de 19,02 μ M, CC₅₀ de 62,08 μ M e SI de 3,26. Ensaio adicionais demonstraram que o CoAtc-Ph inibiu a pós-entrada do CHIKV nas células hospedeiras. Possíveis mecanismos de ação antiviral foram investigados avaliando-se a intercalação desse composto no dsRNA viral e, sua interação com a proteína não-estrutural 4 de chikungunya (nsP4). CoAtc-Ph mostrou forte interação com nsP4, sugerindo que este composto pode inibir o ciclo de CHIKV através dessa interação. Também análises *in silico* demonstraram que o CoAtc-Ph interage com o nsP4 através de ligações de hidrogênio, interações hidrofóbicas e eletrostáticas. Considerando a interação do CoAtc-Ph com a nsP4 e sua interação através de análises *in silico*, podemos sugerir que este pode ser o mecanismo de ação deste composto.

Palavras-chave: Arbovirose. Virus Chikungunya. Antiviral. Tiossemicarbazona. Nsp4.

Abstract

Chikungunya virus (CHIKV) is a single positive strand RNA virus from the *Togoviridae* family that is transmitted through the bite of the female mosquito *Aedes* sp. CHIKV infected patients are affected by Chikungunya fever disease which presents fever and joint pains as clinical symptoms. Additionally, patients may develop a chronic disease that can lead to a disability. Currently, there is no antiviral treatment against CHIKV, demonstrating the necessity to develop novel antiviral agents. In this context, some studies have shown that thiosemicarbazones and their metal complexes possess pharmacological properties as antitumor, antiprotozoal, antibacterial and antiviral activity. The synthesis of these compounds is simple, clean, versatile and results in high yields. Although there is no anti-CHIKV activity described in the available literature of this compounds against CHIKV. Therefore, this work aimed to evaluate the antiviral activity of Cobalt (III) - conjugated thiosemicarbazone (CoAtc-Ph) and its metal precursors against CHIKV *in vitro*. For this, BHK 21 cells were infected with CHIKV *nanoluciferase* (M.O.I 0.01) in the presence or absence of each compound at non-cytotoxicity concentration for 16 hours. Through this assay, CoAtc-Ph at 50 μ M inhibited 94% of CHIKV infection. Using a wider range of concentrations, it was determined that the compound has an EC_{50} of 19.02 μ M, CC_{50} of 62.08 μ M and SI of 3.26. Additional assays demonstrated that CoAtc-Ph inhibited CHIKV post entry to the host cells. Possible mechanisms of antiviral action were investigated by evaluating the intercalation of this compound into the viral dsRNA and, its interaction with chikungunya non-structural protein 4 (nsP4). CoAtc-Ph did shown strong interaction with nsP4 suggesting that this compound may inhibits CHIKV cycle through this. Also *in silico* analyses demonstrated that CoAtc-Ph intereracted with nsP4 through hydrogen bonds, hydrophobic and electrostatic interactions. Considering the interaction of CoAtc-Ph with nsP4 and its interaction through *in silico* analyses we can suggest that this may be the action mechanism of this compound

Keywords: Arboviruses. Chikungunya virus. Antiviral. Thiosemicarbazone. Nsp4

Lista de de ilustrações

Figura 1. Esquema da infecção pelo CHIKV	13
Figura2. Distribuição mundial do mosquito <i>Aedes albopictus</i>	15
Figura 3. Distribuição mundial do mosquito <i>Aedes aegypti</i>	15
Figura 4. Organização do Genoma viral do Chikungunya	16
Figura 5. Esquema Representativo do ciclo replicativo de CHIKV	19
Figura 6. Mapa da distribuição mundial do CHIKV	20
Figura 7. Possíveis casos de febre CHIKV no Brasil	21

ANEXO A

Figure 1. Schematic representation of CHIKV replication cycle. Natural compounds with antiviral activity against CHIKV are indicated in each step of virus replication cycle.	60
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ANEXO B

Figure 1. <i>In vitro</i> screening of 12 MCCB compounds selected from docking analyses.	94
Figure 2. Structure of compounds C5, C13 and analogues.	95
Figure 3. Activity of selected compounds and analogues against CHIKV replication.	96
Figure 4. Effect of C13 and C5 at different stages of CHIKV replication.	97
Figure 5. Activity of C5 and C1 in RD and C2C12 cell lines.	98

ANEXO C

Figure 1. Schematic representation of Zika Virus replicative cycle.	101
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Lista de abreviatura

CC₅₀ – Concentração citotóxica de 50 %

CDC – *Centers for disease control and prevention* (Centro de controle e prevenção de doenças)

CHIKV – Vírus Chikungunya

CMC – Carboximetilcelulose

CO₂ – Dióxido de Carbono

DENV – Vírus Dengue

DMEM – *Dulbecco's Modified Eagle Medium* (Meio Eagle modificado por Dulbecco)

DMSO – Dimetil-sulfóxido

DNA – Ácido desoxirribunucleico

DOX – Doxorrubicina

dsRNA – *Double-Stranded RNA* (Dupla fita de RNA)

EC₅₀ – Concentração efetiva de 50%

ECSA – *East-Central-South African* (Leste-Centro-Sul Africano)

EGFP – *Green fluorescence protein* (Proteína de fluorescência verde)

FTIR - *Fourier-transform infrared spectroscopy* (Espectroscopia no infravermelho por transformada de Fourier)

GAG- Glicosiaminoglicanos

h.p.i – Hours post infection (horas depois da infecção)

HIV – Vírus da imunodeficiência humana

HSV – Herpes vírus

IFN – Interferon

IL – Interleucina

IOL – *Indian-Ocean* (Oceano-Índico)

kb – kilobase

MAYV – Vírus Mayaro

M.O.I – *Multiplicity of infection* (multiplicidade de infecção)

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]

nsP – *non-structural protein* (proteína não estrutural)

OMS – Organização Mundial da Saúde

ORF – *open read frame* (fase de leitura aberta)

PBS – *Phosphate-buffered saline* (Tampão fosfato salino)

PCR – *Polymerase chain reaction* (Reação de polimerase em cadeia)

PFU – *Plate forming-unit* (Unidade formadora de palca)

PHB – proibitina

PtdSer- fosfatidilserina

RE – Retículo endoplasmático

Rluc – Renilla luciferase

RNA – Ácido ribonucleico

SI – Índice de seleção

SRV – Vírus do sarcoma de Rous

UTR – *Untranslated region* (região não traduzível)

WA – *West African* (Oeste Africano)

ZIKV – Vírus Zik

SUMÁRIO

1. Introdução	11
1.1. Arboviroses.....	11
1.2. Febre Chikungunya	11
1.3. Patogênese	12
1.4. Diagnóstico.....	14
1.5. O vetor	14
1.6. Vírus Chikungunya.....	16
1.7. Ciclo replicativo	18
1.8. Epidemiologia.....	19
1.9. Tratamento.....	22
1.10. Atividade biológica de tiossemicarbazonas.....	22
1.11 Objetivo	23
1.12 Objetivos específicos.....	23
Referências	24
2. Cobalt-conjugated Thiosemicarbazone inhibits chikungunya replication <i>in vitro</i> by interacting with RNA-dependent RNA polymerase (nsP4)	33
2.1 Introduction	35
2.2 Material and methods	37
2.2.1 Compounds	37
2.2.2 Cell culture.....	37
2.2.3 Virus.....	37
2.2.4 Cell viability through MTT assay	38
2.2.5 Antiviral activity assays.....	38
2.2.6 Intercalation assay.....	39
2.2.7 CHIKV nsP4 cloning, overexpression and purification:	39
2.2.8 Microscale Thermophoresis (MTS).....	39
2.2.9 <i>In silico</i> analyses	40
2.2.10 Statistical analysis.....	41
2.3 Results	41
2.4 Discussion.....	43
References.....	45
APÊNDICE A. Estrutura dos compostos, sequência CHIKV nanoluc, gráfico ensaio de viabilidade e infecção geral.	49
APÊNDICE B. Gráfico ensaio de EC ₅₀ e CC ₅₀	51
APÊNDICE C. Gráfico e esquema do ensaio para testar a atividade do composto em diferentes etapas da infecção viral.	53
APÊNDICE D. Ensaio de Intercalação de dsRNA.....	55

APÊNDICE E. Purificação e expressão de nsP4 de CHIKV. Interação biofísica e in silico de nsP4 com tiossemicarbazona conjugada com Co(III)	57
ANEXO A: REVIEW ARTICLE ANTIVIRALS AGAINST CHIKUNGUNYA VIRUS: IS THE SOLUTION IN NATURE?.....	59
ANEXO B: IS THE ADP RIBOSE SITE OF THE CHIKUNGUNYA VIRUS NSP3 MACRO DOMAIN A TARGET FOR ANTIVIRAL APPROACHES?	79
ANEXO C: A REVIEW OF THE ONGOING RESEARCH ON ZIKA VIRUS TREATMENT.....	99

1. Introdução

1.1. Arboviroses

Segundo a Organização Mundial da Saúde (OMS), arboviroses são doenças causadas por vírus que são mantidos na natureza e transmitidos entre hospedeiros vertebrados suscetíveis por meio de artrópodes hematófagos (WHO., 1967).

As arboviroses afetam principalmente os países que estão entre os trópicos do hemisfério sul, isso porque estas regiões possuem parâmetros climáticos favoráveis para procriação de vetores artrópodes (LIANG; GAO; GOULD, 2015; MORENS; FAUCI, 2014). Os mosquitos do gênero *Aedes* estão entre os principais vetores artrópodes responsáveis pela transmissão dos vírus Dengue (DENV), Chikungunya (CHIKV), Zika (ZIKV) e Mayaro (MAYV) e Febre Amarela (YFV) (PATTERSON; SAMMON; GARG, 2016). Os mosquitos *Aedes aegypti* e *Aedes albopictus* desenvolvem todo o seu ciclo de vida em áreas urbanas e dessa maneira passaram a se alimentar de sangue humano, mesmo existindo a presença de outros mamíferos nas proximidades. Estes vetores se tornaram transmissores de patógenos que utilizam do mosquito como ponte para o hospedeiro final (DELATTE et al., 2010; KAMGANG et al., 2012; PONLAWAT et al., 2005). Kraemer e colaboradores (2015) mostraram que o mosquito também se adapta em diferentes regiões climáticas, o que aumenta sua ocupação geográfica e consequentemente a possibilidade de transmissão de arboviroses para humanos (KRAEMER et al., 2015).

Nos últimos anos diversos surtos relacionados à arboviroses vêm ocorrendo em todo o mundo (PETERSEN; POWERS, 2016). O aumento destes surtos tem sido motivo de preocupação para órgãos governamentais e pesquisadores, impulsionou pesquisas direcionadas ao desenvolvimento de antivirais específicos e imunizantes contra arboviroses que diminuem a qualidade de vida dos afetados ou até mesmo os levam à óbito (GOULD et al., 2017)

1.2. Febre Chikungunya

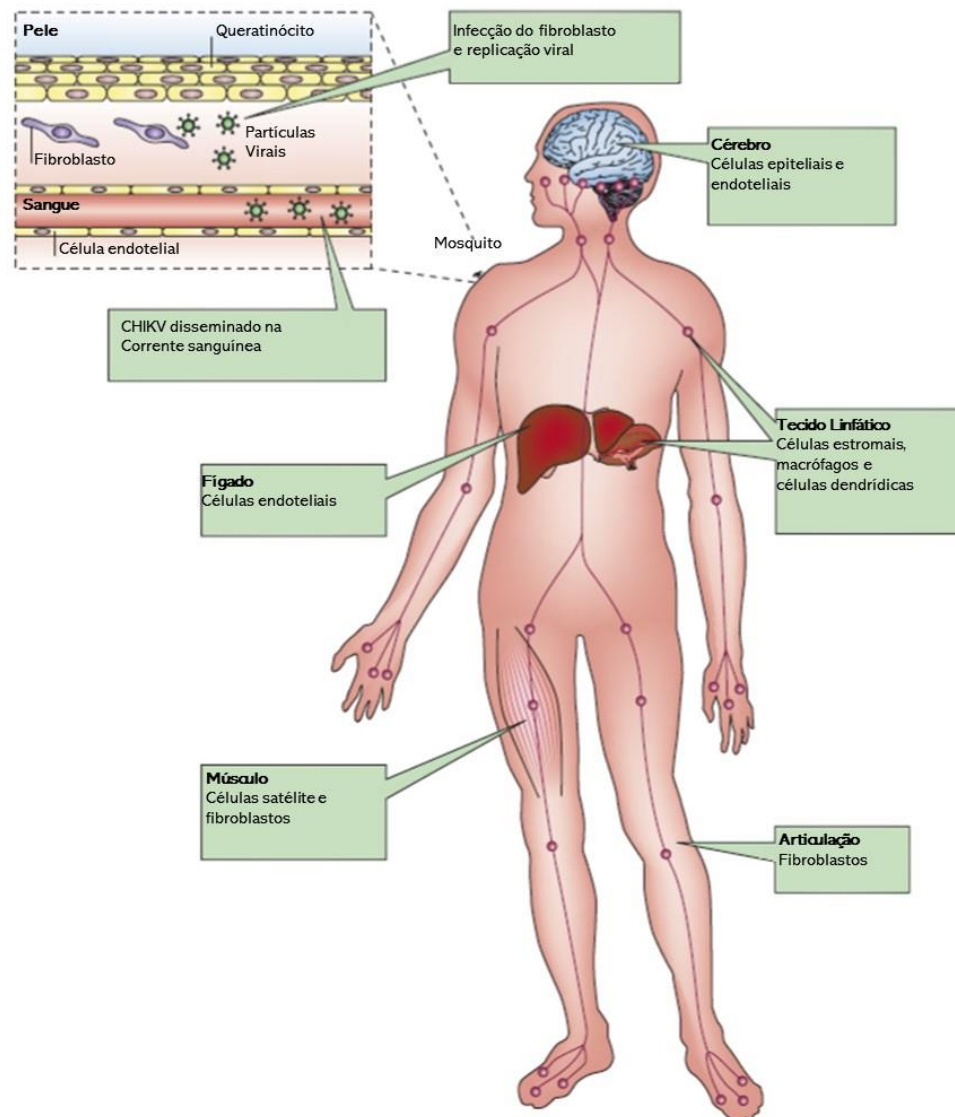
A Febre Chikungunya é a doença causada pelo CHIKV, transmitido principalmente pelos mosquitos do gênero *Aedes* (WEAVER, 2006). Inicialmente, as infecções causadas pelo CHIKV eram confundidas com as causadas pelo vírus da Dengue (LUMSDEN, 1955). Apenas anos mais tarde o CHIKV foi considerado uma arbovirose, quando o vírus foi isolado do soro de pacientes infectados, bem como de *Aedes aegypti* e

Culex (ROSS, 1956; Calisher & Karabtsos, 1988). Atualmente, o vírus é classificado como um Alphavirus pertencente à família *Togoviridae* (ICTV, 2019), e apresenta as linhagens Oeste Africano (WA-*West African*); Leste, Central e Sul-africano (ECSA – *East, Central and Southern African*), Oceano Índico (IOL –*Indian Ocean*) e Asiático(*Asian*) (LANGSJOEN et al., 2018). A palavra Chikungunya no dialeto africano Makonde significa “aqueles que se dobram”, referindo-se a postura dos acometidos pela infecção, devido a fortes dores nas articulações, o sintoma clínico mais característico da infecção pelo CHIKV, que reduzem de 1 a 3 semanas após os episódios de febre (MOHAN et al., 2010). As manifestações clínicas da doença podem começar de 1 a 12 dias após a infecção que é caracterizada por febre alta, erupções cutâneas, dores nas costas e mialgia. Além dessas manifestações clínicas, as infecções podem estar relacionadas à sintomas neurológicos com encefalites, meningite e pode até desencadear a síndrome de Guillain-Barré (MOHAN et al., 2010; STEGMANN-PLANCHARD et al., 2019a). Esta última, é uma doença autoimune que ataca parte do sistema nervoso, inicialmente caracterizada por dormência ou queimação nos membros inferiores, seguido dos membros superiores. Progressivamente, os músculos do paciente vão se enfraquecendo, começando pelas pernas, tronco e face. Alguns pacientes desenvolvem insuficiência respiratória, podendo chegar a óbito (STEGMANN-PLANCHARD et al., 2019b; WHO, 2017).

1.3. Patogênese

Logo após a picada, o CHIKV se multiplica nos fibroblastos de maneira limitada. Quando atinge a corrente sanguínea é transportado até os linfonodos onde há uma alta taxa de replicação do CHIKV com até 10^8 PFU (*plate forming unit* – unidade formadora de placa)/mL. Nas fases iniciais da doença, o vírus se multiplica extensivamente nos macrófagos, ajudando na disseminação do vírus até outras partes do corpo como músculos, articulações, fígado e células do cérebro (HER et al., 2010) (Figura 1) .

Figura 1. Esquema da infecção pelo CHIKV.



Fonte: Figura adaptada de (SCHWARTZ; ALBERT, 2010).

Durante esse processo, a resposta imune inata é ativada a partir do reconhecimento de receptores padrões e a produção de interferon I (IFN I) é iniciada (SCHWARTZ; ALBERT, 2010). A maior taxa de replicação do vírus ocorre nos monócitos presentes nas articulações, e por isso um dos sintomas clássicos da febre Chikungunya são fortes dores nessas regiões. Um dos agravantes da Febre Chikungunya é a possibilidade de cronificação da infecção, levando os indivíduos infectados à incapacitação. Nos pacientes crônicos, os sintomas são caracterizados principalmente pelas fortes dores nas articulações, mesmo após 3 meses da infecção aguda. Também são observados nesses

pacientes altos níveis de interleucina 6 e 1b (IL-6 e IL-1b). Existem poucos dados relacionados às taxas de cronificação, entretanto, os dados mais sólidos demonstram que aproximadamente 43% dos pacientes desenvolvem a doença crônica após 3 meses da infecção, e 21% após 1 ano. Não existem dados substanciais que associem o processo de cronificação à linhagens virais específicas, mas algumas análises sugerem que seja mais prevalente em pacientes infectados com a linhagens ECSA (50%), seguido da linhagem asiática (36%) (PAIXÃO et al., 2018).

1.4. Diagnóstico

Como as arboviroses possuem sintomas iniciais muito semelhantes, o diagnóstico não pode ser dado apenas pelas manifestações clínicas apresentadas pelos infectados, até mesmo porque muitas arboviroses coexistem na mesma região. O diagnóstico laboratorial diferencial é essencial para definir condutas no tratamento do acometido pela infecção. Segundo o Manual de Manejo Clínico do Chikungunya disponibilizado pelo Ministério da Saúde, o diagnóstico pode ser realizado de forma direta, por meio do isolamento viral e da pesquisa de RNA viral em uma amostra do paciente suspeito, ou de forma indireta por intermédio da pesquisa de anticorpos específicos.

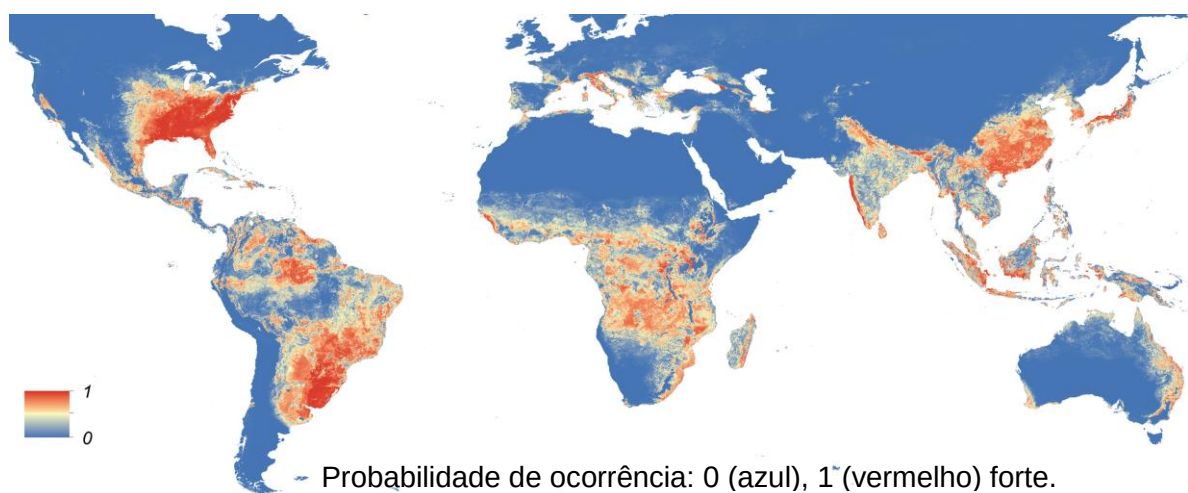
Para pesquisa de RNA viral é utilizado a técnica de reação em cadeia da polimerase (PCR) ou qRTP (*Real time PCR* – PCR em tempo real). Essas técnicas são citadas como padrão ouro devido a alta sensibilidade e especificidade, sendo possível detectar o material até 8 dias após o início dos sintomas. Para pesquisa de anticorpos específicos (POWERS, 2010) as principais técnicas utilizadas são teste de ELISA e imunocromatografia, que permitem a detecção de anticorpos IgM e IgG, que podem ser detectados a partir do segundo dia após o aparecimento dos sintomas. Para uma maior confiabilidade nos resultados é recomendado para detecção de IgM a amostra seja submetida ao teste a partir do quinto dia desde o início dos sintomas, já para IgG a partir do sexto dia. (PANNING et al., 2008). (DASH; MOHANTY; PADHI, 2011). A maneira (PYNDIAH et al., 2012).

1.5. O vetor

Vários mosquitos do gênero *Aedes* são responsáveis pela transmissão do CHIKV na África, Ásia e Oceânia (DIALLO et al., 1999), sendo que os principais são o *A. albopictus* e o *A. aegypti*. O *A. albopictus* possui maior alcance geográfico (Figura 2),

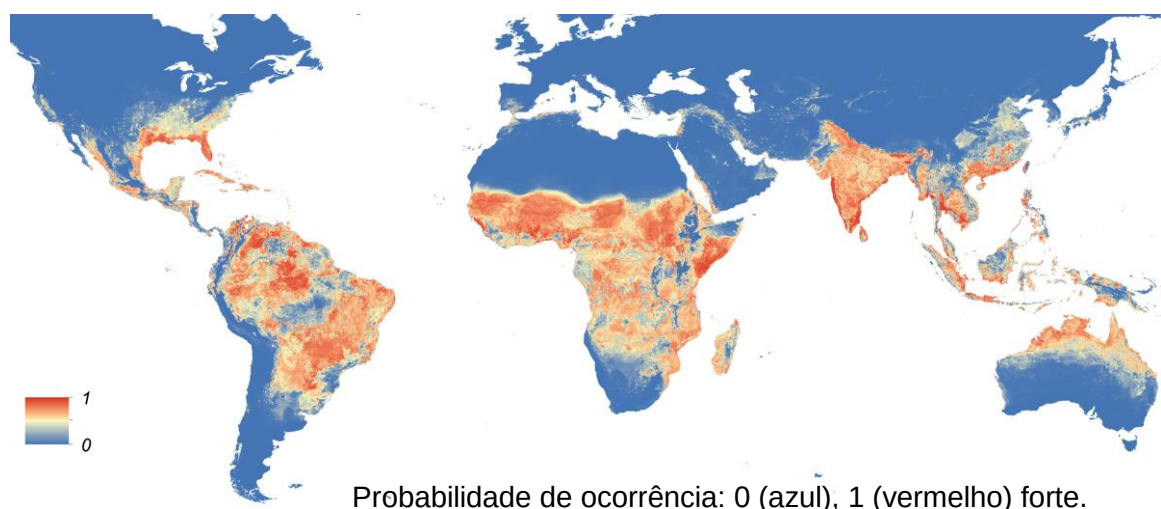
sendo responsável pela transmissão do vírus nos Estados Unidos da América (EUA). Os ovos são estáveis em ambientes rurais e urbanos, e o mosquito consegue voar de 400 a 600 m e sobrevivem de 4 a 8 semanas (CHEVILLON et al., 2008). Por outro lado, o *A. aegypti* possui uma distribuição geográfica menor (Figura 3), habitando mais as regiões urbanas. Esse mosquito afeta mais as regiões marcadamente tropicais do Brasil, como Norte, Nordeste e Centro-oeste, enquanto o *A. albopictus* é mais encontrado nas regiões Sul (CARVALHO; LOURENÇO-DE-OLIVEIRA; BRAGA, 2014; MONTEIRO et al., 2014)

Figura 2. Distribuição mundial do mosquito *Aedes albopictus*.



Fonte: Adaptada de (KRAEMER et al., 2015)

Figura 3. Distribuição mundial do mosquito *Aedes aegypti*.

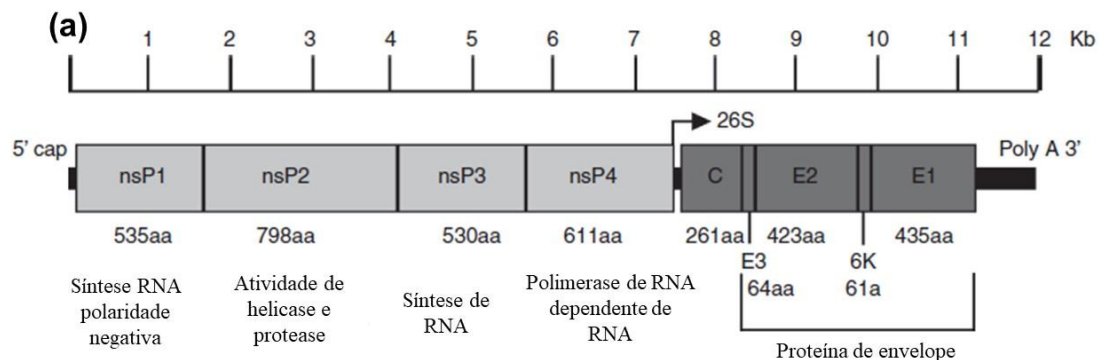


Fonte: Adaptada de (KRAEMER et al., 2015).

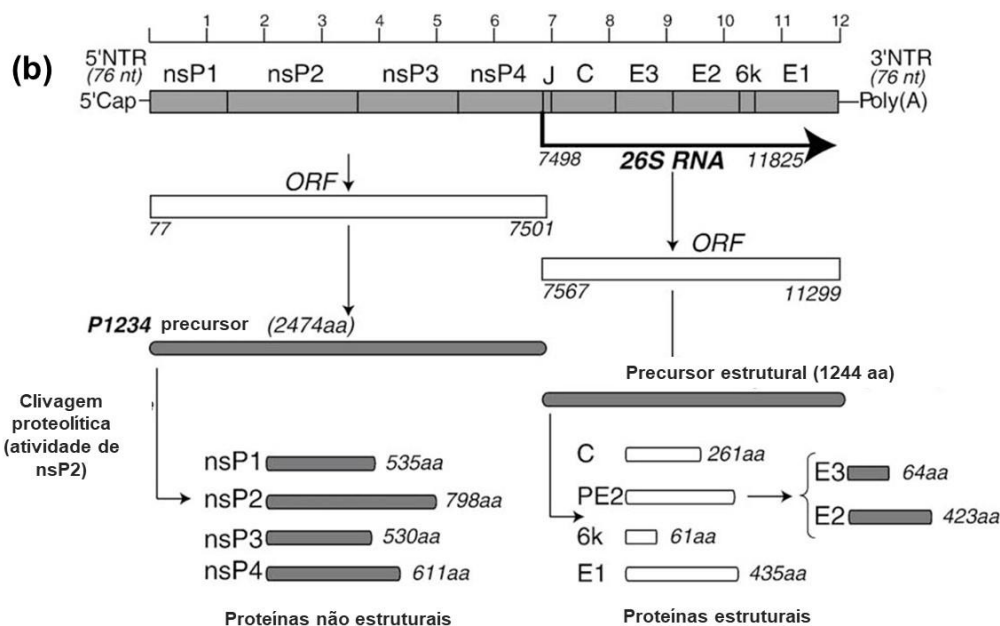
1.6. Vírus Chikungunya

O CHIKV é um vírus de aproximado de 60-79 nm de tamanho, envelopado, de capsídeo icosaédrico, e genoma de RNA polaridade positiva com aproximadamente 12 kb. O genoma viral possui duas *Open Read Frame* (ORF) que codificam as proteínas estruturais e não estruturais do vírus (Figuras 4a e 4b) (KHAN et al., 2002). A primeira ORF codificadas a proteínas não estruturais 1, 2, 3 e 4, e a segunda (segmento denominado RNA 26 S) codifica as proteínas estruturais do capsídeo, E3, 6K e E1. Além dessas regiões de leitura, o genoma possui 3 regiões não traduzíveis (*UTR – Non-Translated Regions*) (KHAN et al., 2002; THIBERVILLE et al., 2013).

Figura 4. Organização do genoma viral do CHIKV. (A) São representadas em ordem as proteínas não estruturais e estruturais e suas funções específicas. Figura retirada e adaptada de (GALÁN-HUERTA et al., 2015). **(B)** Sequência do genoma do CHIKV com as ORF's indicadas, a divisão de cada proteína e o número de aminoácidos.



Fonte: Figura adaptada de (SOLIGNAT et al., 2009).



Fonte: Figura adaptada de (SOLIGNAT et al., 2009).

As proteínas estruturais compreendem a proteína do capsídeo e as glicoproteínas do envelope (Figura 4). O envelope do vírus é formado pelo heterodímero E1-E2 em associação com a proteína 6K (GAEDIGK-NITSCHKO; SCHLESINGER, 1990; LUSA; GAROFF; LILJESTRÖM, 1991; STRAUSS; STRAUSS, 1994). A proteína E1 é responsável por facilitar a fusão do vírus na membrana da célula hospedeira e a E2 facilita a ligação do vírus aos receptores celulares. A proteína 6K é classificada como uma porina viral (*viroporin*), que são proteínas multifuncionais que modificam a estrutura da membrana da célula infectada, facilitando a liberação de novas partículas virais formadas durante o ciclo replicativo. O papel dessa proteína ainda não é muito bem esclarecido no ciclo do CHIKV, mas trabalhos recentes indicam que a 6K é uma proteína formadora de canal de íons quando associada com a membrana do retículo endoplasmático (RE), estando relacionada à liberação de novas partículas virais (DEY et al., 2019).

O vírus possui a sequência para quatro proteínas não estruturais denominadas nsP's (Figura 4). A nsP1 é uma metil e guaniltransferase responsável pela ligação do complexo replicativo às membranas. A nsP2 tem função helicase e protease e é responsável pelos processos de clivagem da poliproteína formada após a tradução do mRNA recém desnudado. A nsP3 é dividida em 3 domínios e sua atividade ainda é pouco esclarecida na literatura. O que se sabe é que no macro domínio dessa proteína se liga

ADP-ribose, que ainda possui um domínio de ligação ao zinco e um domínio que parece ser responsável pela regulação de proteínas celulares. A nsP4 é uma RNA polimerase dependente de RNA e é responsável pela formação de fitas de senso negativo, que servirão de moldes para produção de novas moléculas de RNA senso positivo, que serão empacotadas nas novas partículas virais provenientes do processo infeccioso (AHOLA; MERITS, 2016; MALET et al., 2009; RUPP et al., 2015).

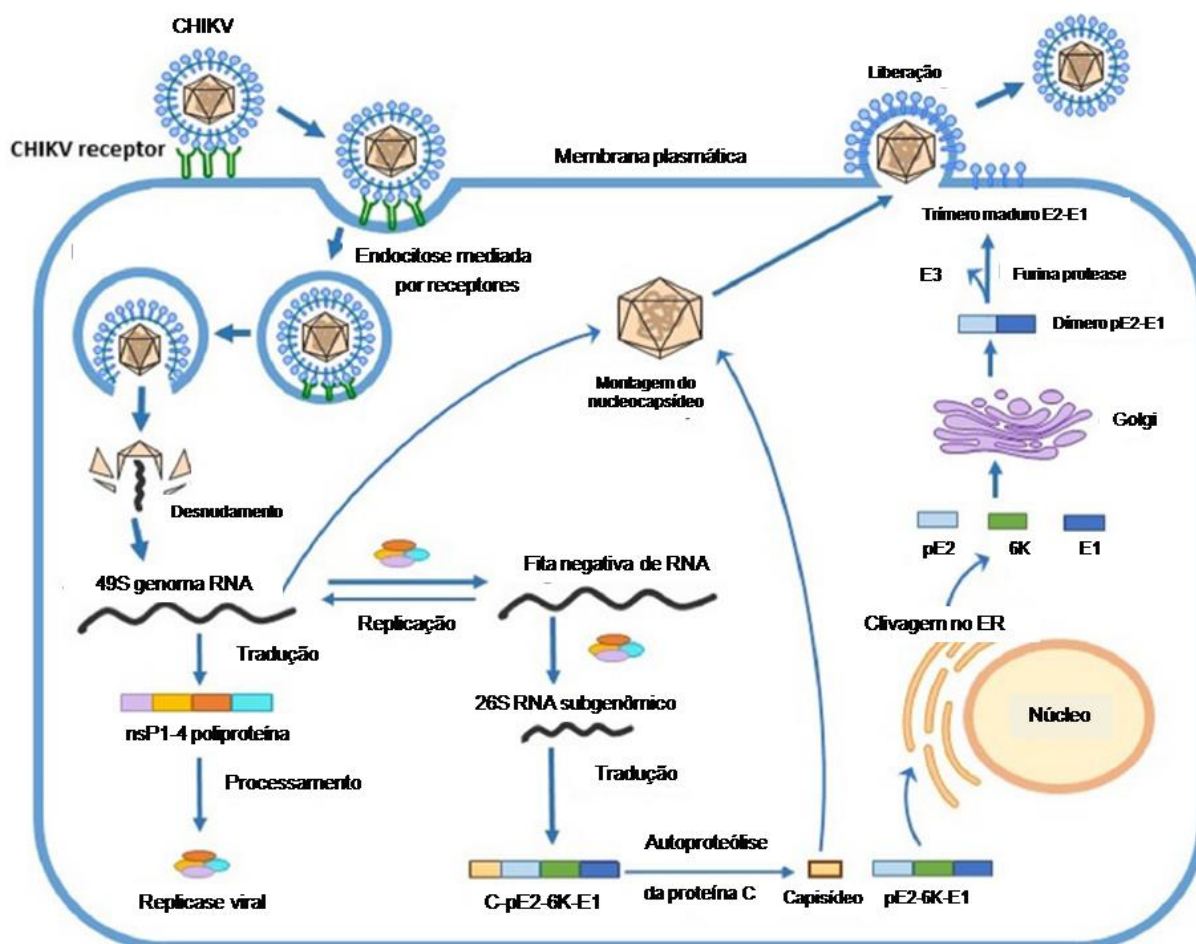
1.7. Ciclo replicativo

O ciclo replicativo se inicia com a ligação da glicoproteína de envelope do vírus com receptores de superfície celular. Em mamíferos, esses receptores são as proibitinas (PHB), fosfatidilserinas (PtdSer) e os glicosaminoglicanos (GAGs), e em células de mosquitos, as ATPsintase β , após essa ligação há a formação de um poro que leva à internalização do vírus por meio de endocitose mediada por clatrin formando um endossomo (BERNARD et al., 2010). Após modificações do pH endosomal, o capsídeo viral é desnudado, liberando o material genético do vírus no citoplasma das células do hospedeiro (HOORNWEG et al., 2016). O genoma viral é diretamente traduzido em uma poliproteína contendo todas as proteínas não estruturais, que sofre um processo de clivagem pela nsP2, formando o complexo replicativo. Esse complexo será responsável pela síntese de uma fita de senso negativo, que servirá de molde para a síntese de novas fitas de senso positivo, que serão traduzidas em proteínas estruturais, e utilizadas para montagem das novas partículas virais (ABDELNABI; NEYTS; DELANG, 2015; GOULD et al., 2010). Após tradução das proteínas estruturais (do RNA 26S), o heterodímero PE2-E1 é transportado para o complexo de golgi e direcionado para a parte interna da membrana citoplasmática. Durante esse processo, a PE2 é clivada por uma furina para formar E2 e E3. Paralelamente, ocorre a tradução das proteínas do capsídeo, que possuem uma região altamente conservada em *Alphavirus*, que formam um dobramento em α -helice, onde ocorre a ligação do RNA para incorporação ao nucleocapsídeo.

Então, o nucleocapsídeo é direcionado para a membrana da célula hospedeira, onde se liga as proteínas de envelope E1 e E2 para formar a dupla camada lipídica (EKSTRÖM; LILJESTRÖM; GAROFF, 1994; FULLER, 1987), formando o complexo capsídeo, RNA e envelope. Em seguida, o complexo é fosforilado (LIU et al., 1996) e

ocorre o brotamento das novas partículas virais que irão infectar novas células. Todo esquema do ciclo está representado na figura 5 (SOLIGNAT et al., 2009)

Figura 5. Esquema representativo do ciclo replicativo do CHIKV

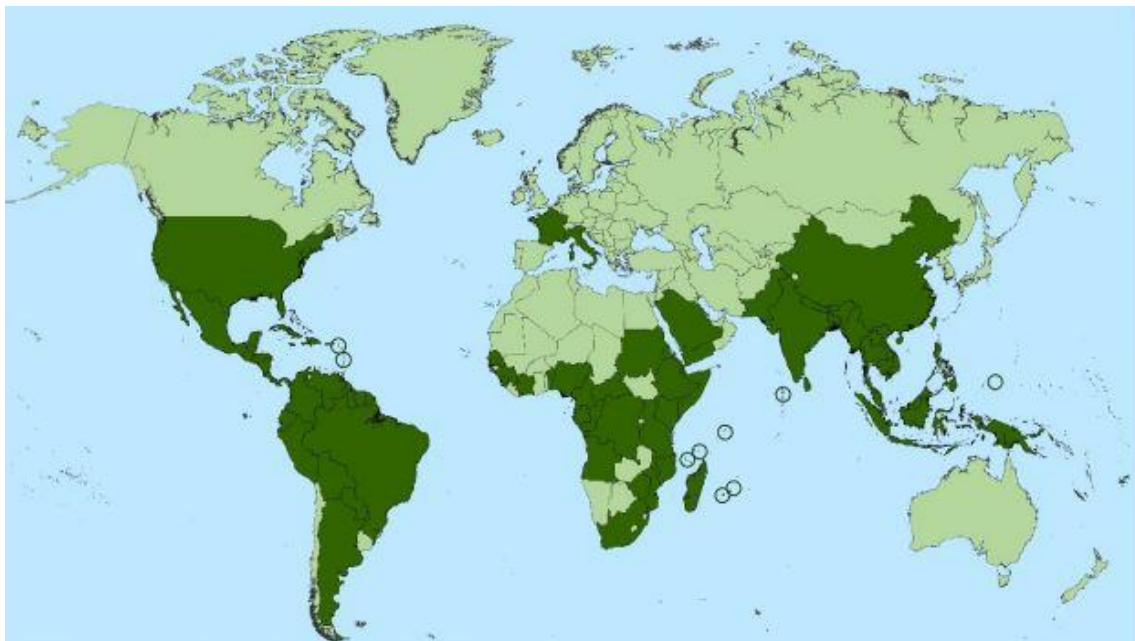


Fonte: Figura adaptada de (ABDELNABI; NEYTS; DELANG, 2015).

1.8. Epidemiologia

Nos anos 60, o CHIKV era responsável por pequenos surtos na África e Ásia (ZELLER; VAN BORTEL; SUDRE, 2016) que tomaram novas proporções em 2004, com um aumento no número de infectados (MCSWEEGAN et al., 2015). Em 2005, um surto de CHIKV atingiu ilhas do oceano Índico (SCHUFFENECKER et al., 2006a), e em 2007, infecções com CHIKV foram registradas na França e Itália

Figura 6. Mapa da distribuição mundial do CHIKV. Esquema representando os países que já registraram casos de Febre Chikungunya de acordo com o CDC marcados em verde escuro.

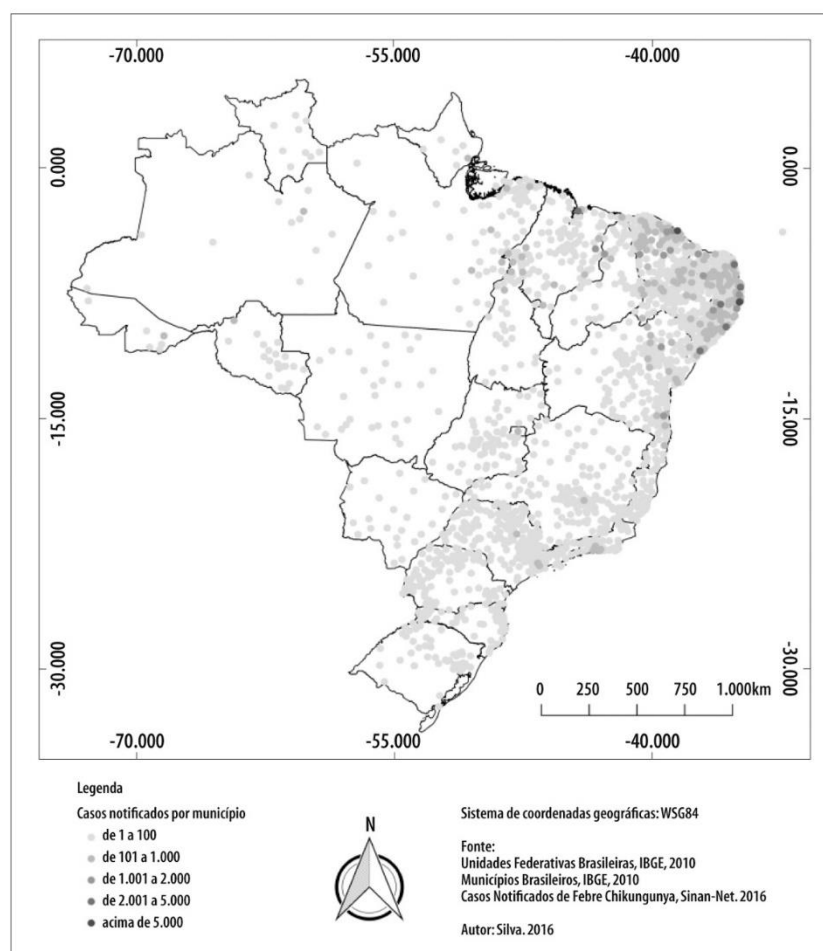


Estudos demonstraram que os surtos no sudoeste dos EUA e no Caribe estavam relacionados a uma mutação do genoma do vírus, no aminoácido 226V da proteína de envelope 1 (EV-1), que melhorou o *fitness* da replicação viral em mosquitos *A. albopictus*, facilitando a transmissão do vírus para o órgãos secundários do mosquito e a transmissão durante a hematofagia (TSETTSARKIN et al., 2007).

20

Salvador, Guiana Francesa, Nicarágua, Porto Rico e Suriname registraram vários casos de CHIKV e 169 mortes foram atribuídas à doença (WHO, 2013). Já no Brasil, de acordo com o boletim de monitoramento de arboviroses urbanas, foram registrados 132.205 casos prováveis de febre Chikungunya com uma taxa de incidência de 62,9 casos/100 mil habitantes no ano de 2019. Nordeste e Sudeste foram as regiões com maiores taxas de incidência, sendo 104,6 e 59,4 casos/ 100 mil habitantes, respectivamente. Rio de Janeiro e Rio Grande do Norte concentram 75,6% dos casos prováveis. No mesmo ano foram confirmados 92 óbitos por Chikungunya, sendo observada uma maior taxa de letalidade em idosos com mais de 60 anos (SAÚDE, 2020a). Em 2020, em meio ao surto de COVID-19, os estados brasileiros Bahia, Mato Grosso, Espírito Santo e Rio de Janeiro apresentaram um aumento nos casos prováveis de Chikungunya, de acordo com o último boletim lançado pela secretaria de vigilância. Bahia e Espírito Santo apresentam um aumento de 551,7 % e 1528 % no número de casos prováveis, respectivamente, se comparado ao mesmo período de 2019 (semana 1 a semana 14) (SAÚDE, 2020b).

Figura 7. Possíveis casos de Febre Chikungunya no Brasil. Notificação de possíveis casos de febre Chikungunya no Brasil registrados no SINAN-net (O Sistema de Informação de Agravos de Notificação).



Fonte: Retirado e adaptado de (SILVA et al., 2018)

1.9. Tratamento

Atualmente não há tratamento antiviral específico licenciado contra a febre Chikungunya. A única via terapêutica existente é paliativa e visa amenizar os sintomas provocados pela infecção. De acordo com a OMS, o medicamento mais indicado é o paracetamol 1 g de 3 a 4 vezes por dia para adultos, 50 a 60 mg por kg por dia para crianças (WHO, 2008). Outros analgésicos não esteroides também são recomendados, exceto a aspirina que pode provocar disfunções plaquetárias (PANNING et al., 2008). A ausência de um tratamento antiviral específico aponta a necessidade de estudos na pesquisa de moléculas com potencial para tratamento da Febre Chikungunya.

1.10. Atividade biológica de tiossemicarbazonas

Tiossemicarbazonas é uma classe de compostos conhecida por possuir uma síntese versátil, baixo custo de produção e pouco desperdício de átomos, visto que usualmente apenas moléculas de água são liberadas durante reações de síntese desses compostos (DU et al, 2002). Também são conhecidas por possuir uma excelente afinidade por metais como platina, cobre e cobalto, se ligando facilmente, e permitindo a síntese de organometálicos quelantes de alta qualidade (ANTONINI et al, 1989; CASAS, GARCÍA-TASENDE, SORDO, 2000; OLIVEIRA et al., 2014).

Dentre as atividades biológicas descritas na literatura em relação à essa classe de compostos associadas a metais, incluem a atividade antitumoral (CORY, 1988; LOUIE, MEADE, 1999), antimicrobiana (KASUGA et al, 2003), antiparasitária (BHARTI et al, 2002) e antiviral (TEITZ et al, 1994). Na maioria dos casos, as tiossemicarbazonas estão combinadas ao Cobre (II), um íon essencial, biologicamente ativo, com atividade quelante (KRÁL'OVÁ et al., 2004) e potencial redox positivo, que permite participação nas reações de transporte biológico (KRÁL'OVÁ et al., 2004). Além disso, o cobre (II) participa da formação do centro ativo de algumas metaloproteínas (BANASZAK, 2000). A combinação de íons metálicos com compostos ativos é defendida, uma vez que o cobre é mais tolerado no organismo devido as vias biológicas preexistentes que regulam os níveis do metal no corpo (WEST et al., 1993).

Levinson e colaboradores (1973) demonstraram que um complexo de tiossemicarbazona conjugado com cobre (II) é capaz de inibir a RNA polimerase

dependente de DNA e transformar o ciclo replicativo do vírus do Sarcoma de Rous (RSV) (LEVINSON et al., 1973). Um dos mecanismos de inibição de tiossemicarbazonas conjugadas com cobre parece estar relacionado a capacidade destes compostos inibirem a ribonucleotídeo redutase, uma enzima que participa da reação de síntese de DNA (LEVINSON et al., 1973; WEST et al., 1993).

O uso de cobalto (III) apresenta vantagem, uma vez que este metal de transição já está presente no organismo humano, e existem vias biológicas de regulação dos níveis deste metal. Como exemplo, o cobalto faz parte da estrutura da vitamina B12, que é essencial para o corpo humano, inclusive para síntese de DNA durante a divisão celular (BERTINI et al., 1994). Tiossemicarbazonas conjugadas já demonstraram atividade antitumoral (FAN et al., 2013), antioxidantes, com interação com DNA de células tumorais (RAMACHANDRAN et al., 2012), atividade antimicrobiana (HOFFMAN et al., 2013; MONDELLI et al., 2013), dentre outras. Até o momento, na literatura disponível não foram encontrados estudos que tenham demonstrado atividade antiviral contra o vírus Chikungunya para essa classe de compostos.

1.11 Objetivo

O presente trabalho teve como objetivo avaliar a atividade antiviral de Tiossemicarbazonas conjugadas com cobalto (III) no ciclo replicativo do vírus Chikungunya *in vitro* e possíveis mecanismos de ação.

1.12 Objetivos específicos

- Avaliar a viabilidade celular no tratamento com tiossemicarbazona e seus precursores nas linhagens celulares Vero, Huh 7.0 e Células de Rim de Hamster bebe (BHK21), por ensaio de viabilidade celular (MTT), e estabelecer as concentrações viáveis para os ensaios antivirais.
- Avaliar a atividade antiviral do composto e seus precursores no ciclo replicativo do CHIKV *in vitro* utilizando a máxima concentração não tóxica;
- Determinar a concentração efetiva que inibe 50 % do CHIKV (EC_{50}), concentração citotóxica que mata 50 % das células (CC_{50}) e índice de seletividade ($SI = CC_{50}/EC_{50}$), para determinar uma faixa de concentração ótima para o tratamento;

- Determinar a etapa do ciclo replicativo inibida e os possíveis mecanismos de ação das moléculas ativas.

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2. Cobalt-conjugated Thiosemicarbazone inhibits chikungunya replication *in vitro* by interacting with RNA-dependent RNA polymerase (nsP4)

Daniel Oliveira Silva Martins^{1,2}, Laísa de Paula Oliveira³; Marjorie Caroline Liberato Cavalcanti Freire⁴, Nathalya Cristina de Moraes Roso Mesquita⁴, Nilson Nicolau Junior⁵, Raphael Enoque Ferraz de Paiva⁶, Robinson Sabino da Silva⁷; Andres Merits⁸; Mark Harris⁹; Carolina Gonçalves Oliveira³; Ana Carolina Gomes Jardim^{1,2*}

¹São Paulo State University, IBILCE, São José do Rio Preto, SP, Brazil

²Laboratory of Virology, Institute of Biomedical Science, Federal University of Uberlândia, MG, Brazil

³Bioinorganic Chemistry Group, Institute of Chemistry, Federal University of Uberlândia, MG, Brazil

⁴Physics Institute of São Carlos, University of São Paulo, São Carlos, SP, Brazil.

⁵Molecular Modeling Laboratory, Institute of Biotechnology, Federal University of Uberlândia, Uberlândia, Brazil

⁶Department of Fundamental Chemistry, Institute of Chemistry, University of São Paulo, SP, Brazil.

⁷Department of Physiology, Institute of Biomedical Sciences, Federal University of Uberlândia, Minas Gerais, Brazil

⁸Institute of Technology, University of Tartu, Tartu, Estonia

⁹School of Molecular and Cellular Biology, Faculty of Biological Sciences and Atsburry Centre for Structural Molecular Biology, University of Leeds, Leeds, United Kingdom

***Corresponding author:** Professor Ana Carolina Gomes Jardim, Institute of Biomedical Science, ICBIM/UFU, Avenida Amazonas, 4C- Room 216, Umuarama, Uberlândia, Minas Gerais, Brazil, CEP: 38405-302. **Tel:** +55 (34) 3225—8682 **E-mail:** jardim@ufu.br

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Abstract

Chikungunya virus (CHIKV) is a single positive strand RNA virus from the *Togoviridae* family that is transmitted through the bite of the female mosquito *Aedes* sp. CHIKV infected patients are affected by Chikungunya fever disease which presents fever and joint pains as clinical symptoms. Additionally, patients may develop a chronic disease that can lead to a disability. Currently, there is no antiviral treatment against CHIKV, demonstrating the necessity to develop novel antiviral agents. In this context, some studies have shown that thiosemicarbazones and their metal complexes possess pharmacological properties as antitumor, antiprotozoal, antibacterial and antiviral activity. The synthesis of these compounds is simple, clean, versatile and results in high yields. Although there is no anti-CHIKV activity described in the available literature of this compounds against CHIKV. Therefore, this work aimed to evaluate the antiviral activity of Cobalt (III) - conjugated thiosemicarbazone (CoAtc-Ph) and its metal precursors against CHIKV *in vitro*. For this, BHK 21 cells were infected with CHIKV *nanoluciferase* (M.O.I 0.01) in the presence or absence of each compound at non-cytotoxicity concentration for 16 hours. Through this assay, CoAtc-Ph at 50 μM inhibited 94% of CHIKV infection. Using a wider range of concentrations, it was determined that the compound has an EC_{50} of 19.02 μM , CC_{50} of 62.08 μM and SI of 3.26. Additional assays demonstrated that CoAtc-Ph inhibited CHIKV post entry to the host cells. Possible mechanisms of antiviral action were investigated by evaluating the intercalation of this compound into the viral dsRNA and, its interaction with chikungunya non-structural protein 4 (nsP4). CoAtc-Ph did shown strong interaction with nsP4 suggesting that this compound may inhibits CHIKV cycle through this. Also *in silico* analyses demonstrated that CoAtc-Ph intereracted with nsP4 through hydrogen bonds, hydrophobic and electrostatic interactions. Considering the interaction of CoAtc-Ph with nsP4 and its interaction through *in silico* analyses we can suggest that this may be the action mechanism of this compound.

2.1 Introduction

The Chikungunya virus (CHIKV) is the etiological agent of the chikungunya fever, a tropical disease characterized by high fever, rash, joint pain and arthralgia (GOULD et al., 2017). Infection caused by this virus can progress to a disabling chronic disease or cause neurological complications as Guillain-Barre syndrome (PAIXÃO et al., 2018; STEGMANN-PLANCHARD et al., 2019).

CHIKV is a mosquito-borne Alphavirus that belongs to the family *Togaviridae* (ICTV, 2019). It is an enveloped virus of icosahedral capsid and a positive strand RNA genome of approximately 12 kb (KHAN et al., 2002). CHIKV genome contains two open reading frames (ORFs)(KHAN et al., 2002). The 5' ORF encodes four non-structural proteins (nsP1 – nsP4) and the 3' ORF encodes a subgenomic RNA which encodes the five structural proteins (C, E1, E2, E3 and 6K) (Kumar et al., 2015;Voss et al., 2010)).

The virus is transmitted through the bite of *Aedes* mosquitoes, it was first isolated in Tanzania in 1952 and for a while, cases related to CHIKV occurred in the Southeast Asian regions, Africa and Oceania (ROSS, 1956). In 2006 an outbreak was documented in some Indian Ocean islands (SCHUFFENECKER et al., 2006) and, a year later, France and Italy reported cases of this infection (GRANDADAM et al., 2011; REZZA et al., 2007). In 2013 an outbreak occurred in the Central America, in the Caribbean Islands (MOWATT; JACKSON, 2014; YACTAYO et al., 2016). Then, the virus spread through the North and South America including Brazil and United States of America (VEGA-RUA et al., 2014). In Brazil, according to the Urban Arboviruses Monitoring Report, in 2019 132,205 cases of CHIK fever were registered with an incidence rate of 62.9cases/100,000 inhabitants in 2019. Northeast and Southeast were the regions with the highest incidence rates, 104.6 and 59.4 cases/100,000 inhabitants, respectively. In the same year 92 deaths were confirmed by Chikungunya, and a higher lethality rate was observed in elderly people over 60 (SAÚDE., 2020a). In 2020, amid the outbreak of COVID-19, the Brazilian states of Bahia, Mato Grosso, Espírito Santo and Rio de Janeiro showed an increase in likely cases of Chikungunya, according to the latest bulletin released by the surveillance secretary (SAÚDE., 2020b) According to CDC (Centers for Disease Control) more than 90 countries reported CHIKV infection cases(CDC, 2019).

Currently there is no licensed vaccine or treatment against CHIKV showing the need for molecules that possess antiviral activity (DEY et al., 2019). The treatment of infected patients is palliative and aims to relief the symptoms caused by the infection. According to the World

Health Organization (WHO), the most appropriate drug is acetaminophen 1 g 3-4 times per day for adults, 50-60 mg per kg per day for children (WHO, 2008). Other non-steroidal analgesics are also recommended, except aspirin which can cause platelet dysfunction (PANNING et al., 2008).

Thiosemicarbazones is a class of synthetic compounds known to have a versatile synthesis, low production costs and few atoms waste (only molecules of water are usually released during synthesis reactions) (DU et al., 2002). These compounds are also known as excellent metal binders, providing high quality chelating organometallics (ANTONINI et al., 1981; CASAS; GARCÍA-TASENDE; SORDO, 2000). This class is interesting for chemical and biological studies due to the aspects described previously, but also because they present ribonucleotide reductase inhibitory activity (RR). This enzyme is being highly studied as a target for cancer treatment (CORY, 1988; LOUIE; MEADE, 1999).

Thiosemicarbazones are also known to possess antimicrobial (KASUGA et al., 2003), antiprotozoal (BHARTI et al., 2002) and antiviral activities (TEITZ et al., 1994). Teitz and coworkers (1994) described Human Immunodeficiency virus (HIV) antiviral activity of N-methylisatin- β 4':4'-diethylthiosemicarbazone, a compound that demonstrated to disrupt the synthesis of HIV structural proteins. This class of compound has also antiviral activity described against herpes simplex virus (HSV), affecting expression of structural proteins of HSV, suppressing capsid assembly and inhibiting cell-to-cell spread (GENOVA et al., 2004). Usually thiosemicarbazones are conjugated with transition metals such as copper (II) and cobalt (III). These metals are present in the human organism, therefore, there are pre-existing biological pathways that will regulate the levels of these metals. It can increase the bioavailability of these conjugated compounds, decrease the cytotoxicity and even improve their biological activity for which it is proposed (WEST et al., 1993).

Considering the absence of antiviral treatment against chikungunya, this work aimed to evaluate the antiviral activity of thiosemicarbazones conjugated with cobalt (III) on CHIKV infection *in vitro*. Our data showed that a Cobalt (III) - conjugated thiosemicarbazone may provide an interested source of compounds to the development of future antivirals to treat Chikungunya fever.

2.2 Material and methods

2.2.1 Compounds

The synthesis of the thiosemicarbazone and its precursors evaluated in this work was previously described (OLIVEIRA et al., 2014). In summary, a reaction with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (**Figure 1A**) and 4-phenyl-3-thiosemicarbazide (HaTc-Ph) (**Figure 1A**) was performed aiming to produce cobalt (III) conjugated thiosemicarbazone (CoAtc-Ph) (**Figure 1A**). The reaction product was characterized by Fourier-transform infrared spectroscopy (FTIR), elemental analysis, UV visible and ^1H NMR spectroscopies, cyclic voltammetry (CV), conductimetry measurements and single crystal X-Ray diffractometry according to (OLIVEIRA et al., 2014). The compounds were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C . Dilutions of the compounds in complete medium were made immediately prior to the experiments to reach a maximum final concentration of 0.1% DMSO. For all the assays performed, control cells were treated with medium added of DMSO at the final concentration of 0.1%.

2.2.2 Cell culture

Baby Hamster Kidney (BHK 21- ATCC® CCL-10™) cells, which are susceptible to CHIKV infection (DAVIS; HODGE; CAMPBELL, 1971), were maintained in Dulbecco's modified Eagl's medium (DMEM; Sigma-Aldrich) supplemented with 100U/mL of penicillin (Hyclone Laboratories, USA), 100 mg/mL of streptomycin (Hyclone Laboratories, USA), 1% of non-essential aminoacids (Hyclone Laboratories, USA) and 1% of fetal bovine serum (FBS, Hyclone Laboratoires, USA) in a humidified 5% CO_2 incubator at 37°C .

2.2.3 Virus

The CHIKV-Nanoluciferase (CHIKV *nanoluc*) construct (**Figure 1B**) used for the antiviral assays was designed from a CHIKV sequence based on CHIKV LR (Lá-reunion) added of CMV promoter and *nanoluciferase* protein sequence (MATKOVIC et al., 2018; POHJALA et al., 2011). . For virus production, 2.3×10^7 BHK 21 cells seeded in a cell culture flask were transfected with 1.5 μg of CHIKV-CMV-*nanoluc* plasmid, using lipofectamine 3000 ® and Opti-Mem medium to produce CHIKV *nanoluc*. Forty-eight hours post transfection the supernatant was collected and stored at -80°C .

To determine viral titer, 5×10^5 BHK 21 cells were seeded in each of 6 wells plate 24 hours prior to the infection. Then, the cells were infected with 10-fold serially diluted of CHIKV *nanoluc* for 1h at 37°C. The inoculum was removed and the cells were washed with phosphate buffered saline (PBS) to remove the unbound virus and added of cell culture media supplemented with 1% penicillin, 1% streptomycin, 2% FBS and 1% carboxymethyl cellulose (CMC). Infected cells were incubated for 2 days in a humidified 5% CO₂ incubator at 37°C, followed by fixation with 3% formaldehyde and stained with 0.5% violet crystal and the viral foci were counted to determine CHIKV *nanoluc* titer (BAER; KEHN-HALL, 2014).

2.2.4 Cell viability through MTT assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) assay. BHK 21 cells were cultured in 96well plate and treated with concentrations 50 μ M, 10 μ M e 2 μ M of each compound for 16h at 37°C with 5% of CO₂. Twenty-four hours post treatment, compound-containing media was removed and MTT solution at 1 mg/mL was added to each well, incubated for 1 hour and replaced with 100 μ L of DMSO to solubilize the formazan crystals.. The absorbance was measured at 560 nm on Glomax microplate reader (Promega, USA). Cell viability was calculated according to the equation $(T/C) \times 100\%$, which T and C represented the optical density of the treated well and control groups, respectively. DMSO was used as untreated control. The cytotoxic concentration of 50% (CC₅₀) was calculated using Prims 8.0 (Graph Pad).

2.2.5 Antiviral activity assays

All experiments were conducted with M.O.I of 0.01, to access the antiviral activity of compounds, BHK 21 cells were seeded at density of 5×10^4 cells per well into 48-well plates 24 h prior to the infection. CHIKV *nanoluc* (MATKOVIC et al., 2018) at a multiplicity of infection (M.O.I) of 0.01 and compounds at the non-citotoxicity concentration were simultaneously added to cells. Samples were harvested in Renilla luciferase lysis buffer (Promega, USA) at 16 hours post-infection (h.p.i.) and virus replication levels were quantified by measuring *nanoluciferase* activity using the Renilla luciferase Assay System (Promega, USA). The effective concentration of 50% inhibition (EC₅₀) was calculated using Prims 8.0

(Graph Pad). The values of CC_{50} and EC_{50} were used to calculate the selectivity index ($SI = CC_{50}/EC_{50}$).

To investigate in which step of CHIKV replicative cycle the compound is active, BHK 21 cells at the density of 5×10^4 were seeded in 48 well plate 24 hours prior to infection and treatment. To evaluate if the compound possesses protective activity to the host cells, cells were treated for 1 hour with the compound at the non-citotoxic concentration selected according to the cell viability assay, before infection, extensively washed with PBS to remove compound and added CHIKV *nanoluc*. The effect on early stages was analyzed by incubating virus and compound simultaneously with BHK 21 cells for one hour. Then, compound was removed and cells added with complete medium. Additionally, to investigate the activity of the compound on post-entry stages of viral replicative cycle, cells were infected with CHIKV for 1 hour, washed extensively with PBS (phosphate buffered saline) to remove unabsorbed virus and added with compound media. M.O.I Sixteen h.p.i., luminescence was measured to analyze the virus replication rates.

2.2.6 Intercalation assay

To investigate whether the compound interacts with the double-stranded RNA (dsRNA), an experiment using the previously described protocol was performed (CAMPOS et al., 2017b). Briefly, fifteen nanograms of the dsRNA were incubated with 50 μ M of CoAtc-Ph at room temperature for 45 min and electrophoresed on a 1% agarose gel prior to analysis by densitometry. DMSO and Doxorubicin (DOX) at 100 μ M were used as negative and positive control, respectively.

2.2.7 CHIKV nsP4 cloning, overexpression and purification:

Aiming to access the interaction between chikungunya non-structural protein 4 (nsP4), the coding region of nsP4 was cloned into pET-SUMO expression vector, generating the nsP4_pET-SUMO/LIC expression vector. Rosetta (DE3) *E. coli* (Novagen) cells were transformed with nsP4_pET-M11/LIC and grown in TB medium, supplemented with 50 μ g.ml⁻¹ kanamycin and 34 μ g.ml⁻¹ chloramphenicol at 37 °C, until the OD_{600} reached 1.0. The protein expression was induced by adding 1 mM of Isopropyl β -D-1-thiogalactopyranoside (IPTG), at 18 °C for 16 h. Cells were harvested by centrifugation and cell pellets were resuspended in 50 mM Tris pH 8.0, 500 mM NaCl and 10% glycerol. Cells were lysed by sonication and cell debris was separated by centrifugation. The nsP4 was purified using an AKTA Purifier System (GE Healthcare). The first purification step was an affinity chromatography using a HisTrap

HP 5.0 mL column (GE Healthcare). Concomitantly, the buffer was exchanged through dialysis and the His-tag-SUMO was cleaved by TEV protease during overnight at 4 °C. Another affinity chromatography was performed using the same system to collect the protein after cleavage. The protein was concentrated and a final purification step was done through size-exclusion chromatography on a XK 26/1000 Superdex 200 column (GE Healthcare) pre-equilibrated in buffer 50 mM Tris pH 8.0, 200 mM NaCl and 10% glycerol. The final protein sample was analyzed in SDS-PAGE 12.5% to confirm its purity. Concentration was determined spectrophotometrically in a Nanodrop 1000 spectrophotometer.

2.2.8 MicroScale Thermophoresis (MST)

Experiments were performed on a Monolith® NT.115 (Nanotemper technologies). The nsP4 was labelled on cysteine residues with NT-647-Maleimide dye (Nanotemper Technologies) using the Monolith NTTM Protein Labeling Kit RED-MALEIMIDE as per manufacturer's instructions. The concentration of protein indicated for MicroScale Thermophoresis experiments was 25 nM and a serial dilution of the compound Co(atc-Ph)₂Cl from 500 µM to 0.015 µM (15 nM). The dissociation constant K_d was obtained by fitting the binding curve with the Hill function.

2.2.9 *In silico* analyses

Aiming to simulate the interaction between chikungunya NSP4 and the molecule CoAtc-Ph, a database representative sequence of the NSP4 extracted from the virus polyprotein (uniprot id: Q8JUX6) was modelled using the I-TASSER (ZHANG, 2008) online server. The NSP4 best model (model 1) was chosen and the binding site was predicted using COACH (YANG; ROY; ZHANG, 2013) based on the I-TASSER structure prediction. COACH is a meta-server approach that combines multiple function annotation results generating ligand binding site predictions. The best prediction indicates a binding site similar to the site where the pyridazinone inhibitor interacts with the crystal structure of HCV NS5B polymerase (PDBid: 3CDE). Thus, the modelled protein was docked with the molecule CoAtc-Ph using GOLD program (JONES et al., 1997). GOLD performs a search for the best pose of the chosen molecule in the receptor binding site using genetic algorithm (GA) and the score ChemPL. The binding site was defined by pyridazinone inhibitor position based on the 3CDE structure and extrapolated to the modelled NSP4. The GA run parameters were maintained at default. The poses generated are then ranked and the solution with the best score was chosen. The interaction

on the best solution were analyzed using the program DS Visualizer (BIOVIA, Dassault Systèmes, Discovery Studio Visualizer, v 20.1, San Diego: Dassault Systèmes, 2020).

2.2.10 Statistical analysis

Individual experiments were performed in triplicate and all assays were performed a minimum of three times in order to confirm the reproducibility of the results. Differences between means of readings were compared using analysis of variance (one-way or two-way ANOVA) or Student's t-test using Graph Pad Prims 8.0 software (Graph Pad Software). P values $\leq$ than 0.05 were considered to be statistically significant.

2.3 Results

CoAtc-Ph and its precursor HaTc-Ph inhibit CHIKV infectivity

A thiosemicarbazone (HaTc-Ph), the ligand Cobalt (III) ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) and the conjugated complex (CoAtc-Ph) (**Figure 1A**) were screened by their antiviral activity by using a recombinant CHIKV engineered to express *nanoluciferase* reporter (**Figure 1B**). First, BHK 21 cells were treated with each compound at 50, 10 e 2 μM for 16 hours and cell viability was measured through an MTT assay to access cytotoxicity (**Figure 1C**). Then, BHK 21 cells were infected with CHIKV *nanoluc* at a M.O.I of 0.01 and simultaneously treated with HaTc-Ph at 2 μM , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ at 10 μM or CoAtc-Ph at 50 μM (cell viability $\geq$ 80%). Nanoluciferase activity levels, proportional to viral replication, were assessed 16 hours post infection (h.p.i). The results demonstrated that CoAtc-Ph at 50 μM showed strong antiviral activity inhibiting 94% of CHIKV infection (**Figure 1D**). The anti-CHIKV activity of CoAtc-Ph seems to be related to its precursor HaTc-Ph (**Figure 1D**). however, the results demonstrated that the complexation significantly affected the therapeutic index of compound (favorable ratio of cytotoxicity to antiviral potency) and the complex inhibited CHIKV replication at 94% *in vitro*. It was mainly observed comparing the concentrations of treatment of the free ligand or complexed molecule. It seems that cells tolerated higher concentrations of the HaTc-Ph when complexed to the metal, than as free molecule. This fact suggests that the metal ion play a role on the mechanism of action of the compounds and its delivery to the cell. Therefore, only CoAtc-Ph was selected for extra analysis. Using serial dilutions ranging from 300 to 0.14 μM of CoAtc-Ph, it was determined that the compound has an EC50 of 19.02 μM , CC50 of 62.08 μM and SI of 3.26 (**Figure 2**).

CoAtc-Ph inhibits post-entry of the CHIKV replication cycle

To analyze the effects of CoAtc-Ph on different stages of the CHIKV replicative cycle, virus and compounds were added in BHK 21 cells at different times. First, cells were pre-treated with CoAtc-Ph for 1h, then were washed extensively with PBS to completely remove the compound and were infected with CHIKV-nanoluc. Luminescence levels were measured 16 h.p.i. (**Figure 3A**). For early stages, CHIKV *nanoluc* and CoAtc-Ph were simultaneously added to the BHK 21 cells for 1 hour, following repeated cells washes with PBS and addition of DMEM with 2% of FBS. Luminescence levels were measured 16 h.p.i. (**Figure 3B**). For post-entry steps, cells were first infected with CHIKV *nanoluc* for 1 hour at 37°C, washed extensively to the removal of unbound virus, and then added of media-containing compound. Luminescence levels were measured 16 h.p.i. (**Figure 3C**). The results demonstrated that CoAtc-Ph at 50 μ M inhibited up to 94% of the CHIKV post-entry replication step (**Figure 3D**), but had no effect on early stages or on the host cells due to the pre-treatment with the compound, in these cases the replication levels remained close to 100%. Based on these results, we investigated whether this compound could intercalate into the viral dsRNA or if it has interaction with chikungunya non structural protein 4 (nsP4) *in vitro*..

During the chikungunya replicative cycle, a double-strand RNA (dsRNA) is formed as replicative intermediate molecules. Taking into account that some compounds possess antiviral activity intercalating on this dsRNA (CAMPOS et al., 2017a), CoAtc-Ph was incubated with 15 nM of dsRNA. Doxorubicin (DOX) at 100 μ M and DMSO 0,1% were used as positive and untreated controls, respectively. Obtained RNA/compound complexes were analyzed in 1% agarose gel. Since CoAtc-Ph had no intercalation activity, ethidium bromide was able to intercalate to the dsRNA and the band was visualized as the one in the untreated control (**Figure 4**). Densitometry analysis confirmed that the compound did not intercalate with dsRNA. (

Aiming to assess the binding affinity between Chikungunya non-structural protein 4 (nsP4) we have had synthesized the protein and purify it. The assessment of nsP4 binding affinity for the CoAtc-Ph and HaTc-Ph was performed by MST, using a serial dilution of the compound to obtain information about the dissociation constant (k_d) and estimate the possible interaction between them. The results obtained for Co(atc-Ph)₂Cl (**Figure 5B**), where it is possible to observe the presence of the bound and unbound states well defined, suggesting the occurrence of interaction between the protein and the compound. Through the obtained curve, the estimated $k_d \pm \Delta k_d$ for this interaction was $8.35 \pm 0.681 \mu$ M. However, for the compound Hatc-Ph, it was

not possible to obtain a pattern of bound and unbound states (**supplementary data**), suggesting that there is no interaction between them. In view of these results, it was not possible to adjust the curve or estimate the $k_d \pm \Delta k_d$.

Trying to better understand how this interaction happens, we made a prediction of the nsP4 protein structure using the I-TASSER method. A root mean square deviation (RMSD) result, obtained after modelling by the I-TASSER server (**Figure 5C**), indicates that the *in silico* modelled CHIKV nsP4 has a clear structural similarity (1.81 Å) with the X-ray structure of the NS5B, an also RNA-dependent RNA polymerase (RdRp) from a classical swine fever virus (PDBid: 5Y6R). The nsP4 was then submitted to docking with the CoAtc-Ph ligand and the best scored complex (ChemPL = 57.49) were selected (Figure 2). The complex ligand interaction analysis (**Figure 5D**) showed hydrogen bonds with G507 e K531 amino acids; hydrophobic interaction with V296, A350, C506, K527, K531 and W557; eletrectostatic interactions (pi-charge) with K531 and E553; and pi-sulfur interaction with C506 and W557.

2.4 Discussion

CHIKV is an emerging arbovirus with high impact to the public health in tropical and non-tropical areas. In the recent year, outbreaks have occurred around the world affecting many patients with chikungunya fever (MOHAN et al., 2010; PAIXÃO et al., 2018; QUEYRIAUX et al., 2008).

Currently there is no antiviral therapy against CHIKV. The treatment of infected patients is palliative and aims to relief the symptoms caused by the infection, including rest and hydration (PANNING et al., 2008; WHO, 2008). It demonstrates the need to develop compounds with antiviral activity against CHIKV (DEY et al., 2019). Thiosemicarbazones are known to possess antitumoral, antiprotozoal, antimicrobial and antiviral activity, and there is no data shown on the anti-CHIKV activity of this class of compounds (BHARTI et al., 2002; CASAS; GARCÍA-TASENDE; SORDO, 2000; GENOVA et al., 2004; KASUGA et al., 2003; OLIVEIRA et al., 2014).

In this study we aimed to analyze the antiviral activity of a thiosemicarbazone conjugated to Cobalt (III) metal complexes and its precursors against CHIKV. Our data demonstrated that the thiosemicarbazone CoAtc-Ph showed strong anti-CHIKV, which seems to be related to its precursor HaTc-Ph. The CoAtc-Ph exhibited the best therapeutic index (favourable ratio of cytotoxicity to antiviral potency) and was selected for extra analysis. We investigated the

antiviral activity of this compound on CHIKV early stages and post-entry steps, and also the effect of the pre-treatment on the host cells. The results showed that CoAtc-Ph inhibited the post entry steps of CHIKV. Thiosemicarbazone had their antiviral activity described against HIV and HSV disrupting the synthesis of structural proteins and also capsid assembly (CASAS; GARCÍA-TASENDE; SORDO, 2000; GENOVA et al., 2004). The compound Harringtonine is described to inhibit the early stages of replication of CHIKV *in vitro* inhibiting RNA and structural proteins expression (KAUR et al., 2013).

In order to achieve this compound mechanism of action we performed a dsRNA intercalation assay. During CHIKV replication cycle, a dsRNA is formed as a replicative intermediate molecule during the synthesis of a negative sense sRNA which is a template for the next viral replication stages. Considering that some compounds that acted on post-entry stages of virus replicative cycle demonstrated antiviral activity intercalating in dsRNA species (CAMPOS et al., 2017), we investigated the interference of CoAtc-Ph with a dsRNA, however, CoAtc-Ph did not act by this mechanisms *in vitro*. As previously mentioned, we found that the CoAtc-Ph inhibits chikungunya in post-entry stages. Knowing that replication is a very important step in the virus replication cycle, we decided to test the interaction of the compound with non-structural protein 4 (nsP4). The nsP4 plays a very important role in the replicative cycle of CHIKV, is an RNA-dependent RNA polymerase (RdRp) responsible for the synthesis of new RNA strands that will be incorporated into the viral progeny resulting from the replicative cycle. As it is crucial for viral replication the RdRp it is an important target for antiviral therapies. Many studies showed compound with antiviral activity against CHIKV on replication, but only a few were documented on nsP4 target., for example Benzimidazole (WADA et al., 2017), hespertin-A derivatives (OO et al., 2016) and, favipiravir (DELANG et al., 2014) Aiming to analyse if CoAtc-Ph played a role interaction with nsP4 we have had synthetised and tested its interaction through a MST assay. The results showed that the compound may interact with this CHIKV non-structural protein suggesting that this might be a mechanism of action. *In silico* analysis showed that the compound interact with CHIKV nsP4 through electrostatic hydrogen and electrostatic interactions.

In summary, we have shown that the CoAtc-Ph inhibits post-entry stage of CHIKV infection. For the better of our knowledge, there is no description of the antiviral activity of thiosemicarbazone conjugated with cobalt (III) against CHIKV in the available literature, showing the relevance and ineditism of the data presented in this article. Therefore, data

obtained in this study can contribute to the development of future drugs to treat Chikungunya fever lowering the social and economic impact of this virus in our society.

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APÊNDICE A. Estrutura dos compostos, sequencia CHIKV *nanoluc*, gráfico ensaio de viabilidade e infecção geral.

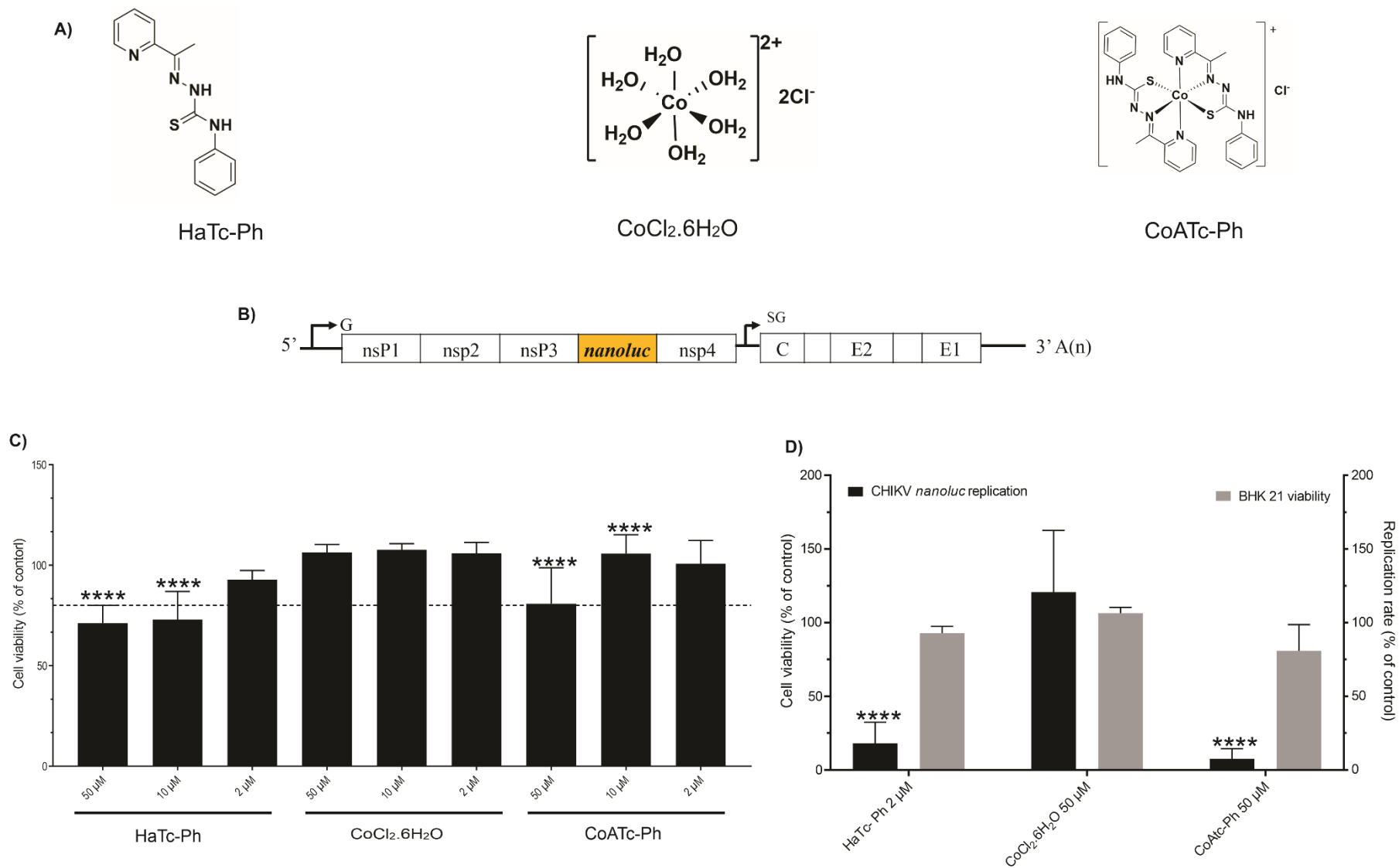


Figure B. Anti-CHIKV activity of CoAtc-Ph and its precursors. (A) Compounds structure, precursors HaTc-Ph and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and the Thiosemicarbazone complex with cobalt (III) CoAtc-Ph . (B) Engineered Chikungunya nanoluciferase (CHIKV nanoluc) sequence. (C) BHK 21 cells were treated with each compound at 50, 10 e 2 μM for 16h. After treatment, the medium with compound was removed and replaced with medium with MTT 1 mg/mL and incubated for 30 min. After incubation, the medium was removed and the crystals solubilized with DMSO. Cell viability was measured by MTT assay (absorbance 490 nm). (D) BHK 21 cells were infected with CHIKV *nanoluc* and simultaneously treated with compound. After 16 h.p.i, cells were lysed and a renilla luciferase assay was performed to assess CHIKV replication rates. In parallel a MTT assay was performed. Mean values of a minimum of three independent experiments each measured in triplicate are represented. **** $P < 0.001$ / * $P < 0.05$ were considered significant.. **From: MARTINS, et al 2020.**

APÊNDICE B. Gráfico ensaio de EC₅₀ e CC₅₀.

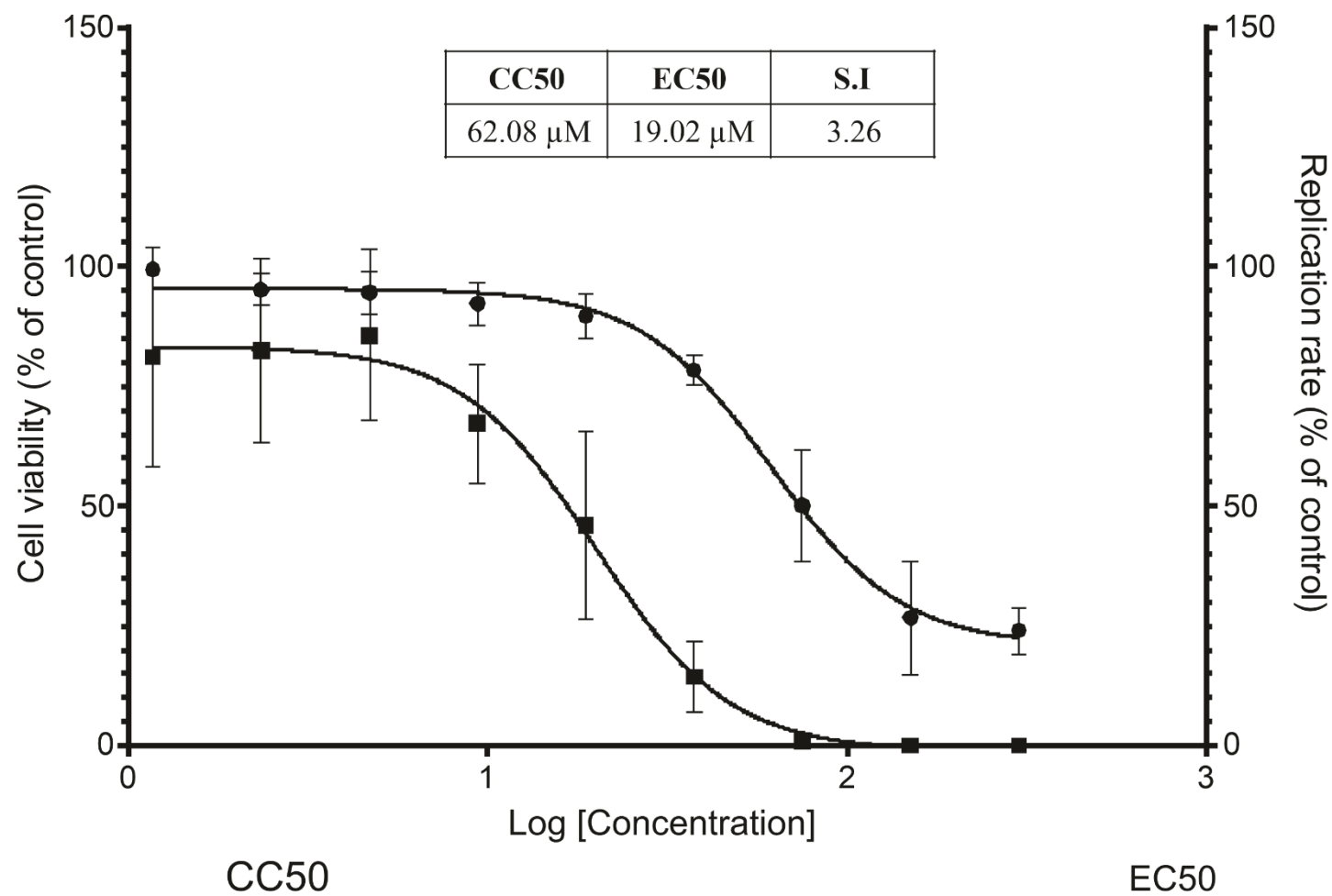


Figure D. The effective concentration (EC₅₀) and cytotoxic concentration (CC₅₀) of CoAtc-Ph. BHK-21 cells were treated with increasing concentrations of CoAtc-Ph ranging from 0.14 to 300 µM. CHIKV replication was measured by luciferase assay (indicated by □) and cellular viability measured using an MTT assay (indicated by •). Mean values of three independent experiments each measured in quadruple including the standard deviation are shown. ***From: MARTINS, et al 2020.***

APÊNDICE C. Gráfico e esquema do ensaio para testar a atividade do composto em diferentes etapas da infecção viral.

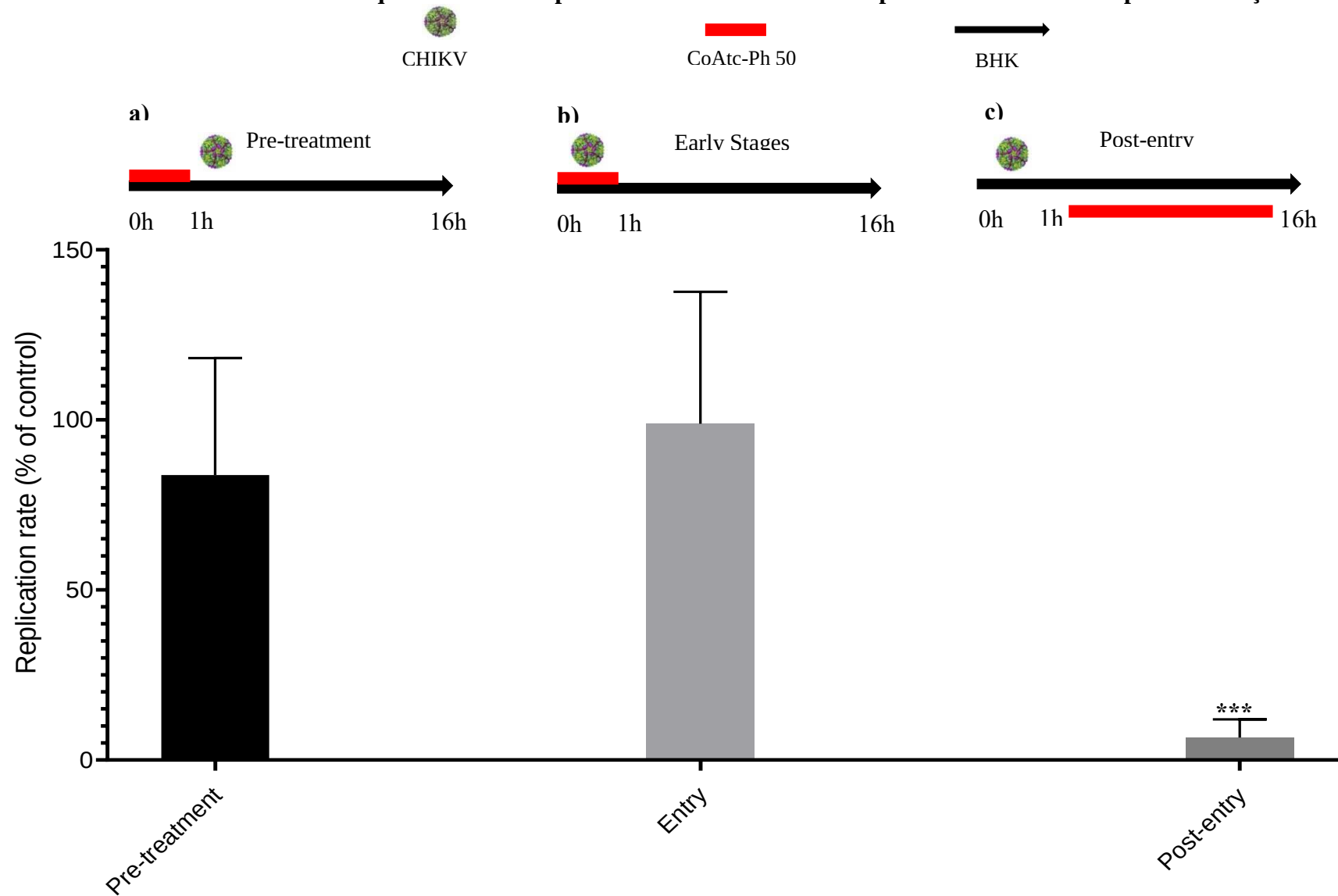
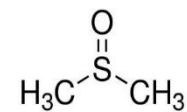
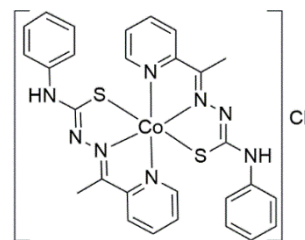
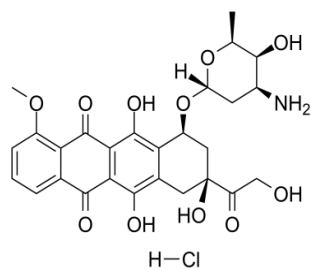
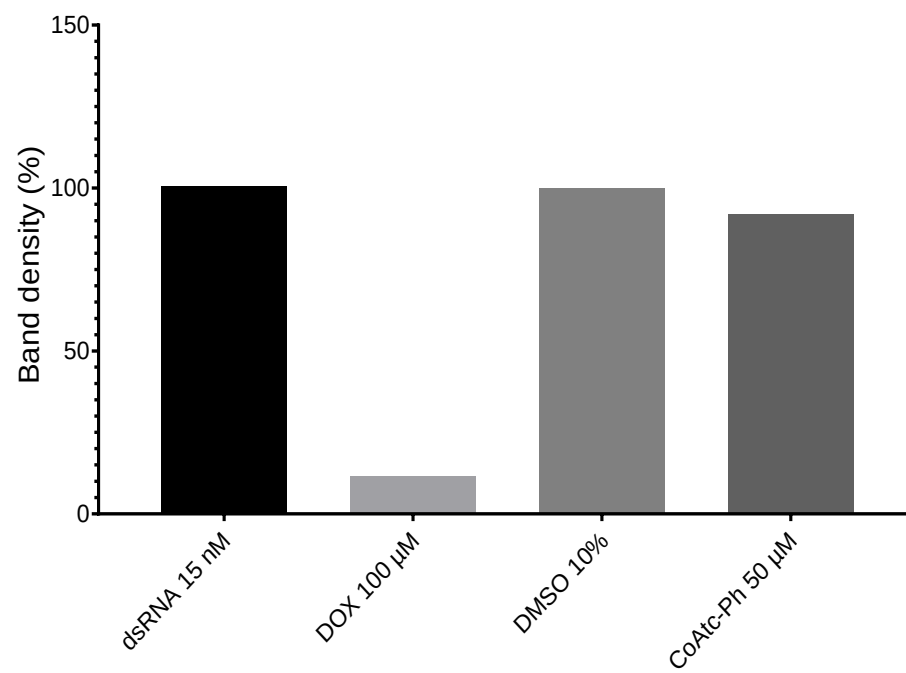


Figure F. CoAtc-Ph activity on different stages of the CHIKV replicative cycle. (A) BHK 21 cells were pre-treated with CoAtc-Ph for 1h, then were washed to completely remove the compounds and were infected with CHIKV *nanoluc* (M.O.I = 0.01). Luminescence levels were measured 16 h.p.i (B) BHK 21 cells were infected with CHIKV *nanoluc* (M.O.I = 0.01) and simultaneously treated with CoAtc-Ph 50 μ M for 1 hour. Then, cells were washed to remove the virus and compound and were replaced with fresh media. (C) BHK 21 cells were infected with CHIKV *nanoluc* (M.O.I 0.01) for 1h, washed to remove unbound virus and added of fresh media with the compound. (D) CHIKV replication rates under the treatment of CoAtc-Ph in different stages of replicative cycle. Mean values of a minimum of three independent experiments each measured in triplicate. $P < 0.001$ were considered significant. **From: MARTINS, et al 2020.**

APÊNDICE D. Ensaio de Intercalação de dsRNA.



dsRNA +



dsRNA +



dsRNA +

Figure 4. dsRNA CoAtc-Ph intercalation assay. CoAtc-Ph was incubated with 15 nM of dsRNA for 45min. DMSO 0,1% and Doxorubicin (DOX) at (100 μ M was used as untreated and positive controls, respectively. Samples were submitted to a 1% agarose (TAE) stained with ethidium bromide electrophoresis gel followed by densitometry analysis. ***From: MARTINS, et al 2020.***

APÊNDICE E. Purificação e expressão de nsP4 de CHIKV. Interação biofísica e in silico de nsP4 com tiossemicarbazona conjugada com Co(III)

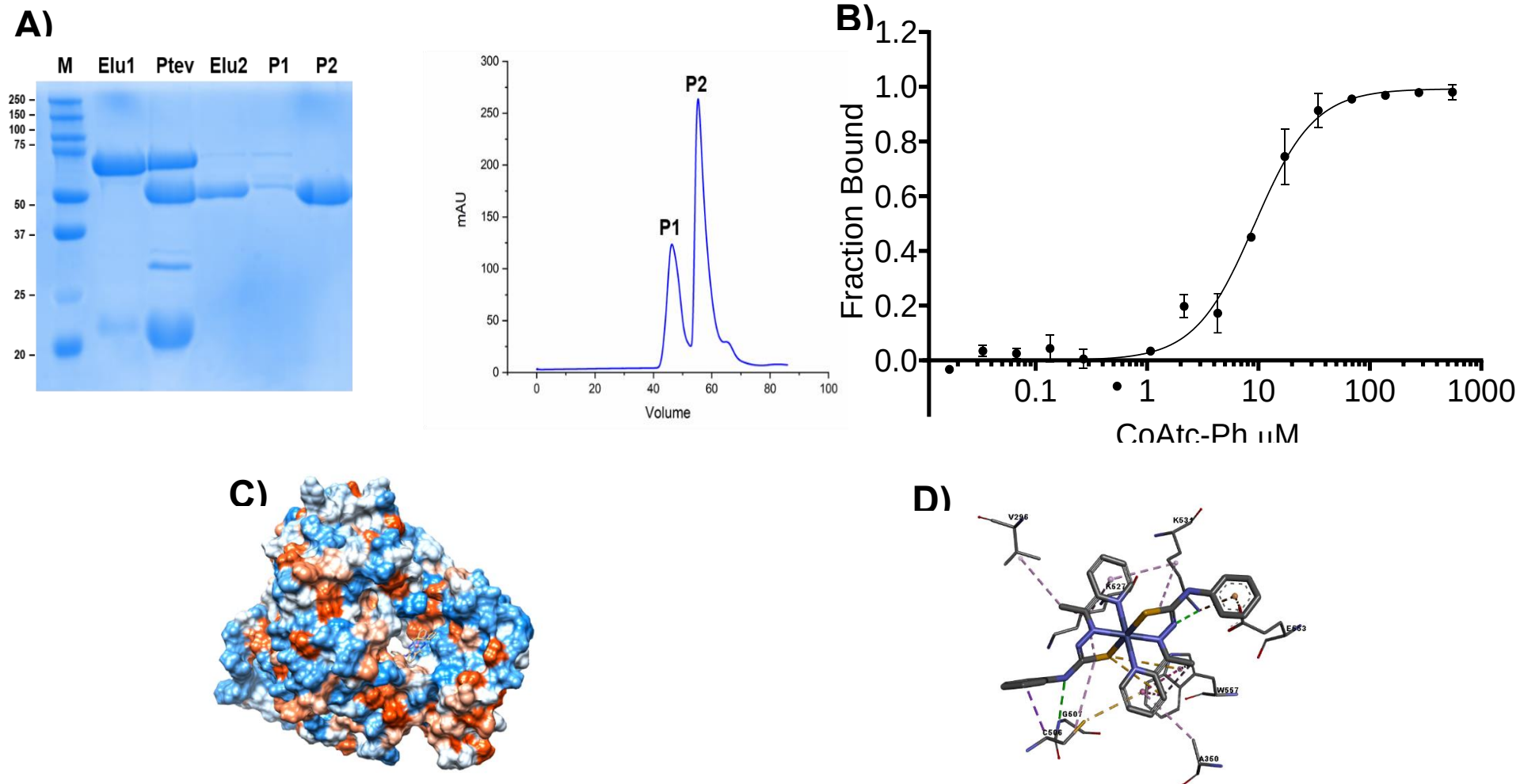


Figure 5. CoAtc-Ph interaction with Chikungunya nsP4. (A) M: Molecular weight marker; Elu1: Elution step with 50% buffer B (250 mM Imidazole); Ptev: sample after cleavage using TEV protease; Elu2: Elution step after TEV protease cleavage; P1: peak 1; P2: peak 2. **B)** Binding affinity of nsP4 and Co(atc-Ph)₂Cl. Estimated $k_d \pm \Delta k_d = 8.35 \pm 0.681 \mu\text{M}$. (C) Complex CHKV nsP4 and CoAtc-Ph interaction. **(D)** CHKV NSP4 and CoPh atomic interactions. Aminoacids are in 1-letter code, green dashed lines indicates hydrogen bonds, magenta dashed lines indicates hydrophobic intecations, brown dashde lines indicates electrostatic interactions and yelow dashed lines indicates pi-sulfur intecations. **From: MARTINS, et al 2020.**

ANEXO A: REVIEW ARTICLE ANTIVIRALS AGAINST CHIKUNGUNYA VIRUS: IS THE SOLUTION IN NATURE?

Review

Daniel Oliveira Silva Martins ^{1,2,†}, **Igor de Andrade Santos** ^{1,†}, **Débora Moraes de Oliveira** ¹,
Victória Riquena Grosche ¹ and **Ana Carolina Gomes Jardim** ^{1,2,*}

¹ Laboratory of Virology, Institute of Biomedical Science, ICBIM, Federal University of Uberlândia, Uberlândia, MG 38408-100, Brazil; danielosmartins@gmail.com (D.O.S.M.); igoras244@gmail.com (I.A.S.); deboramoraes@hotmail.com (D.M.d.O.); victoriagrosche@live.com (V.R.G.)

² São Paulo State University, Institute of Biosciences, Letters and Exact Sciences (IBILCE), State University of São Paulo, São José do Rio Preto, SP 15054-000, Brazil

* Correspondence: jardim@ufu.br; Tel.: +55-(34)-3225-8679

† These authors contributed equally to this work.

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Abstract: The worldwide outbreaks of the chikungunya virus (CHIKV) in the last years demonstrated the need for studies to screen antivirals against CHIKV. The virus was first isolated in Tanzania in 1952 and was responsible for outbreaks in Africa and Southwest Asia in subsequent years. Between 2007 and 2014, some cases were documented in Europe and America. The infection is associated with low rates of death; however, it can progress to a chronic disease characterized by severe arthralgias in infected patients. This infection is also associated with Guillain–Barré syndrome. There is no specific antiviral against CHIKV. Treatment of infected patients is palliative and based on analgesics and non-steroidal anti-inflammatory drugs to reduce arthralgias. Several natural molecules have been described as antiviruses against viruses such as dengue, yellow fever, hepatitis C, and influenza. This review aims to summarize the natural compounds that have demonstrated antiviral activity against chikungunya virus in vitro.

Keywords: chikungunya virus; antiviral; natural compounds

1. Introduction

Chikungunya fever is a tropical disease caused by the chikungunya virus (CHIKV) which is transmitted to humans by the bite of an infected mosquito of *Aedes* sp. The first case of chikungunya fever was reported in 1952 in Tanzania [1]. In February 2005, a major outbreak of chikungunya occurred on the islands of the Indian Ocean [2]. A large number of cases occurred in Europe and India in 2006 and 2007, respectively [2]. Several other countries in Southeast Asia were also affected [3]. In December 2013, autochthonous cases were confirmed in the French part of the Caribbean island of St Maarten [4]. Since then, local transmission has been confirmed in over 60 countries in Asia, Africa, Europe, and the Americas. In 2014, more than 1 million suspected cases were reported in the Americas, with 1,379,788 suspected cases and 191 deaths in the Caribbean islands, Latin American countries, and the United States of America (USA) [5]. Canada, Mexico, and USA have also recorded imported cases. The countries reporting the most cases were Brazil (265,000 suspected cases), and Bolivia and Colombia (19,000 suspected cases each) [6]. The first autochthonous transmission of

chikungunya reported in Argentina occurred in 2016 following an outbreak of more than 1000 suspected cases [7]. In the African region, Kenya reported an outbreak of chikungunya resulting in more than 1700 suspected cases. In 2017, Pakistan continues to respond to an outbreak which started in 2016 [8]. These virus outbreaks have raised concerns on studies of CHIKV epidemiology and antiviral research [9].

CHIKV belongs to the Alphavirus genus and the Togaviridae family. It is a positive-sense, single-stranded RNA (12 kb in length) virus, with an enveloped icosahedral capsid [10]. The virus lifecycle starts via the attachment of the viral glycoproteins to the cell membrane receptors, mainly to MXRA8 [11,12] but also to prohibitin (PHB) [13], phosphatidylserine (PtdSer) [14], and glycosaminoglycans (GAGs) [15] receptors in mammalian and to ATP synthase β in mosquito cells [16], forming a pore. Then, a virus capsid is released into the cytoplasm, where the replication process takes place. Viral genome is uncoated and directly translated into nonstructural (NS) proteins nP1–4. The NS proteins form the viral replicase complex that catalyzes the synthesis of a negative strand, a template to synthesize the full-length positive sense genome, and the subgenomic mRNA. The subgenomic mRNA is translated in a polyprotein, which is cleaved to produce the structural proteins C, E3, E2, 6k, and E1, followed by the assembly of the viral components and virus release (Figure 1) [17,18].

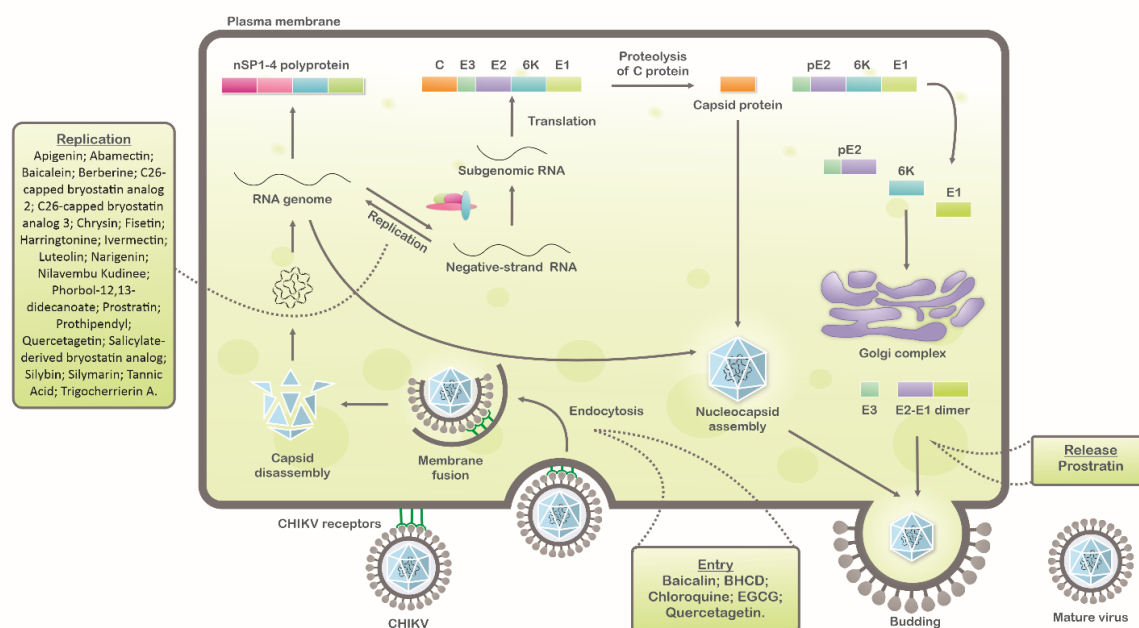


Figure 1. Schematic representation of chikungunya virus (CHIKV) replication cycle: Natural compounds with antiviral activity against CHIKV are indicated in each step of virus replication cycle (entry, replication, and release). **From: MARTINS, et al 2020.**

Chikungunya fever is characterized by strong fever, arthralgia, backache, headache, and fatigue. In some cases, cutaneous manifestation and neurological complications can occur [19,20]. There is no Food and Drug Administration (FDA) approved specific antiviral or vaccine against CHIKV. Therefore, the treatment of infected patients is based on palliative care, using analgesics for pain and non-steroidal anti-inflammatory drugs to reduce arthralgia in chronic infections [10].

Due to the lack of efficient anti-CHIKV therapy, researches have been developed to identify new drug candidates for the future treatment of chikungunya fever [21]. Among them, antiviral research based on natural molecules is a potential approach. Many natural compounds showed antiviral activity against a variety of human viruses such as dengue (DENV) [22–25], yellow fever (YFV) [25–27], hepatitis C (HCV) [28–32], influenza [33,34], and zika (ZIKV) [33,35,36]. Here, we aim to summarize the natural compounds previously described to possess anti-CHIKV activity.

2. Inhibitors of CHIKV Replicative Cycle

2.1. Epigallocatechin Gallate (Green Tea)

Epigallocatechin gallate (EGCG) is the major catechin constituent in green tea that has shown antiviral activity against CHIKV in vitro [37]. HEK 293T cells (human kidney cells) were infected with the pseudo particles CHIKV-mCherry-490 with a multiplicity of infection of 1 (M.O.I = 1) in the presence or absence of EGCG at 10 µg/mL, which blocked up to 60% of CHIKV entry. Through lentiviral expression of CHIKV glycoprotein, the authors evaluated the antiviral activity of EGCG on entry steps and suggested that EGCG interferes with CHIKV entry due to their effect on CHIKV envelope protein [37].

2.2. Chloroquine

According to the studies of Khan and coworkers, a synthetic compound derived from the natural Chloroquine used to treat malaria infection has shown antiviral activity against CHIKV [38]. To do this, Vero cells were infected with the African East-Central-South (ECSA) CHIKV genotype, DRE-06 strain, and incubated with the compound at 5, 10, or 20 µM to evaluate its antiviral activity. Three treatment strategies were used for the plaque assay: 1) pretreatment of the cells 24 h before infection; 2) concurrent treatment by simultaneously adding virus and chloroquine; and 3) treatment of cells up to 6 h post-CHIKV infection of Vero cells. Chloroquine at 20 µM was nontoxic to the cells and inhibited CHIKV entry by approximately 94% when cells were pretreated, 70% in the concurrent treatment, and 65% in the post-infection treatment. The results suggested that this compound presents strong antiviral activity, mainly when administered 24 h prior to infection [38].

2.3. Apigenin, Chrysin, Luteonin, Narigerin, Silybin, and Prothipendyl

Pohjala and colleagues demonstrated the anti-CHIKV activity of five natural compounds by using either a replicon cell line expressing the nonstructural proteins of CHIKV and the eGFP and Renilla luciferase (Rluc) markers or the full-length virus genetically modified with the reporter Rluc. Firstly, BHK21 (baby hamster kidney) cells were infected with the full length CHIKV-Rluc (M.O.I = 0.001) and simultaneously treated with different concentrations of each compound ranging from 0.01 to 100 µM for 16 h. The compounds apigenin (inhibitory concentration (IC₅₀) = 70.8 µM), chrysin (IC₅₀ = 126.6 µM), narigenin (IC₅₀ = 118.4 µM), silybin (IC₅₀ = 92.3 µM), and prothipendyl (IC₅₀ = 97.3 µM) significantly inhibited CHIKV-Rluc replication [39].

In addition, Muralli and coworkers also tested the antiviral activity of apigenin and luteonin ethanolic fraction from *Cynodon dactylon* in Vero cells and found that the fractions inhibited 98% of CHIKV activity at concentration of 50 µg/mL through the cytopathic effect [40]. Using a reverse transcriptase polymerase chain-reaction (RT-PCR) the authors also demonstrated that virus RNA levels decreased

under treatment. In another study, apigenin and luteonin were isolated from a fraction of the *Cynodon dactylon* plant, obtained from the National Institute of Virology of India, and were used to assess the cytotoxicity and antiviral activity in Vero cells. Results showed that concentrations ranging from 5 to 200 µg/mL were nontoxic as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay (MTT assay). In addition, treatment of cells at 10, 25, and 50 µg/mL showed a reduction of viral activity by decreasing 68%, 88%, and 98% of the cytopathic effect of the virus, respectively [39,40].

2.4. Flavaglines

As CHIKV uses prohibitin as a receptor to entry into mammalian cells [13], Wintachai and colleagues investigated the anti-CHIKV activity of the plant-derived compounds sulfonyl amidines 1M and the flavaglines FL3 and FL23 [41], previously reported to interact with this receptor. These compounds demonstrated antiviral activity against the CHIKV strain E1:226V East-Central-South-Africa (ECSA) genotype of a Thai isolate. The cell line HEK-293T/17 was added to each compound at specific concentrations (1, 5, 10, and 20 nM) for one hour and then infected with 10 pfu/cell of CHIKV. After 20 h, cell pellets were submitted to flow cytometry and the supernatant to a plaque assay to measure CHIKV titers. All three compounds significantly reduced the percentage of viral production in the infected cells at 10 and 20 nM concentrations. Sulfonyl amidine 1M and FL23 at 20 nM reduced viral cytopathic effect by approximately 40%, and FL3 at 20 nM reduced viral yield by 50% [41].

2.5. Compounds from *Tectona grandis*

The antiviral activity of three isolated and characterized compounds from *Tectona grandis* had its antiviral activity tested against the CHIKV strains ECSA KC 969208 and Asian KC969207 in Vero cells [42]. The authors determined IC₅₀ of the compounds 2-(butoxycarbonyl) benzoic acid (BCB), 3,7,11,15-tetramethyl-1-hexadecanol (THD), and benzene-1-carboxylic acid-2-hexadecanate (BHCD). They demonstrated that the most potent anti-CHIKV activity was observed for BHCD with selectivity index (SI) of 116 for the Asian strain and 4.66 for ECSA. In silico analyses were performed and showed that the compound possessed strong interactions with CHIKV envelope protein 1 (E1) and poor interactions with nonstructural proteins (nSP) that may suggest that this compound could act on CHIKV entry [42].

2.6. Trigocherrierin A

The work of Bourjot and colleagues showed that compounds isolated from the *Trigonostemon cherrieri* presented inhibitory activity against CHIKV replication [43]. Vero cells were used in cell proliferation assay (MTS) to evaluate the anti-CHIKV activity of compounds by decreasing the cell death induced by the virus infection [43]. Among the isolated compounds, trigocherrierin A inhibited death of cells caused by the virus with a concentration that induced half of the maximum effect (EC₅₀) of 0.6 ± 0.1 µM, CC₅₀ of 43 ± 16 µM, and the SI of 71.7. Thus, trigocherrierin A has been shown to be the most potent tested compound against CHIKV replication in this study [43].

2.7. Harringtonine

Harringtonine, a natural compound derived from the Japanese plant *Cephalotaxus harringtonia*, demonstrated antiviral activity against CHIKV replication [44]. The authors investigated the anti-

CHIKV activity of this compound by using the cell lines BHK-21, C6/36 (embryonic tissue cells of the *Aedes albopictus* mosquito), and HSMM (human skeletal muscle myoblasts) and the virus strains CHIKV-0708 (Singapore 07/2008, lacking the A226V mutation in E1 protein) and CHIKV-122508 (SGEHICHD 122508, having the A226V mutation in the E1 protein) [44]. In BHK-21 cells, harringtonine at 1 and 10 μ M showed potent anti-CHIKV action, inhibiting up to 90% of viral replication with cell viability higher than 80%. Aiming to investigate the harringtonine mechanism of action, the authors performed a time addition assay. Compounds were added at different concentrations, prior to infection ($\sim$ 2 h) and at 0, 2, 6, 12, and 16 hours post infection (h.p.i.). Treatments showed inhibition of CHIKV replication at 2 h.p.i, indicating that harringtonine inhibits the early steps of the CHIKV replicative cycle. Additionally, cells were infected and treated for 6 h, and western blot and qRT-PCR assays were performed. The results showed that harringtonine reduced negative- and positive-sense RNAs of CHIKV and the production of nSP3 and E2 proteins [44].

2.8. Diterpene Ester (phorbol-12,13-didecanoate)

Twenty-nine diterpenoids isolated from Euphorbiaceae species had their antiviral activity tested against CHIKV (Indian Ocean strain 899) in vitro through MTS assay [45,66]. First, media with serial dilutions of each compound was added to empty 96-well microplate, and then, each well was added of media containing Vero cells (2.5×10^3 cells per well) and CHIKV for 6–7 days. Among the tested compounds, phorbol-12,13-didecanoate was shown to be the strongest candidate as an antiviral against CHIKV replication, with an $EC_{50} 6.0 \pm 0.9$ nM [45,66].

2.9. Daphnanane Diterpenoid Ortho Esters

A panel of diterpenoids or thioesters isolated from *Trigonostemon cherrieri* was used to evaluate the antiviral activity against CHIKV [46]. Vero cells were used to determine the cytotoxicity of compounds, and antiviral properties were accessed by plaque assay. Among the tested compounds, Trigoocherrins A, B, and F were shown to be potent inhibitors of CHIKV replication with SIs of 23, 36, and 8, respectively [45].

2.10. Aplysiatoxin-Related Compounds

Five bioactive compounds from the cyanobacteria *Trichodesmium erythraeum* had their antiviral activity evaluated [46]. Cell viability was measured and a dose-dependent anti-CHIKV assay was performed to access the antiviral activity of the compounds under pre- or post-treatment conditions. The Debromo analogues 2 and 5 showed significant antiviral activity in post-treatment of infected BHK 21 cells with EC_{50} of 1.3 and 2.7 μ M and SI of 10.9 and 9.2, respectively. The authors suggested that the antiviral activity of these compounds blocks the replication step of the CHIKV replicative cycle [46].

2.11. Tannic Acid

Tannic acid (TA) is a compound found in different species of plants, but its structure varies according to their sources. It previously demonstrated antiviral activity against viruses as Herpes (HSV) and HCV [47,48]. The anti-CHIKV activity of TA was investigated by KONISHI and HOTTA by performing plaque reduction assay using BHK-21 cells [49]. TA reduced 50% of the virus infectivity

in lower concentrations and demonstrated inhibition of virus post-entry steps in BHK-21 cells. To investigate which chemical group of TA is associated with its antiviral activity, the authors tested TA analogues on their virus-inhibiting capacities. The results demonstrated that phenolic hydroxyl groups may be related to the antiviral activity, since the displacement of these groups make the molecule ineffective [49].

2.12. Silymarin

Silymarin is a polyphenolic compound from flavonoids family, is extracted from *Silybum marianum*, and is described to possess antiviral activity against HCV [50]. A study tested the activity of silymarin on CHIKV genotype ECSA with A226V mutation in E1 protein from a clinical strain isolated in an outbreak in 2008. BHK-21 and Vero cells were used to evaluate different steps of the viral replicative cycle, and silymarin showed inhibition of post-entry stages of CHIKV with an EC₅₀ of 16.9 µg/mL and SI of 25.1. By using a stable cell line expressing CHIKV replicon and EGFP and Rluc markers [39], it was demonstrated that silymarin suppressed 93.4% of CHIKV replication. Western blot assay was performed, showing that silymarin treatment decreased the amounts of nSP1, nSP3, and E2 proteins [51].

2.13. Baicalein, Fisetin, and Quercetagenin

Baicalein, fisetin, and quercetagenin are compounds from the flavonoids family that exhibited antiviral activity against DENV [22] and enterovirus A71 [52]. Lani and colleagues infected Vero cells with the CHIKV genotype ECSA strain from the outbreak of 2008 and evaluated their effects in reducing the cytopathic effect resulting from viral infection [53]. All three compounds were found to inhibit CHIKV replication in a dose-dependent manner and reduced E2, nSP1, and nSP3 protein synthesis, as showed by Western blot analysis. Baicalein and quercetagenin showed anti-CHIKV activity by inactivating the virus, preventing the attachment of the virus to the host cells and blocking post-entry stages, with EC₅₀ of 1.891 µg/mL and 13.85 µg/mL, respectively. Fisetin only inhibited post-entry steps with EC₅₀ of 8.44 µg/mL [53].

2.14. Bryostatin

Bryostatin is a macrolide lactone derived from a marine animal named *Bugula neritina* [54]. It was described by the antineoplastic activity [55], affects Alzheimer's disease [56], and has been related to the eradication of human immunodeficiency virus reservoirs [57]. The anti-CHIKV activities of the Bryostatin analogs salicylate-derived analog 1, C26-capped analog 2, and C26-capped analog 3 were assessed by evaluating the cytopathic effect (CPE) caused by CHIKV Indian Ocean lineage strain 899 replication under treatment with these three compounds [58]. All of the Bryostatin analogs inhibited the CHIKV replicative cycle, decreasing infectious progeny and viral RNA copies, confirmed by supernatant titration and RT-PCR. A time-addition assay showed that these compounds inhibited late stages of CHIKV replication, with EC₅₀ rates of 4 µM, 8 µM, and 7.5 µM, respectively. Additionally, salicylate-derived analog 1 but not the other compounds blocked entry of CHIKV pseudoparticles into Buffalo green monkey kidney cells (BGM) [58].

2.15. Prostatin

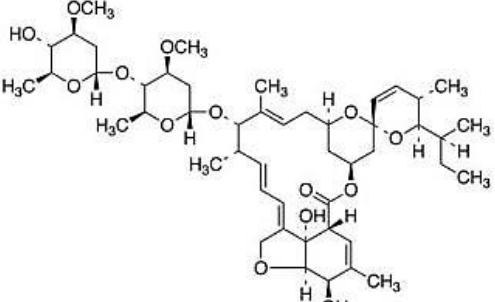
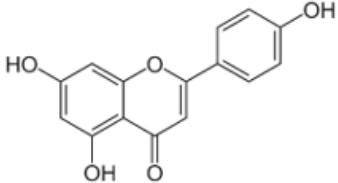
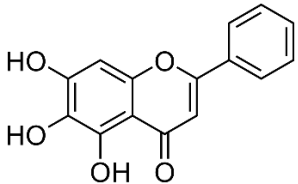
Bourjot and coworkers described the effect of prostratin, a compound derived from *Trigonostemon howii*, on CHIKV infection in Vero cells by a CPE assay ($EC_{50} = 2.6 \mu M$) [59]. Another work used CHIKV lineage Indian Ocean 899 to infect Vero, BGM, or Human embryonic lung fibroblasts (HEL) cells at M.O.I of 0.001 under the treatment with prostratin and obtained EC_{50} of $8 \mu M$, $7.6 \mu M$, and $7.1 \mu M$, respectively. Using a delay treatment associated with a RT-PCR or CHIKV pseudoparticle techniques, it was demonstrated that prostratin decreased both the number of CHIKV genome copies and the production of infectious progeny virus particles. A western blot assay was used to detect CHIKV proteins and showed that prostratin also reduced the accumulation of nSP1 and capsid proteins [59].

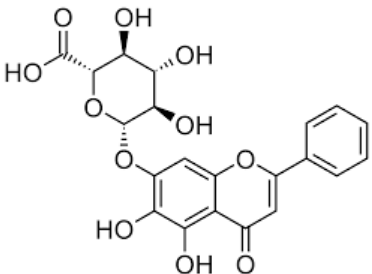
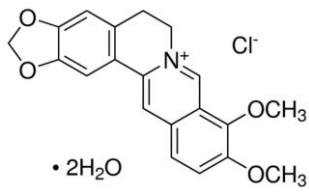
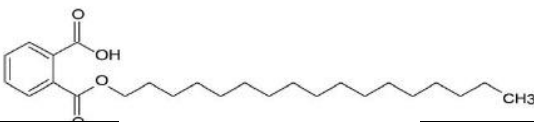
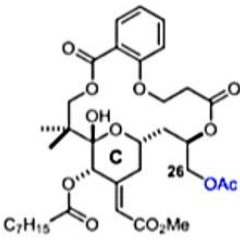
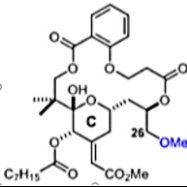
2.16. Berberine

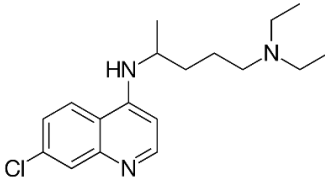
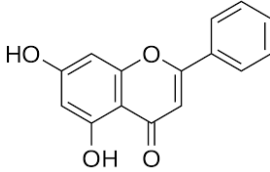
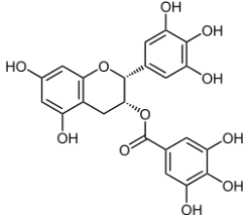
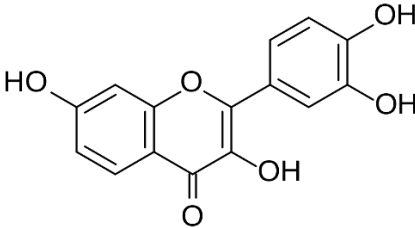
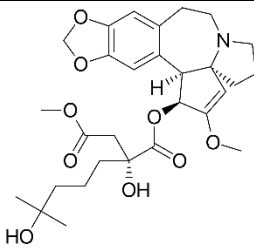
Berberine is a compound found in plants from the *Berberis* genus, family Berberidaceae, that previously demonstrated antiviral activity against other viruses [60]. Varghese and colleagues analyzed the antiviral effect of berberine on the CHIKV replication cycle using the CHIKV lineage LR2006 OPY1 with the Rluc marker to infect HEK-293T, HOS (human bone osteosarcoma), and CRL-2522 cells. The berberine EC_{50} for each cell line were 4.5, 12.2, and $35.3 \mu M$, respectively. This compound was also active against the different CHIKV strains LR2006 OPY1, SGP11, and CNR20235, showing EC_{50} of 37.6, 44.2, and $50.9 \mu M$, respectively. Berberine showed no inhibition on CHIKV entry or replication but decreased viral RNA and viral protein synthesis, suggesting that berberine is indirectly perturbing CHIKV replication by affecting host components [60].

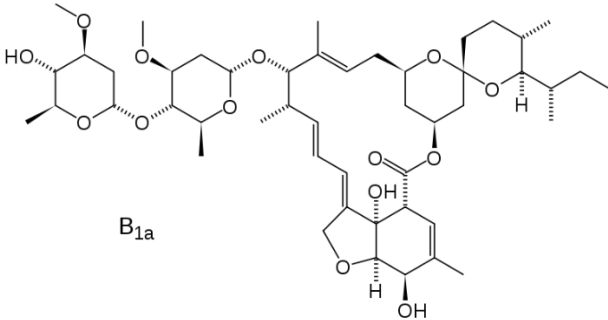
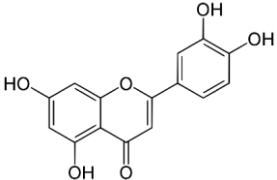
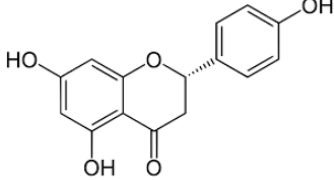
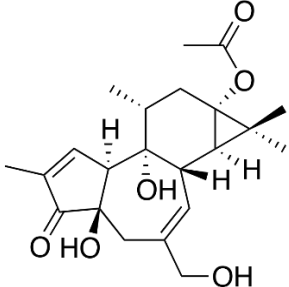
2.17. Avermectin derivatives

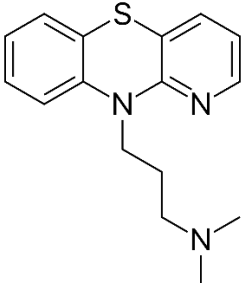
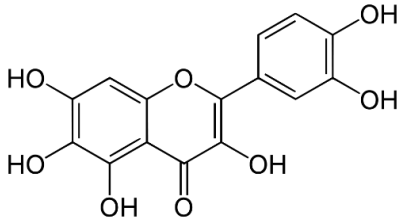
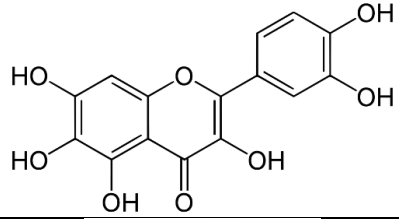
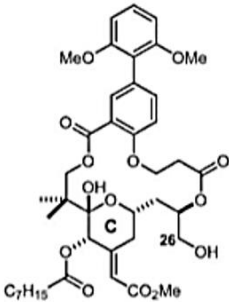
Avermectin is naturally produced in *Streptomyces avermitilis* bacteria and showed different biological properties including antiparasitic [61], antiviral [63], and antibacterial [63,64] activities. Ivermectin (IVN) and abamectin (ABN) are chemically modified derivatives of avermectin. The activity of these derivatives on the CHIKV replication cycle was described in a study that used BHK-21 with CHIKV containing the Rluc gene [65]. IVN and ABN demonstrated EC_{50} of $0.6 \mu M$ and $1.5 \mu M$, respectively, and strongly reduced nSP1 and nSP3 even in high M.O.Is. A time-of-addition assay demonstrated that IVN and ABN interfered in earlier stages of CHIKV cycle but not when cells were pretreated. Alternatively, the activity of these compounds was decreased in the later stages of the CHIKV replicative cycle [65].

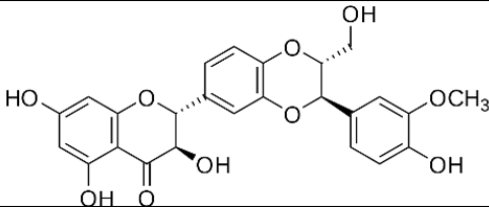
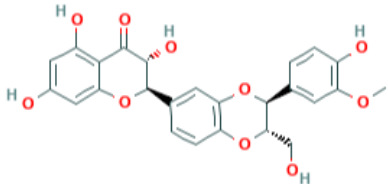
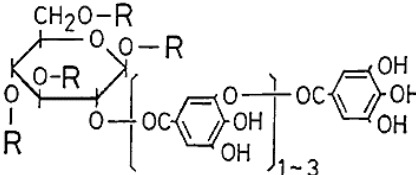
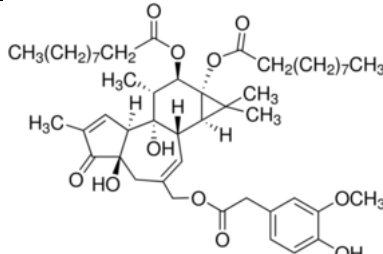
Compound	Structure	Inhibition	SI or EC ₅₀	Cell Line
Abamectin [65]		Replication	1.5 μ M	BHK-21
Apigenin [40,39]		Infection/Replication	70.8 μ M	BHK 21
Baicalein [53]		Infection and replication	1.891 μ g/mL	BHK-21

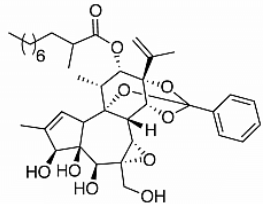
Baicalein [53]		Entry, binding	6.997 μ M	Vero
Berberine [60]		Replication (interfering in host components)	$\leq 35.3 \mu$ M	CRL-2522, HEK-293T, and HOS
BHCD [42]		Entry	116 (Asian strain) and 4.66 (ECSA)	Vero and in silico
C26-capped bryostatin analog 2 [58]		Replication	8 μ M	Vero
C26-capped bryostatin analog 3 [58]		Replication	7.5 μ M	Vero

Chloroquine [38]		Entry	37.14	Vero
Chrysin [39]		Infection	126.6 μ M	BHK 21
EGCG [37]		Entry steps; cell attachment	6.54 μ g/mL	HEK 293T
Fisetin [53]		Replication	8.44 μ g/mL	BHK-21
Harringtonine [44]		Early stages of replication	0.24 μ M	BHK 21

Ivermectin [65]	 B_{1a}	Replication	0.6 μ M	BHK-21
Luteolin [40]		Replication	NS	Vero
Narigenin [39]		Infection	118.4 μ M	BHK 21
Prostratin [59]		Replication and release	2,6 μ M and $\pm$ 8 μ M	Vero, BGM, and HEL

Prothipendyl [39]		Replication	97.3 μ M	BHK 21
Quercetagenin [53]		Entry and binding	43.52 μ M	Vero
Quercetagenin [53]		Entry and replication	13.85 μ g/mL	BHK-21
Salicylate-derived bryostatin analog [58]		Entry and replication	4 μ M	Vero

Silybin [39]		Infection	92.3 μ M	BHK 21
Silymarin [51]		Replication	16.9 μ g/mL	BHK-21 and Vero
Tannic Acid [49]		Replication	NS	BHK-21
Phorbol-12,13-didecanoate [66]		Replication	6 $\pm$ 0.9 nM	Vero

Trigocherrierin [43]		Replication	$0.6 \pm 0.1 \mu\text{M}$	Vero
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NS = Not shown, data not shown Table: **From: MARTINS, et al 2020.**

1. Prospects

The aim of this review was to si data from literature concerning the natural compounds described to possess anti-CHIKV activity. Altogether, data is heterogeneous since authors developed a variety of assays using cell lines and CHIKV strains or replicons. Some studies did not elucidate the mechanism of action (MOA) of the compound, retaining their information as EC50, CC50, and/or SI. For the compounds presented in this review, it would be desirable to demonstrate the MOA in order to elucidate the biochemical and molecular basis of the compound–virus or compound–cell interactions and to be able to predict and promote strategies for pharmacological outcomes in further studies [67]. Also, the investigation of the effects of each compound in cell lines would provide information concerning the effects of these compounds on the host cells [68,69]. Besides that, all data summarized here represent a relevant source of knowledge concerning the antiviral potential of molecules isolated from nature.

From the natural compounds cited in this review, chloroquine was the only compound tested in vivo, in non-human primates, and in human clinical trials. Chloroquine is already used for the treatment of [70]. However, despite the in vitro results, chloroquine demonstrated no relevant results in vivo in decreasing viremia or in reducing clinical manifestations during acute stage of CHIKV infection [71]. Therefore, the results demonstrated by in vitro analysis were not correlated with the in vivo analysis that showed that chloroquine was not suitable for patients with CHIKV. Additionally, the remaining compounds described here have not been tested in vivo yet, representing a delay in anti-CHIKV drug development.

Apart from the chloroquine case, all compounds that demonstrated antiviral activity have the potential to be further investigated by their therapeutically properties against chikungunya fever. Furthermore, natural compounds may as a source of molecules with biological activities that could be used as templates to the development of novel antivirals.

4. Conclusion

The spread of CHIKV in the last years demonstrated the need to develop effective antiviruses to treat chikungunya fever and to prevent future outbreaks. In this, natural compounds have shown antiviral activity against a range of viruses. This review summarized the natural compounds described to possess anti-CHIKV activity by blocking early and/or late stages of virus replication in vitro. Apart from the great antiviral activity of the described compounds, further research is needed for the development of future treatments.

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Conflicts of Interest: The authors declare no conflict of interest.

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ANEXO B: IS THE ADP RIBOSE SITE OF THE CHIKUNGUNYA VIRUS NSP3 MACRO DOMAIN A TARGET FOR ANTIVIRAL APPROACHES?

Jacqueline Farinha Shimizu^{a,b}; Daniel Oliveira Silva Martins^{a,b}; Martin J. McPhillie^c, Grace C. Roberts^d; Carsten Zothner^d, Andres Merits^e; Mark Harris^d; Ana Carolina Gomes Jardim^{a,b*}

^a São Paulo State University, IBILCE, S. José do Rio Preto, SP, Brazil

^b Laboratory of Virology, Institute of Biomedical Science, ICBIM, Federal University of Uberlândia, Uberlândia, MG, Brazil

^c School of Chemistry, Faculty of Engineering & Physical Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

^d School of Molecular and Cellular Biology, Faculty of Biological Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds LS2 9JT, United Kingdom

^e Institute of Technology, University of Tartu, Nooruse 1, 50411 Tartu, Estonia

***Corresponding author:** Prof Dr Ana Carolina Gomes Jardim, Institute of Biomedical Science, ICBIM/UFU, Avenida Amazonas, Bloco 4C – sala 216. Umuarama. Uberlândia, MG, Brazil. CEP: 38405-302. Tel.: +55 (34) 3225-8682.

E-mail address: jardim@ufu.br

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Highlights

- Virtual screening identifies molecules that binding to ADP-ribose site of CHIKV
- Two selected compounds inhibited CHIKV replication in vitro
- Compounds are probably interfering in the early CHIKV replication

Abstract

Chikungunya virus (CHIKV) is a mosquito-transmitted virus of special concern as it causes Chikungunya fever, characterized by an acute febrile illness, rash, and arthralgia that can progress to chronic and debilitating arthritic symptoms. The effects of climate change on the geographic distribution of the mosquito vector has the potential to expose more of the globe to this virus. No antiviral agents or vaccines are currently available against CHIKV infection and the development of novel therapies that may lead to a future treatment is therefore necessary. In this context, the ADP-ribose binding site of the CHIKV nsP3 macro domain has been reported as a potential target for the development of antivirals. Mutations in the ADP-ribose binding site demonstrated decreased viral replication in cell culture and reduced virulence. In this study, 48,750 small molecules were screened *in silico* for their ability to bind to the ADP-ribose binding site of the CHIKV nsP3 macro domain. From this *in silico* analysis, 12 molecules were selected for *in vitro* analysis using a CHIKV subgenomic replicon in Huh-7 cells. Cell viability and CHIKV replication were evaluated and molecules C5 and C13 demonstrated 53 and 66% inhibition of CHIKV replication, respectively. By using a CHIKV-Dual luciferase replicon contain two reporter genes, we also demonstrated that the treatment with either compounds are probably interfering in the early replication rather than after RNA replication has occurred.

Introduction

Chikungunya virus (CHIKV) is an arbovirus that belongs to Alphavirus genus and Togaviridae (Lumsden, 1955). CHIKV infection can cause fever, skin rash, and arthralgia, 15 % of CHIKV infections can be asymptomatic (Miner et al., 2015). The infection can progress to severe symptoms and persist for months or years, leading to an economical burden for many countries (WHO, 2017).

According to the Pan American Health Organization, 349,936 suspected cases were reported in 2016 in the Americas, with 146,914 cases confirmed by laboratory analysis. No antiviral or vaccine is available against CHIKV infection. Therefore, the development of an antiviral is needed for the treatment of infected patients.

CHIKV is an enveloped virus with a positive single strand RNA genome, containing two open frames (ORFs). The 5' ORF encodes four non-structural proteins (nsP1 – nsP4) and the 3' ORF encodes a subgenomic RNA which encodes the five structural proteins (C, E1, E2, E3 and 6K) (Kumar et al., 2015; Voss et al., 2010).

Of the CHIKV proteins, nsP3 is the most 'enigmatic' since its functions in the virus life cycle remain uncertain (Götte et al., 2018). nsP3 comprises 3 domains: an N-terminal region macro domain, followed by the alphavirus unique domain (AUD), and the C-terminal hypervariable region (Malet et al., 2009; Shin et al., 2012). The nsP3 macro domain is conserved among alphaviruses and other pathogenic positive single strand RNA viruses (Eckel et al., 2017). Recently, the ADP-ribosylhydrolase activity of CHIKV nsP3 macro domain was shown to be critical for virus replication and virulence (McPherson et al., 2017).

Previously, the macro domain of CHIKV nsP3 has been described as a potential target in the development of anti-alphavirus compounds (Nguyen et al., 2014; Subudhi et al., 2018; Vijayasri and Hopper, 2017). Despite the interest in the macro domain of nsP3, there is a lack of in vitro or in vivo studies on drugs that bind to this region of nsP3. Most are computational studies, with no further in vitro or in vivo validation (Nguyen et al., 2014; Vijayasri and Hopper, 2017). Computational methods can be useful to refine the search for new antiviral drugs, reducing efforts and costs to identify antivirals, however, the validation of these analysis is essential.

In this study, we combined a virtual screening cascade to identify small molecules that putatively bind to ADP-ribose site of the macro domain of nsP3 and further investigated the antiviral activity of the selected molecules in vitro.

Material and Methods

In silico screening

An in-house library containing small synthetic molecules from the Medicinal Chemistry/Chemical Biology (MCCB) group (School of Chemistry, Faculty of Engineering & Physical Sciences – University of Leeds) was docked to the ADP-ribose binding site of the CHIKV nsP3 macro domain (Protein Data Bank – 3GPO) (Malet et al., 2009) using the docking algorithm Glide (vHTS mode). Molecules were ranked according to predicted binding affinity and the top compounds were re-docked using Glide XP mode (Maestro, Schrödinger, LLC, New York, NY, 2017). Then the compounds were visually inspected. Based on these parameters, 12 compounds were selected for in vitro activity assays.

Compounds

The 12 compounds selected by in silico screening were kindly provided by MCCB group. Compounds were stored at 14 °C in 10 mM DMSO stock solutions, in 0% humidity cabinets. After initial in vitro assays, new stocks of compounds C5 (ChemDiv V029-0567), C13 (ChemDiv V028-7674) and their respective analogs were purchased (ChemDiv inc, San Diego – US).

Cell lines and replicons

The human hepatoma (Huh-7) and rhabdomyosarcoma (RD) cell lines were grown in DMEM, supplemented with 10% FBS, 0.5 mM non-essential amino acids and 100 units/mL penicillin-streptomycin. Murine myoblast cells (C2C12) were grown in DMEM supplemented with 20% FBS and 100 units/mL penicillin-streptomycin. All cells were maintained at 37 °C with 5% CO₂ in a humidified incubator.

The subgenomic replicons CHIKV wild type nsP3 firefly luciferase (CHIKV-Fluc SGR) and CHIKV dual luciferase (CHIKV-D-Luc SGR) were derived from the ECSA genotype LR2006-OPY1 (Pohjala et al., 2011).

Cell viability assay

Cell viability was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma–Aldrich) assay. 24 hours post treatment, compound-containing media was removed from 96 well plates and MTT at 1 mg/mL solution was added to each well, incubated for 1 hour and replaced with 100 μ L of DMSO to solubilize the formazan crystals. The absorbance was measured at 560 nm on Infinite F50 microplate reader (Tecan). Cell viability was calculated according to the equation $(T/C) \times 100\%$, which T and C represented the optical density of the treated well and control groups, respectively. DMSO was used as non-treated control. The cytotoxic concentration of 50% (CC50) was calculated using Prims 8.0 (Graph Pad).

RNA Transcription and electroporation

Plasmids were linearized using restriction enzyme NotI-HF (New England Biolabs), prior to in vitro transcription using the mMESSAGE mMACHINE™ SP6 Transcription Kit (Invitrogen) following the manufacturer's protocol. RNA was purified using the LiCl precipitation protocol. For electroporation, 3×10^6 cells were electroporated with 2 μ g of RNA in 4 mm cuvette, 260 V, 25 ms, 1 pulse, using GenePulser Xcell (Bio-Rad) (Roberts et al., 2017). Cells were plated in 48 well plates (6×10^4 cells/well) for replication assays and 96 well plates (2×10^4 cells/well) for cell viability assays. Four hours post-electroporation (h.p.e.) the media was removed, replaced with compound-containing media in concentrations and incubated for 24 hours.

Replication assay

To measure CHIKV replication levels, compound-containing media was removed 24 hours after treatment, cells were washed with PBS, and harvested by lysis with Passive Lysis Buffer (Promega, USA). Samples were stored at -20°C and thawed prior to measurement of luminescence levels on a FLUOStar optima microplate reader (BMG Labtech) using the Luciferase Assay System (Promega, USA) for CHIKV-Fluc SGR and the Dual-Luciferase® Reporter system (DLR™) (Promega, USA) for CHIKV-D-Luc SGR. The effective concentration of 50% (EC50) was calculated using Prims 8.0 (Graph Pad). Replication assays were carried out in parallel with cell viability assays.

Statistical Analysis. Individual experiments were performed in triplicate and all assays were performed a minimum of three times in order to confirm the reproducibility of the results.

Statistical analysis was performed using GraphPad Prims 8.0 software. A Kruskal-Wallis test followed by post hoc analysis by Dunn's test was used for multiple comparisons

P values of less than 0.05 (indicated by asterisks) were considered statistically significant.

Results

In silico identification of ligands to the Macro domain of CHIKV-nsP3

To identify potential inhibitors against the macro domain of CHIKV nsP3, the structure of CHIKV nsP3 complexed with ADP-ribose (Protein Data Bank – 3GPO) was used. The ADP-ribose substrate was manually removed from the ADP-ribose binding site and a library containing 48,750 small synthetic molecules were docked to this region of the protein with Schrodinger's docking tool Glide in vHTS mode using default parameters. The compounds were ranked based on Glide docking scores, a prediction of binding affinity, and the top 1000 scoring compounds were re-docked using Glide XP mode, a higher precision scoring function. After that, the top 50 compounds were visually inspected. And 12 compounds were selected for biological testing based on the docking score (Table 1); inspection of hydrogen bonds between ADP-ribose binding site of CHIKV nsP3 and the compounds; the conformation of each docking pose and the position of the compound inside the binding site relative to the ADP-ribose.

Activity of compounds against CHIKV replication

Initial in vitro activity of compounds selected by docking analysis

The 12 compounds selected by docking analysis were evaluated in vitro by their antiviral activity using the CHIKV-Fluc SGR system. Huh-7 cells were electroporated with CHIKV-Fluc SGR RNA, seed in 48 well plates and treated with each compound at 20 μ M, 10 μ M and 1 μ M at 4 h.p.e. to assess the effect of these compounds on both CHIKV replication and cell viability. Figure 1 presents data obtained from the treatment with the maximum non-toxic concentration of each compound. The results demonstrated that at 20 μ M compounds C5 and C13 inhibited CHIKV replication by 53% and 66% respectively, and retained 94% of cell viability (Figure 1, Table 1). Other compounds did not demonstrate significant activity against CHIKV replication.

Compounds with anti-CHIKV activity and related analogues

In silico analysis showed predicted hydrogen bonding interactions of compounds C5 and C13 with residue Tyr114 and van der Waals interactions/hydrophobic effect with residue Val33 and Asn24 (Figure 2^a). After initial in vitro screening, compounds C5 and C13 were selected for further assays and structural analogues were purchased from ChemDiv (Figure 2B, Table 2).

The EC₅₀ and CC₅₀ of C5, C13 and their analogues were determined by performing replication and viability assays in cells treated with a range of concentrations of each compound. Huh-7 cells were electroporated with CHIKV-Fluc SGR RNA, seeded in 48 well plates and treated with a two-fold dilution (ranging from 1.5-100 μ M) of each compound at 4 h.p.e. Cell viability and CHIKV replication levels were measured 24 h post treatment (Figure 3). The selective index (SI) was calculated by dividing the value of CC₅₀ by EC₅₀ (CC₅₀/EC₅₀). The EC₅₀ and CC₅₀ values and SI of C5, C13 and their analogues are shown in Table 3. Compound C5 showed EC₅₀ of 12.7 μ M and CC₅₀ of 38.9 μ M, and demonstrated lower EC₅₀ value than its analogues 1C5, 2C5, 3C5 and 4C5. Compound C13 presented a slightly higher EC₅₀ (11.9 μ M) and CC₅₀ (36.8 μ M). While the C13 analogue 1C13 showed a decrease in EC₅₀, the value of CC₅₀ decreased almost by a half.

Investigation of C5 and C13 compounds mechanisms

Compounds C5 and C13 presented the highest SI and their effects on CHIKV replication were further investigated. To this end, the CHIKV-D-Luc replicon containing two genes, the renilla luciferase (Rluc) and firefly luciferase (Fluc) was used, the replicon is described in

(Roberts et al., 2017). The Rluc is in the hypervariable region of nsP3 of CHIKV and its indicate both input translation and early replication levels. Alternatively, the Fluc replaces the virus structural genes and it is only expressed from a subgenomic RNA, thus indicating that RNA replication has occurred (Figure 4^a). In order to investigate a possible mechanism of C5 and C13 were acting during early or late replication, Huh-7 cells were electroporated with CHIKV-D-Luc and treated with C5 (Figure 4B) or C13 (Figure 4C) at non-toxic concentrations (concentrations above 80% of cell viability were considered non-toxic). The data obtained demonstrated that the treatment with C5 (Figure 4B) and C13 (Figure 4C) compound significantly decreased Rluc levels, but not Fluc . Therefore, suggesting that these compounds are most likely interfering with early replication events.

Finally, the effects of C5 and C13 were also investigated in muscle derived cell lines. For this, human rhabdomyosarcoma (RD) cells and murine myoblast (C2C12) cells were used. However, the results demonstrated that C5 and C13 were only able to inhibit CHIKV replication at concentrations that also demonstrated some cell toxicity (Figure 5). At 100 μ M, C5 inhibited 100% and 70% of CHIKV replication in RD and C2C12, respectively (cell viability 65% and 49%) (Figure 5^a and 5B). C13 inhibited CHIKV replication in 95% and 64% in RD and C2C12, respectively (cell viability 70% and 51%) (Figure 5B and 5D).

Discussion

Virtual screening has the advantage of reducing costs/time to identify hit compounds when compared to screening large numbers of compounds in a HTS approach (Piccirillo and Amaral, 2018). In this study, the macro domain of CHIKV nsP3 was investigated since this protein has been reviewed and described to participate in several processes of the viral cycle.

The macro domain of CHIKV nsP3 possesses ADP-ribose binding and ADP-ribose hydrolase activity, removing ADP-ribose from mono(ADP-ribosyl)ated proteins, this activity has been shown to be for viral replication (McPherson et al., 2017). CHIKV mutants with reduced hydrolase activity showed slower virus replication and decrease of virulence, and mutants with no hydrolase activity were unable to replicate in mammalian or mosquito cells (McPherson et al., 2017). Therefore, a compound that can block CHIKV nsP3 hydrolase activity is proposed to be a target for antiviral therapy. Additionally, the macro domain of nsP3 is a well conserved region in the Alphavirus genus and other positive single strand RNA viruses, demonstrating the potential to be a broad spectrum antiviral (Eckei et al., 2017; Gregor et al., 2016).

A library of 48,750 small molecules was initially screened *in silico* to predict their ability to bind to ADP-ribose binding site of nsP3. Twelve selected compounds were further investigated *in vitro* for both their toxicity to human hepatoma cells and antiviral activity on CHIKV replication. Compounds C5 and C13 presented the highest selective index (ratio between the cell viability and the replication inhibition) (5.2 and 31, respectively), and demonstrated to share a similar mode of binding to ADP-ribose binding site, in terms of shape and electrostatic potential. Therefore, analogues of C5 and C13 were also investigated, however, they were less active than the original compounds. Moreover, C5 and C13 demonstrated interference in the early steps of CHIKV replication (for example formation of the replication complex) rather than after RNA replication has occurred.

However, the computer-aided virtual screening studies based on CHIKV nsP3 describe only in silico data (Pérez-Pérez et al., 2019). Nguyen and collaborators used the database Diversity Set II chemical library of the National Cancer Institute (NCI) to virtually screen 1541 compounds in conjunction with the structure of the CHIKV nsP3 macro domain from the Protein Data Bank (PDB id: 3GPG) and selected five potential ligands (Nguyen et al., 2014). Vijayasri and collaborators also used the same CHIKV nsP3 macro domain structure to dock 150 phytochemicals from various plant sources, among them fisetin and quercetin (Vijayasri and Hopper, 2017). Later, the phytochemical fisetin and quercetagenin, compound similar to quercetin, showed in vitro anti-CHIKV activity by inhibiting RNA production and viral protein. (Lani et al., 2016). Baicalein was also considered a potential antiviral candidate against CHIKV nsP3 in a computational study (Seyedi et al., 2016). In 2018, Oo and collaborators showed that baicalein also exhibited binding affinity to CHIKV envelope protein E2. Baicalein higher inhibition on virus entry than replication. The authors suggested a possible interaction between the compound and cellular factors (Oo et al., 2018).

ADP-ribosylation is a common post-translational modification in cellular proteins (Hottiger et al., 2010). In this regard, the tested compounds showed considerable cytotoxicity, this might be because they were selected to bind to the ADP-ribose binding site of nsP3. By definition this will be similar to the ADP-ribose binding site of cellular macrodomains (Schleicher et al., 2018). ADP-ribosylation is known to regulate cell DNA repair, cell proliferation, transcription and cell death (Barkauskaite et al., 2015; Munnur and Ahel, 2017). Therefore, we suggest that these selected compounds against nsP3 macrodomain may be acting on these other cell pathways affecting cell viability.

In this regard it is interesting to note that compounds C5 and C13 showed higher cytotoxicity in muscle derived cell lines RD and C2C12. Roberts and collaborators, showed that immune cell lines si variance in the levels of replication (Roberts et al., 2017). The cytotoxicity observed for RD and C2C12 cell lines could be related to the immune expression. of these cells, as well as their ability to support CHIKV replication.

In addition interferon treatment induces the immune expression. of several cellular ADP-ribosyltransferases which act as inhibitors of cellular translation and virus replication (Atasheva et al., 2014), leading to the hypothesis that de-ADP-ribosylation activity of macro domains can act as a response to cellular antiviral mechanisms. Inhibition of nsP3 macro domain activity would therefore have a two-pronged effect – direct inhibition of viral RNA replication and

potential blockade of the innate immune response. However, more studies are needed to precisely understand the role(s) of the nsP3 macro domain during the virus lifecycle.

Conclusion

In this study a virtual screening approach has been used to identify putative CHIKV nsP3 inhibitors that target the ADP-ribose binding site. *In vitro* analysis showed that two of the selected compounds inhibited CHIKV replication, probably acting in the early replication. Computer-aided drug design (CADD) is a facilitator in the process of searching for new antivirals, however, the selection of compounds by docking does not eliminate other variables in a living system and needs to be combined with biological assays.

Author contributions

Jacqueline Farinha Shimizu: Methodology, Investigation, Visualization and Writing-Original draft preparation. **Daniel Oliveira Silva Martins:** Writing-Original draft preparation. **Martin J. McPhillie:** Software, Conceptualization, Formal analysis. **Grace C. Roberts:** Methodology. **Carsten Zothner:** Methodology. **Andres Merits:** Methodology, Resources. **Mark Harris:** Funding acquisition, Supervision, Resources and Conceptualization. **Ana Carolina Gomes Jardim:** Writing-Reviewing, Visualization, Editing and Supervision.

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Table 1. Docking scores, physicochemical properties and biological activity of selected ligands docked to ADP-ribose binding site at the macro domain of CHIKV nsP3. HBA = hydrogen bond acceptor. HBD = hydrogen bond donor. MW = molecular weight. PSA = polar surface area.

Compound	Glide Rank	CHIKV %replication 20 uM	% Toxicity (20µM)	Reported Bioactivity	Rotatable bonds	Docking Score	AlogP	logP ZINC15	HBA	HBD	MW	PSA
C9	1	None	None		7	-10,778	1,152		4	2	359,352	91,65
C3	3	None	None		12	-10,371	3,453		5	3	476,548	133,73
C14	8	None	None		12	-9,695	4,193		3	2	484,03	78,51
C1	14	None	None		6	-9,435	1,64		5	1	344,388	114,35
C13	16	66%	None	None	11	-9,388	2,73	4,81	5	1	470,444	84,4
C4	21	None	None		12	-9,153	1,751		6	2	499,531	106,2
C5	30	53%	None	None	11	-8,972	3,025		4	1	466,548	70,84
C17	33	None	None		6	-8,952	1,723		4	2	407,871	117,95
C12	46	None	None		3	-8,751	3,324		2	0	368,857	40,62
C8	48	None	None		9	-8,735	4,065		3	1	413,512	56,15
C16	50	None	None		6	-8,7	1,403		3	2	368,43	81,75

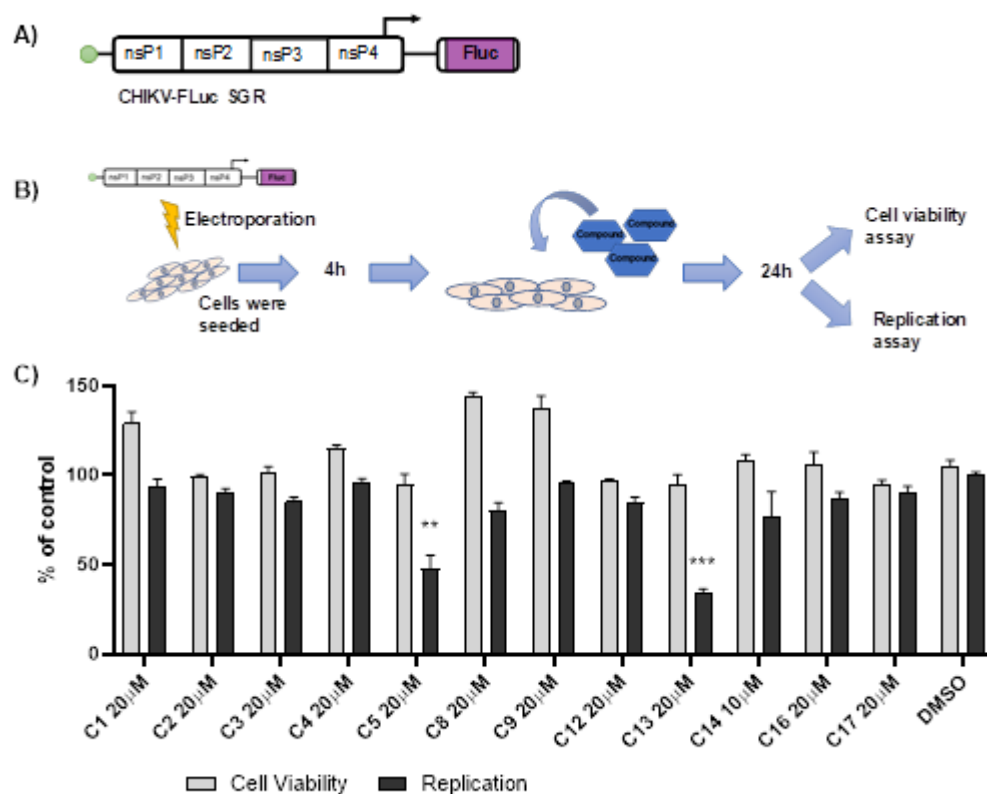


Figure 1: *In vitro* screening of 12 MCCB compounds selected from docking analyses. CHIKV-Fluc SGR replicon scheme **(A)**. Schematic representation of *in vitro* initial trials of MCCB compounds **(B)**. The results of the treatment with maximum non-toxic concentration of Huh-7 cells harbouring CHIKV-Fluc SGR are shown for cell viability and activity against CHIKV replication **(C)**. DMSO was used as non-treated control. The asterisks indicate statistically significant differences between each compound and DMSO control (* $p \leq 0.05$, ** $p \leq 0.01$), calculated using the Kruskal–Wallis test with the Dunn’s post test.

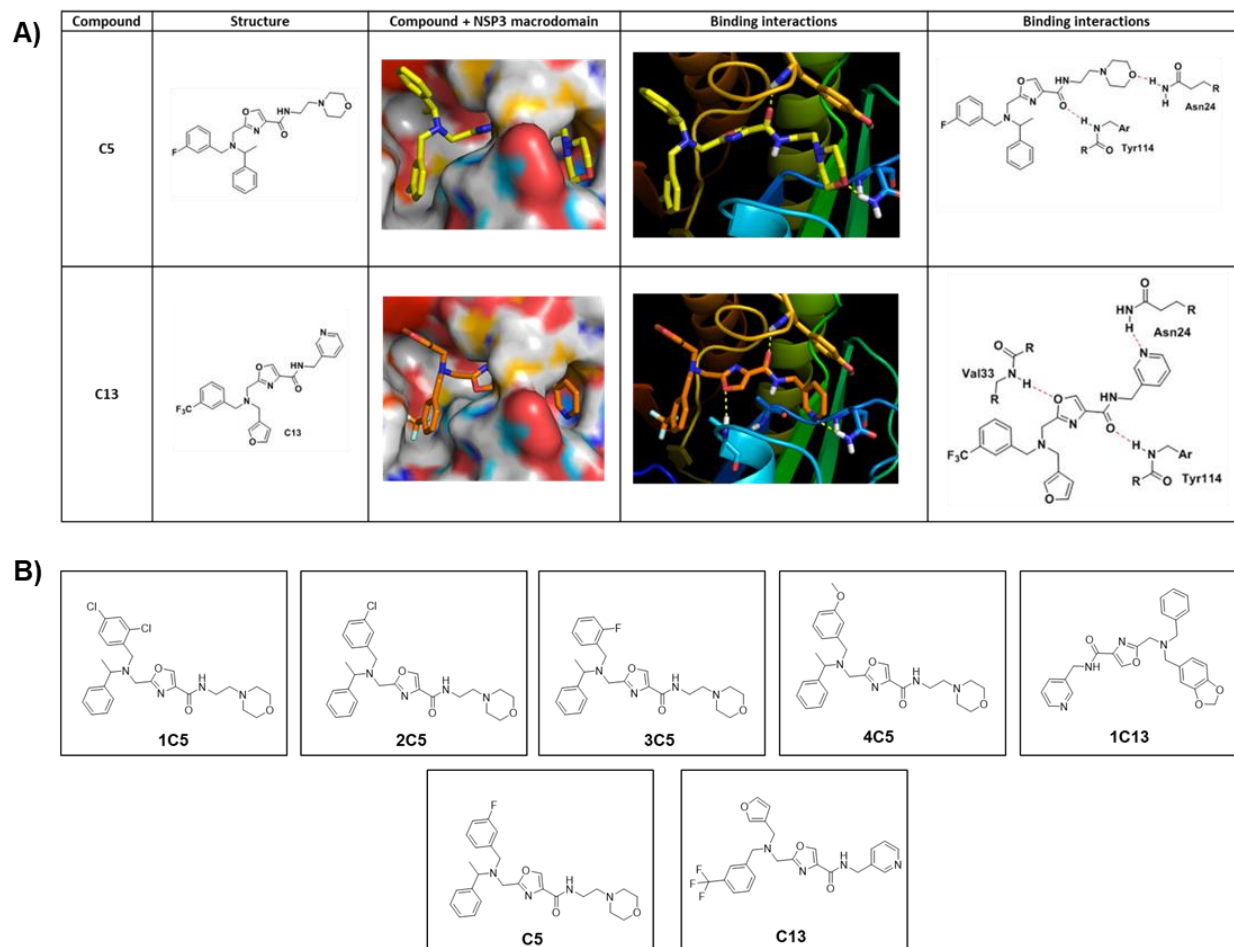


Figure 2. Structure of compounds C5, C13 and analogues. **(A)** Predicted binding conformations of C5 and C13 docked within to the ADP-ribose binding site of CHIKV nsP3 macro domain. Predicted hydrogen bonding interactions between 2D compound structures and CHIKV nsP3 amino acids are shown with a dotted line. **(B)** Molecular structures of the selected analogues (1C5, 2C5, 3C5, 4C5).

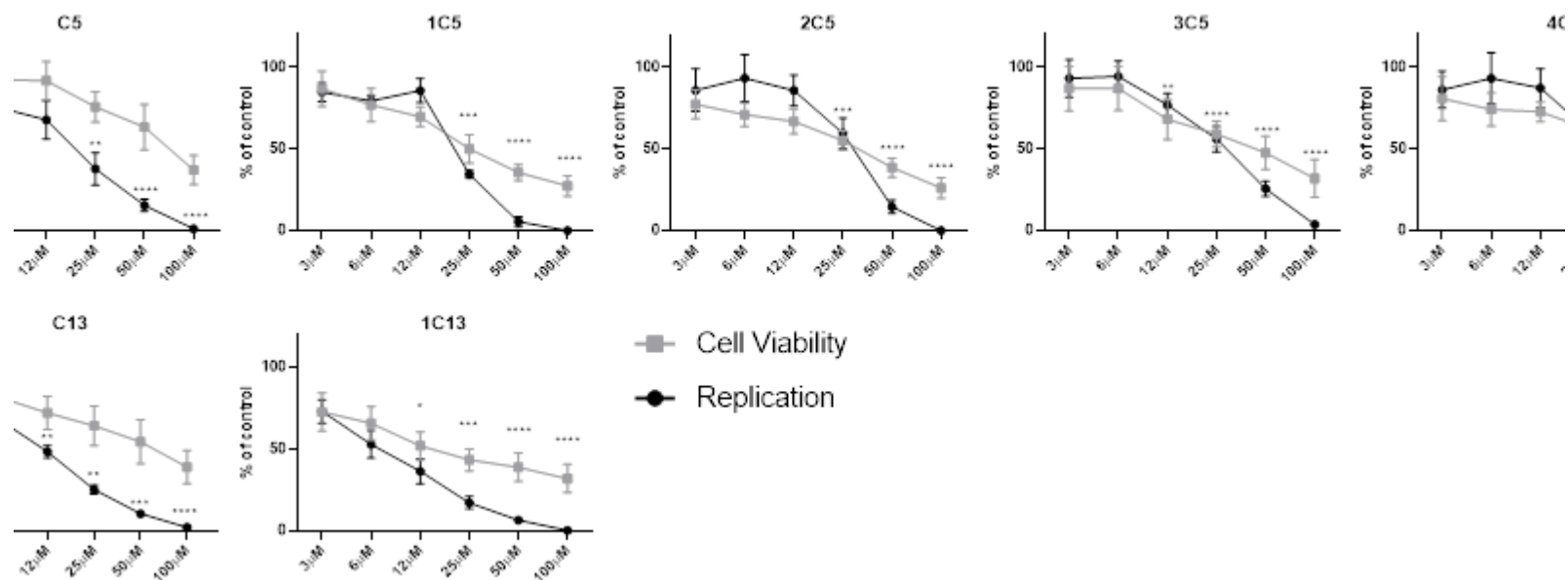


Figure 3: Activity of selected compounds and analogues against CHIKV replication. Huh-7 cells were electroporated with CHIKV-Fluc-SGR RNA, seed and treated 4 h later with compounds at concentrations ranging from 100μM to 3μM. Cell viability (■) was measured by MTT and replication (●) by luciferase assay 24 h post treatment. DMSO was used as non-treated control. P < 0.05 was considered significant, the asterisks indicate statistically significant differences between each compound and DMSO control (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 and ****p ≤ 0.0001), calculated using the Kruskal–Wallis test with the Dunn’s post test.

Table 3. EC₅₀ and CC₅₀ of C5 and C13 analogues.

Compound	EC ₅₀ (μM)	CC ₅₀ (μM)	SI
C5	12.7	38.9	3.1
1C5	19.8	27.5	1.4
2C5	27.3	27.4	1.0
3C5	26.9	40.5	1.5
4C5	29.3	45.2	1.5
C13	11.6	36.8	3.2
1C13	6.9	18.1	2.6

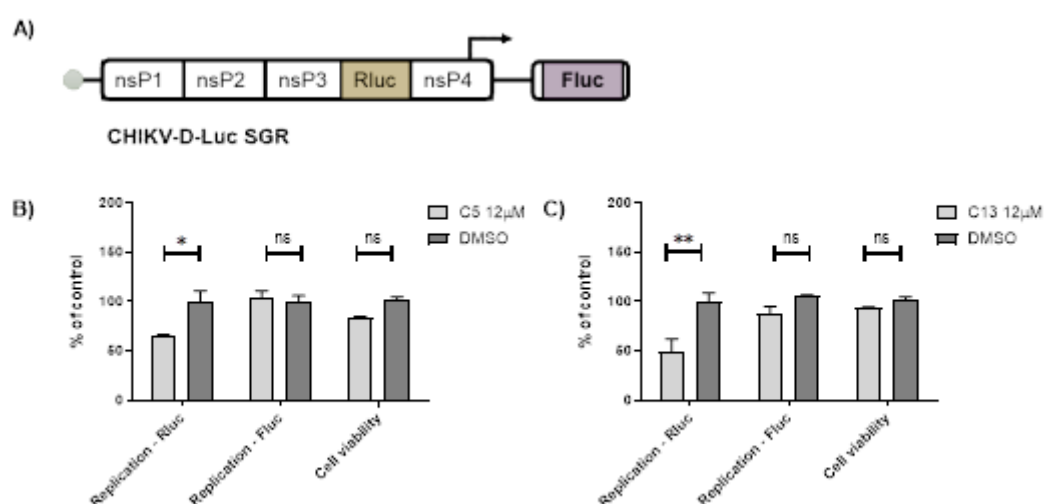


Figure 4: Effect of C13 and C5 at different stages in CHIKV replication. CHIKV-D-Luc wild type RNA (A) were electroporated in Huh-7 cells used to analyse the activity of C5 (B) and C13 (C). DMSO was used as non-treated control. The asterisks indicate statistically significant differences between each compound and DMSO control (* $p \leq 0.05$, ** $p \leq 0.01$). $P < 0.05$ was considered significant calculated using the Kruskal–Wallis test with the Dunn’s post test.

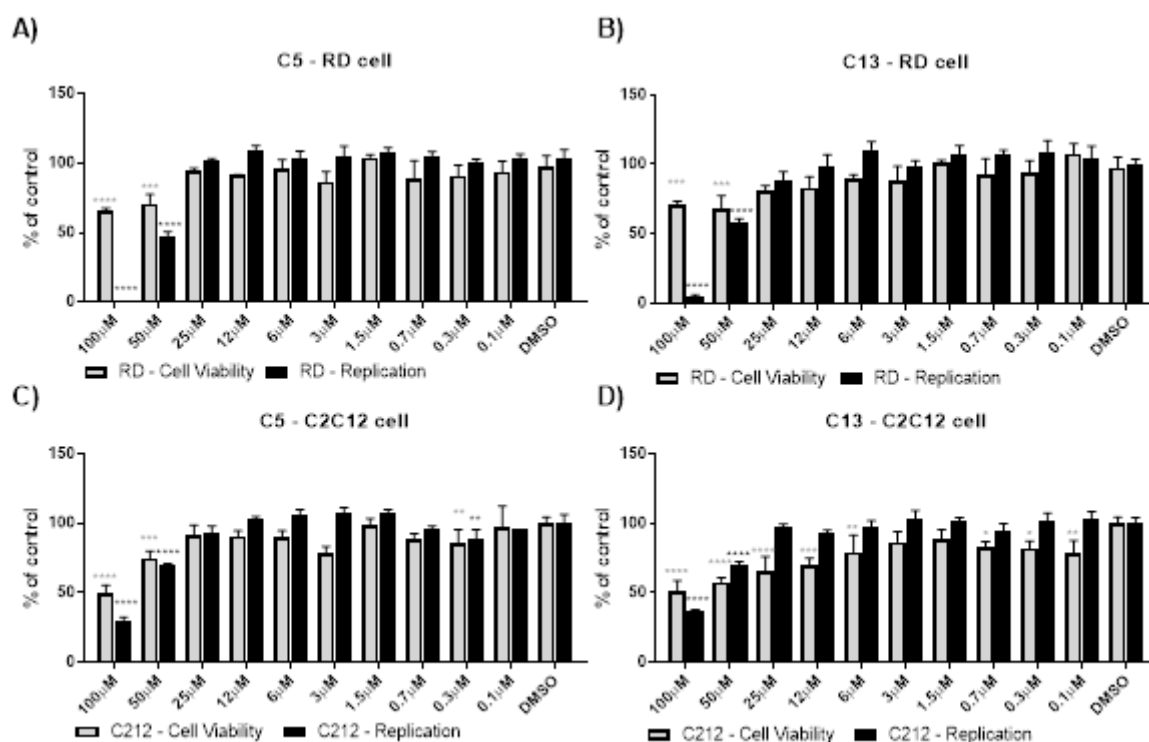


Figure 5. Activity of C5 and C13 in RD and C2C12 cell lines. Cells were electroporated with CHIKV-Fluc RNA, seeded and after 4h treated with C5 or C13. Cell viability and replication were measured in RD (A and B) and C2C12 cells (C and D). DMSO was used as non-treated control. The asterisks indicate statistically significant differences between each compound and DMSO control, cell viability differences (grey asterisks) and replication differences (black asterisks). $P < 0.05$ was considered significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$), calculated using the Kruskal–Wallis test with the Dunn’s post test.

ANEXO C: A REVIEW OF THE ONGOING RESEARCH ON ZIKA VIRUS TREATMENT

A Review of the Ongoing Research on Zika Virus Treatment

Suely da Silva ^{1,2,†}, Daniel Oliveira Silva Martins ^{1,2,†} and Ana Carolina Gomes Jardim ^{1,2,*}

¹Laboratory of Virology, Institute of Biomedical Science, ICBIM, Federal University of Uberlândia, Uberlândia, MG 38405-302, Brazil;

²Genomics Study Laboratory, São Paulo State University, IBILCE, São José do Rio Preto, SP 15054-000, Brazil

suelysilva0712@hotmail.com (S.d.S.);

danielosmartins@gmail.com (D.O.S.M.)

*Correspondence: jardim@ufu.br; Tel.: +55-34-3225-8682

†These authors contributed equally to this work.

Abstract: The Zika fever is an arboviral disease resulting from the infection with Zika virus (ZIKV). The virus is transmitted to humans by the bite of *Aedes* mosquitos, mainly *Aedes aegypti* and *Aedes albopictus*. ZIKV has been detected for decades in African and Asian regions and, since 2007, has spread to other continents; among them, infections are most reported in the Americas. This can be explained by the presence of vectors in highly populated and tropical regions where people are susceptible to contamination. ZIKV has been considered by the World Health Organization a serious public health problem because of the increasing number of cases of congenital malformation and neurological disorders related to its infection, such as microcephaly, Guillain–Barré syndrome, meningoencephalitis, and myelitis. There is no vaccine or specific antiviral against ZIKV. The infection

is best prevented by avoiding mosquito bite, and the treatment of infected patients is palliative. In this context, the search for efficient antivirals is necessary but remains challenging. Here, we aim to review the molecules that have been described to interfere with ZIKV life cycle and discuss their potential use in ZIKV therapy.

Keywords: Zika fever; ZIKV; treatment; antiviral; screen of drugs

Introduction

Zika virus (ZIKV) (strain MR 766) was first isolated [1] from the serum sample of a Rhesus monkey during a research on yellow fever virus (YFV) in the Zika forest, Uganda 1947. In 1948, the virus was isolated from a pool of *Aedes Africanus* (*Stegomyia*) mosquitoes in the same forest [2–4]. The first cases of human infection were reported in 1950 in Africa and later in Asia and remained restricted to these regions until 2007 [2,5,6]. Since then, ZIKV has spread geographically, with reports of infection in the North Pacific [7–9], French Polynesia, and South Pacific [10]. The first records in the Americas date from 2015, when ZIKV spread across the Pacific Ocean to invade Brazil, Suriname, and Columbia [11]. Since 2015, 84 countries reported the new introduction or re-introduction of ZIKV, and, although prevention and control measures are being taken, the risk of infection is still a reality, mainly in regions with a large mosquito vector presence [12].

ZIKV is mainly transmitted by the bite of female mosquitoes of the *Aedes aegypti* and *Aedes albopictus* species. The latter is prevalent in Southeast Asia and, because of its ability to adapt to different environments, it has been found in the Americas, Pacific Islands, Australia, and Africa.

A. albopictus is a potential vector for more than 20 arboviruses, including important members of the Flaviviridae and Togaviridae families such as Dengue (DENV) and Chikungunya (CHIKV), respectively [13]. Both species can co-exist in the same regions. Although previous researches demonstrated the superiority of *A. albopictus*, this species is considered the second main vector of DENV and CHIKV [13,14]. Indeed, the transmission by other species of the *Aedes* genus, including *Aedes africanus*, *Aedes luteocephalus*, *Aedes vitattus*, *Aedes furcifer*, *Aedes hensilli*, and *Aedes apicoargenteus*, should be considered [15]. The successful virus transmission by these species is related to their high capacity of proliferation in tropical and subtropical regions [15–17]. Even though wild primates are the main non-human reservoirs, anti-ZIKV antibodies have been also identified in rodents and domestic animals [18,19]. Additionally, other transmission routes have been confirmed, such as blood transfusion [20], sexual [21], perinatal, and transplacental transmissions [11,22].

Zika fever is considered a mild disease with symptoms as headache, fever, malaise, and joint pain [23]. However, since reemerged, ZIKV has been associated with the increasing number of microcephaly cases, Guillain–Barré syndrome, meningoencephalitis, and myelitis in highly affected areas like the Brazilian North Eastern State [24,25].

The ZIKA Virus

ZIKV belongs to the Flaviviridae family and the genus *Flavivirus*, being phylogenetically related to the dengue virus (DENV), West Nile virus (WNV), and yellow fever virus (YFV) [7]. Recently, the structural similarity among the virions of ZIKV and other *Flavivirus* was demonstrated by cryoelectron microscopy (cryo-EM) [26]. The viral nucleocapsid of *Flavivirus* is surrounded by a bilayer lipid membrane with a 25 to 30 nm diameter derived from the host cells, in which viral glycoproteins are inserted.

Like other flaviviruses, the virus enters the cell by endocytosis driven by the interaction of the envelope proteins and specific receptors on the host cell [27]. The process of internalization is intermediated by clathrin protein in a low pH environment. The viral genome is released into the host cytoplasm where it will be translated. The ZIKV genome is a positive-sense single-stranded RNA approximately 11 kb long that is uncoated immediately after virus entry into the host cells [28]. The viral genome has a single “open reading frame” (ORF) flanked by 5' and 3' untranslated regions of 106 nucleotides and 428 nucleotides, respectively. The polyprotein encoded by this ORF is proteolytically cleaved into three structural proteins (capsid protein, premembrane/membrane protein, and envelope glycoprotein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [8,29,30]. The NS3 and NS5 proteins are essential for viral replication, possessing high catalytic activity [31]. After viral genome translation, the new viral particles are released from the cells and restart the infection process [30] (Figure1).

Phylogenetic analysis of the ZIKV genome indicates the existence of three lineages: West Africa, Asian/American [32,33], and Brazilian ZIKV [34]. Further analyses of Asiatic and African ZIKV strains isolated from infected mosquitoes, monkeys, and humans showed significant amino acid variations throughout the sequence of the viral polyprotein [35]. The same study associated the human strains isolated during the recent outbreaks with the viral strain P6-740 (from Asian mosquito, 1966) and demonstrated that all strains have a common ancestor. However, all the recent strains present a minimum of 400 amino acid mutations when compared

with P6-740, which can interfere with the viral replicative efficiency, fitness, and transmissibility [35].

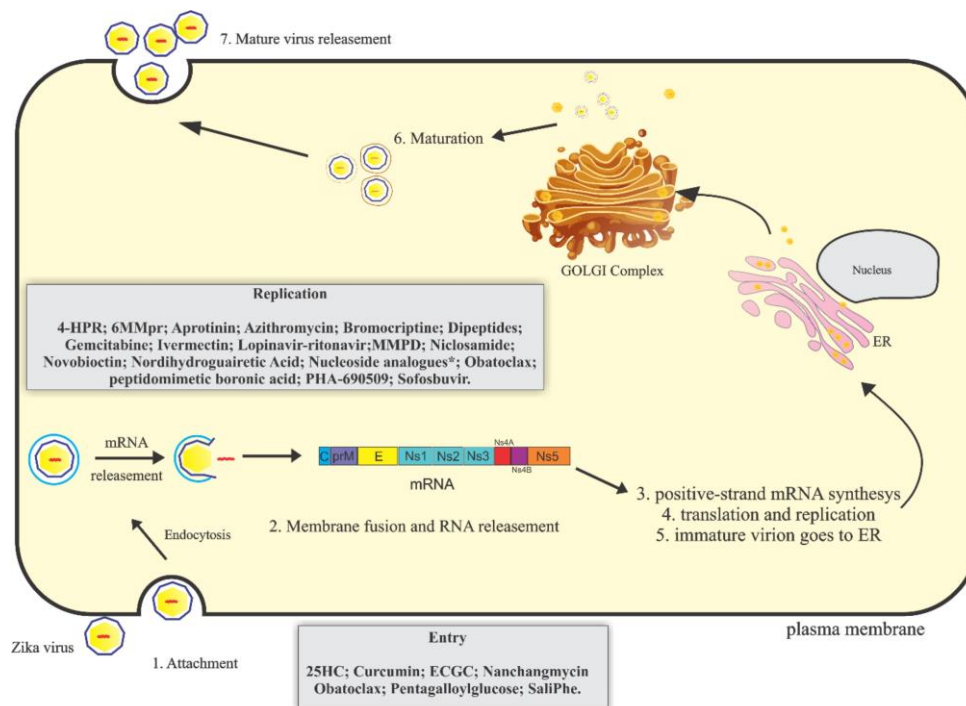


Figure 1. Schematic representation of Zika virus (ZIKV) replicative cycle. ZIKV replicative cycle and inhibitory drugs of viral entry and replication. 4-HPR = N(4-hydroxyphenyl) retinamide; 6MMpr = 6-methylmercaptapurine riboside; MMPD = Merimepodib; 25HC = 25-Hydroxycholesterol ; ECGC = Epigallocatechin galato; ***7-desaza-2^J-C-methyladenosine (7-desaza-2^J-CMA); 2^J-C-methyladenosine (2^J-CMA); 2^J-C-methylcytidine (2^J CMC); 2^J-C-methylguanosine (2^J-CMG) and 2^J-C-methyluridine (2^J-CMU).

Therapeutical Potential of Anti-Zikv Molecules

Currently, there is no approved vaccine or specific antiviral against ZIKV infection. The main strategy for controlling ZIKV is to prevent the breeding of mosquitoes [36]. The treatment is palliative and includes rest and fluids intake. Acetaminophen or paracetamol are used to alleviate the symptoms of headache, fever, and myalgia. The use of aspirin or other salicylates is not recommended in childhood to avoid the risk of Reye's syndrome. Other non-steroidal anti-inflammatory drugs (NSAIDs) should be avoided due to the risk of hemorrhagic complications [37,38]. Here, we aim to review the molecules that have been described to interfere with the ZIKV replication life cycle (Figure1, Table1).

Table 1. Compounds tested with potential anti-ZIKV activity.

	Compound	Validated Cell/Test	Anti-ZIKV Strategy	Reference
Drug Repurposing	Bromocriptine	Vero cells	Inhibition of NS2B-NS3 protease	[39]
	Emricasan	Glioblastoma SBN-19, hNPCS and human astrocytes cells	Reduces cellular apoptosis by inhibition of caspase-3 activities	[40]
	Ivermectin, Daptomycin, Mycophenolic acid (MPA),			
	Sertraline, Pyrimethamine, Cyclosporine A, Azathioprine, Mefloquine	HuH 7 cells	Unknow	[41]
	Nicosamide, PHA-690509	Glioblastoma SBN-19, hNPCS and human astrocytes cells	Inhibit viral replication	[40]
	Chloroquine	Vero, hNSC and hBMEC cells	Inhibits early stages of replicative cycle	[42]
	Bromocriptine	Vero cells	Inhibition of NS2B-NS3 protease	[39]
	Curcumin	HeLa cells	ZIKV entry and virucidal effect	[43]
	Epigallocatequina galato	Vero cells	Inhibits the viral entry	[44]
	GSK126	Telomerase reverse transcriptase-immortalized HFF cells	Strongly inhibited ZIKV infection in pre-treated cells	[45]
Potential Inhibitors of the early and late stages of replication	Heparin	human neural progenitor cells (hNPCs)	Inhibits caspase 3 activity mediated by ZIKV infection.	[46]
	Nanchangmycin	U2OS cells	Blocks viral entry blocking clathrin-mediated endocytosis	[47]
	Obatoclax	Human telomerase reverse transcriptase-immortalized retinal pigment (RPE)	Inhibits endocytic uptake of ZIKV and viral protein synthesisPrevents caspase 8, 3 and 7 activationProtects the phosphorylation status of p27 phosphoprotein	[48]
	Pentagalloylglucose (PPG)	Vero B4	ZIKV entry	[49]
	SaliPhe (Sofosbuvir)	human telomerase reverse transcriptase-immortalized retinal pigment (RPE)	Inhibits endocytic uptake of ZIKV and protein viral synthesisPrevents caspase 8, 3 and 7 activation	[48]
	25-Hydroxycholesterol (25 HC)	BHKK-21	Inhibits ZIKV internalization	[50]
			Nucleoside inhibitor; binds too amino acid	

Table 1. Cont.

Compound	Validated Cell/Test	Anti-ZIKV Strategy	Reference
Novobiocin, lopinavir-ritonavir	Vero/Huh-7/ <i>in silico</i>	Inhibition of NS2B-NS3 protease	[52]
Peptidomimetic boronic acid	Huh-7/ <i>in silico</i>	Inhibition of NS2B-NS3 protease	[53]
Aprotinin	<i>In silico</i>	Inhibition of NS2B-NS3 protease	[54]
<i>N</i> -(4-hydroxyphenyl) retinamide (4-HPR)	Vero cells	Inhibition of viral replication probably interacting with NS5 protein Inhibits ZIKV RNA replication inhibiting	[55]
Merimepodib (MMPD)	Huh7	IMPDH (inosine-5'-monophosphate dehydrogenase)	[56]
Gemcitabine	Human telomerase reverse transcriptase-immortalized retinal pigment (RPE)	Interferes with transcription of viral RNA Inhibits viral protein synthesis Prevents caspase 8, 3 and 7 activation Changes the phosphorylation status of the CREB phosphoprotein affected by ZIKV infection	[48]
Cimiphenol, Cimracemate B, Rosemarinic acid	<i>In silico</i>	high affinity with NS5 methyl transferase 6 MMpr used during viral RNA synthesis	[57]
6-methylmercaptopurine riboside (6 MMpr)	Vero and SII—SY5Y neuronal cells	reducing the viral infectivity caused by mutations and viral replication errors	[58]
4,7-digalloylcatechin, Prenylated chalcone,		High affinity with NS5 RNA-dependent	
2,4,4-trihydroxy-3,3-diprenylchalcone, Bis-indole alkaloid flinderole Lignan di- <i>O</i> -demethyl isoguaiacin	<i>In silico</i>	RNA polymerase	[57]
Bis-indole alkaloids flinderole A and flinderole B	<i>In silico</i>	High affinity with NS2B-NS3 protease	[57]
Cimiphenol, Cimracemate B, Rosemarinic acid	<i>In silico</i>	High affinity with NS5 methyl transferase	[57]
Isoquinoline alkaloid cassiarin D3-Omethyldiplacone, Exiguaflavanone A, Sesqui terpenoid lactucopicrin Aurone kanzonol V, Chalcones vangusticornin BB Balsacone Bkansonol Y lignans hibalactone kaerophyllin	<i>In silico</i>	Inhibition of NS3 helicase ATP site	[57]
Nordihydroguaiaretic Acid	Vero	Disturbs the lipid metabolism	[59]
Nucleoside analogues:			

7- desaza-2^J-C-methyladenosine
 (7-desaza-2^J-CMA)2^J-C-methyladenosine (2^J-
 CMA)2^J-C-methylcytidine (2^J CMC)2^J-C-
 methylguanosine
 (2^J-CMG)2^J-C-methyluridine (2^J-CMU)

Vero, Human neuroblastoma (UKF-NB-4 and
 porcine kidney (PS) cells

Reduce cytopathic effect, cell death and
 inhibit the viral replication

[60]

Drug Repurposing

A research group screened 6000 pharmacologically active compounds against ZIKV infection, measuring caspase-3 activity and cell viability of treated human neural cells. Emricasan, also known as IDN-6556 or PF-03491390, is an inhibitor of the pan-caspase pathway that presented a protective effect against ZIKV-induced cell death in glioblastoma SNB-19 cells. This effect was observed when the cells were previously infected with ZIKV strains MR766 (1947 Ugandan strain), FSS13025 (2010 Cambodian strain), PRVAB659 (2015 Puerto Rican strain) and then treated with 0.13–0.9 μM (IC_{50}) of the compound. Also, Emricasan reduced the number of active (cleaved) caspase-3-expressing forebrain-specific human neural progenitor cells (hNPCs) infected by the FSS13025 strain in both monolayer and 3D organoid cultures. Although cell proliferation was not affected by Emricasan treatment of ZIKV-exposed brain organoids, the data showed that ZIKV antigen persisted in both cultures. Therefore, the authors concluded that Emricasan presented neuroprotective activity for hNPCs but did not inhibited ZIKV-replication [40].

Niclosamide, an anti-helminthic approved by the Food Drug Administration (FDA), and the cyclin-dependent kinase inhibitor PHA-690509 demonstrated to significantly inhibit ZIKV infection in vitro [40]. SNB-19 cells were treated with increasing concentrations of these compounds one hour prior inoculation with the FSS-13025, PRVABC59, or MR766 ZIKV strains (multiplicity of infection (M.O.I) = 1). Twenty-four hours post inoculation, the cells were harvested, and the expression of the protein NS1 was evaluated by western blotting. The results demonstrated that these compounds significantly inhibited infection by the three strains of ZIKV. Additionally, the effects of these compounds on the early and post-entry stages were investigated. The results showed that niclosamide and PHA-690509 inhibited ZIKV infection post-entry, probably blocking viral replication. Additionally, a combined therapy with Emricasan and PHA-690509 presented an additional inhibition effect on caspase activity, showing the synergistic effect of these compounds [40].

Similar analyses were performed by Barrows and coworkers who investigated the anti-ZIKV activity of a library of 774 drugs approved by the Food and Drug Administration (FDA). Huh-7.0 cells were pre-treated with the compounds for one hour, inoculated with ZIKV MEX_I_7/15 (M.O.I = 0.4), fixed twenty-four hours post-infection (h.p.i), and labeled with immunofluorescent antibodies against the viral envelope proteins. The majority of drugs showed no significant effect; however, 30 compounds demonstrated to be effective, decreasing virus infectivity. Eight compounds (ivermectin, daptomycin, mycophenolic acid (MPA), sertraline, pyrimethamine, cyclosporine A, azathioprine, and mefloquine) were selected for tests in human cell lines derived from cervical cancer (HeLa), placental (JEG3), and neural tissues (hNSC). In Hela cells, all the eight compounds reduced the infectivity rates, and four of these compounds presented anti-ZIKV activity in JEG3 cells. For flow cytometry analyses, hNSC cells were maintained in the presence of the drugs and ZIKV (M.O.I = 3) for 96 h. The results showed that MPA, cyclosporine A, and ivermectin significantly inhibited ZIKV MEX_I_7 infection. In human amnion epithelial cells (HAECs), ivermectin, daptomycin, sertraline, and mefloquine inhibited viral infection at the maximum concentration of 16 μM . Ivermectin, sertraline, and mefloquine demonstrated a higher antiviral effect, however, a significant reduction of cell viability was observed. Ivermectin (EC_{50} = 1 μM) and MPA (EC_{50} = 10 μM) demonstrated the most potent action on ZIKV replication [41]. Among these compounds, the most potent inhibitors were ivermectin, mycophenolic acid (MPA), and daptomycin. Ivermectin is an anti-helminthic agent that in previous studies showed a potential activity against in YFV infection [61], while MPA is a nonnucleoside inhibitor of inosine monophosphate (IMP) dehydrogenase whose antiviral properties against DENV have been described [62]. Daptomycin is an antimicrobial whose antiviral properties had not been previously described [41].

The antiviral activity of 2177 compounds approved by the FDA was evaluated as a treatment of ZIKV infection *in vitro*. The Vero and U87 cell lines were treated for a period of 2 h with each compound at 2 μ M and then were infected with the strain ZIKV-BR, with M.O.I = 1, 3, or 10. After 72 h, the antiviral activity was analyzed by luminescence, and, in parallel, cell viability was evaluated. The compounds that inhibited viral infection and resulted in cell viability being 2.5-fold greater than in the untreated control were chosen to be validated by flow cytometry. To validate the antiviral properties, U87 cells were pretreated with different concentrations of the compounds for 1 h and then were infected with the Brazilian viral strains ZIKV-BR or ZIKV-PR. Forty-eight hours post-infection (h.p.i), the cells were fixed, permeabilized, labeled with specific antibodies against the viral envelope protein, and analyzed by flow cytometry. The macrolide antibiotic azithromycin inhibited viral replication at the different M.O.Is = 0.01 (EC50 = 2.1), M.O.I = 0.1 (EC50 = 2.9), and M.O.I = 3 (EC50 = 5.1) and also reduced cell

death triggered by the infection. The authors suggested that this compound may be considered as a potential antiviral to treat the viral infection caused by ZIKV and could be administered to pregnant women [63].

Chloroquine is another compound approved by the FDA to treat malaria that can be administered to pregnant women. The anti-ZIKV properties of chloroquine were tested in Vero cells, human brain microvascular endothelial cells (hBMEC), and hNSCs infected with ZIKV MR766 for 5 days and treated with the compound at concentrations ranging from 6.25 to 50 μ M. The viral envelope E protein was labeled with the 4G2 antibody, specific to flavivirus, to measure viral infectivity. The data obtained showed a decreasing virus infection in a dose-dependent manner. The results were corroborated by immunofluorescence and flow cytometry analyses, which demonstrated that chloroquine at 50 μ M reduced ZIKV infection up to 65% and 50% in Vero cells and hBMEC, respectively, with no effect on cell viability. In hNSCs, chloroquine at 50 μ M reduced ZIKV infection by 57% after 4 days of treatment, with 70% of cell viability. Chloroquine at 25 μ M was also tested in Vero cells infected with the ZIKV Brazilian strain isolate and showed to decrease up to 16 folds the viral RNA levels in the supernatant. Moreover, chloroquine activity was tested in distinct stages of ZIKV infection, demonstrating to block the early stages of the replication cycle [42]. Chufeng Li and coworkers also demonstrated that chloroquine protects fetal mice from microcephaly caused by ZIKV [64].

Bromocriptine is a dopamine D2 and D3 receptor agonist, which is used to treat galactorrhea and Parkinson's disease [65,66] and was also reported to possess anti-viral properties [67]. Bromocriptine was tested for the ability to inhibit viral infection caused by the ZIKV. First, Vero cells were treated with different concentrations of the compound for 2 h. Then, the medium-containing compound was removed, and the cells were infected with ZIKV (Puerto Rico-PRVABC59) at M.O.I = 0.0001 for 1 h. After the adsorption, the inoculum was completely removed, and the cells were supplied with fresh media containing Bromocriptine and incubated for 6 days. The cytopathic effect was examined by microscopy, and cell viability was measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. The data showed the absence of cytopathic effects caused by ZIKV infection. In addition, the supernatant from the infected cells was collected for analysis of viral yield by qRT-PCR, which demonstrated a reduction of the viral titers, suggesting that Bromocriptine is able to inhibit the infection caused by ZIKV. In order to evaluate in which stage of the replicative cycle the compound was acting, a time-of-drug addition assay was performed. The cells were treated with 20 μ M of bromocriptine prior to the infection (−1 to 0 h.p.i), simultaneously with the virus (0 to 1 h.p.i), and after infection (0.3, 6, 9, 12, 14 h.p.i). The cells were infected with ZIKV at M.O.I of 2 and incubated for 18 h. The viral supernatant from each time point was collected and submitted to qRT-PCR for quantification of RNA. The results showed low RNA levels when the treatment was performed 0.3, 9.9, or 12 h.p.i. No significant reduction of RNA was observed in the early phases of the replicative cycle (0–1 h.p.i), in the release step (after 14 h.p.i), or in the pretreatment condition

(−1 h.p.i). In addition, it was analyzed whether the compound Bromocriptine inhibited ZIKV NS2B-NS3 protease in an in vitro system. For this analysis, ZIKV NS2B-NS3 protease containing a fluorescent reporter was previously synthesized, incubated with different concentrations of Bromocriptine for 10 minutes, and then subjected to fluorescence reading. The results showed that Bromocriptine inhibited NS2B-NS3 protease activity in a dose-dependent manner. In addition, in silico analysis showed an intracellular interaction between Bromocriptine and NS2B-NS3 protease. This analysis revealed that Bromocriptine presented high binding affinity with various residues of the NS2B-NS3 complex and could possibly interact with the active site of ZIKV NS2B-NS3 protease and inhibits its activity [39].

The flaviviral NS2B-NS3 protease process the ZIKV polyprotein into seven non-structural and three structural proteins, being crucial for ZIKV replication and demonstrating to be an important antiviral target [68]. The NS3-NS2B gene was cloned into a plasmid, and Rosetta2 (DE3) cells were transformed, cultured, and lysed to extract the protease in a study by Lee and coworkers. The authors performed a high-throughput assay testing 70 synthetic compounds through enzyme reactions, measuring fluorescence intensity. The compounds named 1 and 2 strongly inhibited ZIKV protease with half maximal inhibitory concentration (IC₅₀) values of 5.2 μ M and 4.1 μ M, respectively. Both compounds strongly bound to the protease catalytic site, competitively inhibiting the protein [69]. Other works have also found ZIKV NS2B-NS3 protease inhibitors, such as the peptidomimetic boronic acid [53], dipeptides [70], Aprotinin [54], novobiocin, and lopinavir/ritonavir [52].

In Silico Interaction Analysis and Natural Drugs

Byler and coworkers generated structural models of ZIKV proteins (NS3 helicases, NS5 methyltransferase, RNA-dependent RNA polymerases, and NS2B-NS3 proteases) by homology modeling techniques to analyze in silico the interactions between these viral proteins and molecules derived from plants. A total of 2263 compounds isolated from herbs, including alkaloids, polyphenols, and terpenoids, were screened. For ZIKV NS2B-NS3, only the bis-indole alkaloids flinderole A and flinderole B were in agreement with the Lipinski's rules (experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings) [71]. For ZIKV NS3 helicase, the isoquinoline alkaloids cassiarin D, 3-methyldiplacone, exiguaflavanone A, the sesquiterpenoids lactucopicrin, auronekazonol V, chalcones angusticornin B, balsacone B, and kazonol Y, and the lignans hibalactone and kaerophyllin showed high binding activity to the ATP site. The polyphenolic compounds cimiphenol, cimracemate B, and rose marinic acid presented strong docking to NS5 methyl transferase, while ZIKV NS5 RNA-dependent RNA polymerase presented docking ligand properties to the polyphenolic compounds 4,7-digalloylcatechin, prenylatedchalcone, 2,4,4-trihydroxy-3,3-diprenylchalcone, bis-indole alkaloid flinderole, and lignan di-O-demethylisoguaiacin. All polyphenolic compounds showed to be the best ligands, demonstrating high affinity to several protein sites due to the higher potential to the target viral proteins. Monoterpenoids and triterpenoids showed the lowest binding affinity. Other phytochemicals, such as balsacone B, kazonol V, cinnamoylchinchinanthol, cimiphenol, and rosemarinic acid, isolated from *Populus balsamifera*, *Glycyrrhiza glabra*, *Echinacea* root, *Cimicifuga racemosa* and *Rosmarinus officinalis*, respectively, showed great docking properties to viral proteins, which highlights the antiviral potential of molecules available in nature [57].

Potential Inhibitors of the Early Stages of Replication

Kuivaniemi and coworkers investigated the antiviral activity of the compounds SaliPhe, obataclax, and gemcitabine in human telomerase reverse transcriptase-immortalized retinal pigment (RPE) cells infected with ZIKV FB-GWUH-2016 strain. These three compounds

present anticancer properties and also demonstrated antiviral properties against the Influenza A virus. SaliPhe and obatoclax act to prevent virus entry into cells, while gemcitabine inhibits viral replication [72]. The authors analyzed ZIKV-mediated cellular apoptosis, transcription, signaling, and metabolism. Immunofluorescence assay showed that these compounds, at non-cytotoxic concentrations, inhibited the synthesis of viral proteins 24 h.p.i. The reduction of RNA viral expression and production of infectious viral particles was also confirmed by RT-qPCR or plaque assay 48 h.p.i., respectively. It was also analyzed whether the compounds inhibited ZIKV-mediated cell death. Initially, short treatments were performed with each compound, starting at 0, 2, 4, 6, and 8 h.p.i. The infected cells were maintained in the presence of each compound for 2 h and then supplied with fresh culture medium. A second experiment was performed by adding each compound 0, 2, 4, 6, 8, and 10 h.p.i, but the culture medium containing the compound was not replaced. For both treatments, cell viability was measured 48 h.p.i. The results demonstrated that cellular viability was maintained when the cells were treated up to 4 h.p.i., and gemcitabine protected the cells when added 10 h.p.i without replacing the medium [48]. These compounds also inhibited ZIKV-mediated activation of caspases 3, 7, and 8, responsible for cellular apoptosis. SaliPhe, obatoclax, and gemcitabine were evaluated by their activity on two signaling phosphoproteins which are mainly affected by ZIKV infection, i.e., the transcription factor cAMP response element binding protein (CREB) and the cyclin-dependent kinase inhibitor 1B (p27). For a better understanding of SaliPhe and obatoclax effects on antiviral genes expression, the cells were treated with the compounds at a concentration of 1 μ M and infected with ZIKV at M.O.I = 8,5. After 10 h, the gene expression profiles were analyzed by RNA quantification. Antiviral gene expression was impaired by SaliPhe and obatoclax, but not by gemcitabine. The research results showed that these compounds presented different mechanisms of action, and, therefore, a combined therapy could be a great strategy against ZIKV infection [48].

Epigallocatechin gallate (EGCG) is a natural polyphenol that exists in high abundance in green tea, with antiviral properties described for human immunodeficiency virus (HIV) [73], herpes simplex virus (HSV) [74], influenza virus (FLU) [75], and hepatitis C virus (HCV), consisting in the inhibition of viral entry into cells [76]. The anti-ZIKV potential of the natural compound epigallocatechin gallate (EGCG) was demonstrated in a research conducted by Carneiro and coworkers. They investigated the virucidal potential of EGCG against ZIKV by using Vero cells infected with ZIKV BR or MR766 strains. First, the compound and the virus were simultaneously incubated for 1 h and then added to the cells for 96 h. After incubation, the viral supernatant was titrated by plaque assay. The results demonstrated that EGCG at 200 and 25 μ M reduced the foci number of ZIKV BR and MR766 by a minimum of 90% and 85%, respectively. These data also showed that EGCG acts by strongly inhibiting ZIKV entry into Vero cells [44]. Another study showed that EGCG minimally inhibited the infection caused by ZIKV [77]. This could be explained by the difference in the methodology used by the authors. In this work, Raekiansyah and coworkers investigated EGCG effect on ZIKV by infecting the cells in the presence or absence of EGCG for 5 days and by measuring the levels of viral antigens using an enzyme-linked immunosorbent assay (ELISA). Another interesting factor that may have contributed to the divergence of the results may be related to the different types of strain used in this study [77].

After a high-throughput screening against JEV (Japanese Encephalitis Virus), Wang and coworkers found 28 bioactive compounds which inhibited JEV infection by $\geq 90\%$. Among them, nine compounds preserved cell viability by more than 80% of. These compounds were tested against ZIKV by pretreating Vero cell for 1 h and then infecting them with ZIKV M.O.I 0.2 for 1 h. Forty-eight h.p.i. the cell supernatants were submitted to RNA extraction and qRT-PCR. The cells were lysed, and the viral RNA was quantified. Through these assays, the authors found that manidipine, clinidipine, benidipine, primocrolimus, and nelfinavir mesylate were potential antiviral candidates. Manidipine, clinidipine, and benidipine significantly reduced

ZIKV RNA levels into the infected cells. These and the other compounds also decreased ZIKV RNA levels in the supernatants at concentrations ranging from 4 to 10 μM . The authors explained that more studies are needed to elucidate the mechanism of action of these compounds [78].

Using a microscopy assay, Keiko Rausch and coworkers isolated 38 compounds against ZIKV infection. Among those, nanchangmycin, a natural product isolated from *Streptomyces nanchangensis*, presented a strong anti-ZIKV activity. The authors pretreated U2OS cells with 2 μM of each compound for 1 h, prior to infection with ZIKV (MEX2-81 at a M.O.I = 100) for 30 minutes. The cells were then fixed and submitted to confocal microscopy, by using an antibody against ZIKV glycoprotein (4G2). Their results showed that the virions remained on the cell surface, and that nanchangmycin inhibited viral entry by blocking clathrin-mediated endocytosis [47].

Arbuckle and coworkers assessed the antiviral activity of EEZH2/1 histone methyltransferases inhibitors against ZIKV. Telomerase reverse transcriptase-immortalized human foreskin fibroblasts (HFF) cells were pretreated with GSK 126, GS343, UNC1999, astemizole, and ACV at concentrations ranging from 5 to 100 μM for 5 h and then infected with ZIKV (H/PF/2013; 30,000 FFU/ 2.2×10^4 cells). Forty h.p.i., the cells were fixed and stained with pan-Flavivirus monoclonal antibody E60, and the foci formation was scored by size with Immuno Spot Macronalyzer. The authors conducted a similar assay also with cells treated after ZIKV adsorption. The compound GSK126 strongly inhibited ZIKV infection at concentrations of 15 and 20 μM when the cells were pretreated and also showed antiviral activity in the post-treatment experiment. The authors suggested that the compounds could activate proinflammatory and immune cell recruitment pathways as their mode of action [45].

Cortex Moutan (CM) is the bark of the peony tree root used in Chinese medicine as a drug with hepatoprotective, anti-inflammatory, and antiviral properties. The natural compound Pentagalloylglucose (PPG) is one constituent of CM and has been shown to interfere with the entry of all HCV virus genotypes into human hepatoma cells as well as primary hepatocytes. The antiviral properties of PPG were also tested on ZIKV infectivity. For this, Vero B4 cells were infected with ZIKV Puerto Rico strain and treated with different concentrations of PPG for 48 h. After this time, the supernatants were harvested, and the viral titer in the supernatants was measured by qRT-PCR. The results showed a dose-dependent decrease of the viral titers in the supernatants, with IC₅₀ of

4.1 μM . These data showed that the natural compound PPG not only prevents HCV infection but also has anti-ZIKV properties, showing the potential of PPG as an antiviral against both infections [49].

Li and Deng tested synthetic 25-Hydroxycholesterol (25HC) as an antiviral against ZIKV. BHK21 cells were treated with 25HC (1–5 μM) and infected with ZIKV (GZ01/2016 strain 200 PFU/well) 12 h later at 4 °C for 1 h. After incubation, the cells were washed with cold PBS to remove the unbound viruses and incubated 1 h at 37 °C. The virus titers were measured by plaque assay. The compound 25HC inhibited ZIKV internalization up to 100% at 5 μM . In previous works, the authors suggested that this compound inhibited viral entry by suppressing the fusion between the virus and the cell membranes for VSV, HIV, and Nipah virus [50].

Curcumin is a natural compound extracted from the *Curcuma longa* L. root that has broad therapeutic properties, such as anti-inflammatory, anti-microbial, and anticancer [79]. The antiviral properties of curcumin were also evaluated against ZIKV. HeLa cells were pretreated with the compound at different concentrations (from 1 nM to 5 μM) for 2 h, and fresh medium containing the compound and ZIKV M.O.I = 0.1 was added to the cells for 48 h. The viral supernatant was then collected and titrated by plaque assay. The results showed that concentrations equal to or greater than 100 nM significantly decreased the infection by ZIKV

in a dose-dependent manner. RNA from the supernatants was also extracted and quantitated by qRT-PCR for the determination of RNA levels, and the data obtained showed that 5 μ M of curcumin was able to reduce the viral RNA levels, corroborating the results from the previous assay. To analyze at which stage of the replicative cycle curcumin would be acting, Hela cells were treated with the compound and infected with ZIKV (M.O.I = 0.1) at different times (before and after infection). In addition, an assay was proposed to verify whether curcumin inhibited the infection by interfering directly with the virus and preventing ZIKV entry into the cells. To this end, the virus and the compound (1, 10, or 100 μ M) were incubated for 1 h and then added to the Hela cells for a further 1 h. The cells and the bound virus were collected and lysed, and the RNA was extracted and quantified by qRT-PCR. The results showed a significant dose-dependent reduction of viral RNA, showing that this could be a possible mechanism of action for curcumin. The same assays were performed with the curcumin analog compounds demethoxycurcumin and bisdemethoxycurcumin, which also presented anti-ZIKV activity, exhibiting similar cell toxicity as unmodified curcumin [43].

Potential Inhibitors of the Late Stages of Replication

In the search for effective antivirals, research centers have screened compounds which previously demonstrated to possess antiviral activity against other pathogens for their therapeutic potential against ZIKV infection [40]. As an example, nucleoside analogues showed to inhibit the viral replication of the herpes simplex viruses 1 and 2 (HSV-1 and HSV-2) [80], cytomegalovirus (CMV) [81], hepatitis B virus (HBV) [82], human immunodeficiency virus (HIV) [83–86], and hepatitis C virus [87].

Eyer and coworkers investigated the anti-ZIKV efficacy of a panel of 29 nucleoside analogues by using an infected culture system. Vero cells were infected with ZIKV at a M.O.I of 0.1 in the presence or absence of compounds ranging from 0 to 100 μ M. Five days post-infection (d.p.i.), cell viability was determined by a cytotoxicity assay, and the viral titers were determined by the plaque assay. The nucleoside analogues 7-desaza-2j-C-methyladenosine (7-desaza-2j-CMA), 2j-C-methyladenosine (2j-CMA), 2j-C-methylcytidine (2j CMC), 2j-C-methylguanosine (2j-CMG), and 2j-C-methyluridine (2j-CMU) reduced the cytopathic effect caused by ZIKV infection, demonstrating the anti-ZIKV potential of these compounds [60].

In another work, Vero cells were infected with ZIKV PA259459 strain (M.O.I 1) for 1 h, washed, and supplied with 35 μ M of Nordihydroguaiaretic Acid (NDGA), tetra-O-methyl nordihydroguaiaretic acid (M4N), PF-429242, and fosfatin [59]. Twenty-four h.p.i. a plaque assay demonstrated that the four compounds significantly inhibited ZIKV multiplication. It is known that NDGA disturbs the lipid metabolism, on which the flavivirus is highly dependent; therefore, this represents one of the mechanisms of action of this compound.

The antiviral activity of 6-methylmercaptopurine riboside (6 MMpr) was evaluated through RNA quantification, viral titration, and plaque reduction assay in Vero and SII-SY5Y neuronal cells by Valério and coworkers [58]. Vero and SII-SY5Y cells monolayers were infected with ZIKV PE243 at M.O.I 0.1 for 2 h at 37 °C, washed, and additionated with four concentrations of 6 MMpr. The cell supernatants were collected 120 h.p.i. and submitted to qRT-PCR and virus titration. The results showed that 6 MMpr inhibited viral RNA in a dose-dependent manner, and, at 30.3 μ M, the riboside decreased the viral RNA up to 99%. In addition, 30.3 and 60.5 μ M of 6 MMpr reduced ZIKV titers by 77.24% and 99.45%, respectively. For SII-SY5Y, the cells were submitted to flow cytometry and immunofluorescence 72 h.p.i. The authors concluded that 6 MMpr reduced ZIKV replication by around 70% at 157 μ M; additionally, 6 MMpr reduced ZIKV yields by 99.8%. The authors suggested the use of 6 MMpr during viral RNA synthesis induce mutations and viral replication errors [58].

Sofosbuvir (SOF) is a nucleoside inhibitor of HCV NS5B polymerase used in the treatment of patients who have developed a chronic disease [88,89] Mesci and coworkers investigated the

antiviral activity of SOF in the infection caused by ZIKV in vitro and in vivo [90]. First, both 2D models (human neural progenitor cells (NPC) and Vero cells) and 3D neurosphere models were infected with IBH 30656 or Brazil-ZKV2015 ZIKV strains and treated with SOF at different concentrations. The results showed that SOF at the IC₅₀ of 13.6 μ M and 30.9 μ M in NPC and Vero cells, respectively, was able to inhibit viral replication and consequently reduce cell death induced by ZIKV infection in both models. Then, the antiviral properties of SOF were tested in vivo in mouse models. NOD/SCID mice ($N \geq 6$) were infected with 108 PFU of ZIKV (IBH 30656) and treated with 50 mg/kg SOF daily for 10 days. Then, the mice were sacrificed, and blood samples were collected for analysis. Additionally, 2–3-month-old pregnant female SJL were infected with Pa259459 ZIKV (2×10^5 pfu) and treated for 6 days with SOF at 50 mg/kg. On day 7, the mice were sacrificed to collect fetal samples and the mother's blood. The samples from both assays were processed for serum separation and submitted to plaque assay to determine the viral titer. In addition, viral RNA was extracted from the serum, and qRT-PCR was performed for quantification of the RNA levels. The results of both plaque assay and qRT-PCR demonstrated a reduction in the viral load, supporting the anti-ZIKV activity of SOF [90]. Sacramento and coworkers also tested sofosbuvir in vitro. A yield-reduction assay was performed by infecting Vero cells for 1 h with ZIKV BR at different M.O.I.s and adding different concentrations of sofosbuvir for 24 h. The ZIKV titer in the supernatant was determined by plaque forming. Also, the authors tested if sofosbuvir could inhibit ZIKV RNA polymerase (ZKRP) isolated from BHK-21 infected with ZIKV. For this, a PCR containing ZKRP ribonucleotides, HEPES, MgCl, and 500 ng of ZIKV RNA was performed in the presence or absence of sofosbuvir. The authors concluded that the compound inhibited ZKRP. Also, BHK 21, SH-SY5Y, Huh-7, and Vero cells were infected with ZIKV and treated with different concentrations of sofosbuvir. The compound strongly inhibited ZIKV replication in Huh-7 and SH-5YSY but not in BHK 21 and Vero cells. ZKRP and sofosbuvir interactions were evaluated in silico. The compound and polymerase strongly bound through two amino acid residues which are critical for ribonucleotide incorporation. This was suggested by the authors as one of the mechanisms of action of sofosbuvir [51].

Although the replication of flavivirus occurs in the cytoplasm within the endomembrane system, some papers have shown that NS5 proteins can be found in the nucleus, as has been shown for DENV. NS5 enters the nucleus by a viral replication strategy that occurs through the heterodimeric host transport factor importin (IMP) α and IMP β 1 [91]. Both compounds ivermectin and N-(4-hydroxyphenyl) retinamide (4-HPR) inhibited the viral infection caused by DENV 1–4 by binding to the interaction site of IMP α/β 1 and NS5 protein. Because of the similarity between the NS5 of ZIKV and DENV, it was investigated whether ZIKVNS5 protein could be recognized by IMPs, and if 4-HPR inhibits the transport of NS5 to the nucleus, when it interacts with IMP. In order to measure the interactions between these proteins, an AlphaScreen (amplified luminescent proximity homogeneous assay; PerkinElmer, Waltham, MA, USA) assay was performed. The roboticides was measured using 30 μ M His6-ZikaRdRp and 10 μ M biotinylated IMP α or 10 μ M biotinylated IMP β 1, or ivermectin (positive control) and 4-HPR. The results revealed high affinity of Zika RNA-dependent RNA polymerase (RdRp) for both IMP α and IMP β 1 heterodimers showing that, as observed for DENV NS5 protein, ZIKV NS5 protein is imported into the nucleus by interactions with IMPs. The AlphaScreen assay also showed that 4-HPR is a potent inhibitor of the interaction IMP–ZIKV NS5, with IC₅₀ = 1 μ M. Additionally, the antiviral potential of 4-HPR was evaluated. For this, Vero cells were infected with ZIKV at M.O.I = 0.1 for 2 h and then treated with different concentrations of 4-HPR for 22 h. The supernatant was collected, and the viral RNA was quantified by plaque assay and qRT-PCR. The results showed that 3 μ M of the compound significantly inhibited viral infection, suggesting that the mechanism of inhibition is through the ability of 4-HPR to inhibit the interaction between ZIKV NS5 and IMPs [92]. The chemical nucleus of the compound mefloquine (trifluoromethyl) is present in several compounds with already described therapeutic properties against malaria, cancer, and tuberculosis [41].

Barbosa-Lima et al. synthesized and evaluated the antiviral activity of a number of molecules derived from 2,8-bis (trifluoromethyl) quinoline (N1-(2,8-Bis (trifluoromethyl) quinolin-4-yl) ethane-1,2-diamine 3a, N1-(2,8-Bis (trifluoromethyl) quinolin-4-yl) propane-1,3-diamine 3b, N1-(2,8-bis (trifluoromethyl) quinolin-4-yl) decane-1,10-diamine 3c, N-butyl-2,8-bis (trifluoromethyl) quinolin-4-amine 3d, 2-(2,8-Bis (trifluoromethyl) quinolin-4-yl) amino) ethanol 4, and N-(2-Chloroethyl)-2,8-bis (trifluoromethyl) quinolin-4-amine 5) on ZIKV infection in vitro. For antiviral analysis, Vero cells were infected with ZIKV M.O.I 0.1 for 1 h and then supplied with culture medium containing different concentrations of the compounds. After 24 h of incubation, the viral supernatants were collected, and the viral RNA was extracted and quantified by qRT-PCR. Compounds 3a, 3b, 4, and 5 showed antiviral activity similar to mefloquine. The compounds showed a minimum of 75% reduction of the viral RNA levels, showing to be promising anti-ZIKV molecules [55].

Merimepodib (MMPD) demonstrated to interfere with ZIKV RNA replication by inhibiting IMPDH (inosine-5j-monophosphate dehydrogenase). Tong and coworkers pretreated Huh7 cells with serial dilutions of MMPD, 12 h before infection with ZIKV MR766 at M.O.I of 0.06. Two days post-infection, the RNA levels were measured from the cell culture supernatants by qRT-PCR. The researchers also assessed cytotoxicity using the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) (MTS) protocol to determine half maximal effective concentration (EC₅₀) and 50% cytotoxic concentration (CC₅₀). MMPD inhibited ZIKV RNA replication, with EC₅₀ = 0.6 ± 0.2 µM, CC₅₀ > 10 µM, and SI > 17, showing to be a potent antiviral against ZIKV infection [56].

Concluding Remarks

Since the ZIKV infection is currently a serious public health problem, many research centers have directed efforts in the search for a drug with potent anti-ZIKV activity. In this review, we have discussed some approaches that have been performed to this purpose, being most of the considered drugs either pre-approved by FDA or mainly based on molecules previously documented to possess antiviral effects against other diseases, such as Ebola, Giardíase, Malaria, Hepatitis C, Herpes, Hepatitis B, and syndrome of the human immunodeficiency. Some molecules, such as niclosamide, emricasam, ivermectin, MPA, and daptomycin, have shown to be effective on ZIKV infection in vitro by inhibiting viral entry into the cells and/or replication, or by decreasing cell death induced by infection. Natural compounds, synthetic compounds, and nucleoside analogs have been the subject of research in the search for new molecules that inhibit the infection caused by ZIKV, and some studies have demonstrated the therapeutic potential of these different approaches. Other tools, like the use of molecular docking, have optimized the discovery of new molecules that may turn out to be potential antivirals. Efforts to uncover antiviral drugs that treat ZIKV infection continue since the ZIKV outbreak of the 2016. Most of the tests described above were performed in vitro, and clinical trials I and II are required to prove the efficacy of these compounds in humans. Currently, few compounds are in phase I trials, such as Sofosbuvir, 7-DMA, and NITD008, and there is still no treatment against ZIKV infection. Hence, further research is clearly needed for the development of effective and safe antivirals that could be used for the control of ZIKV infection with no significant side effects.

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