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Maria Letícia Duarte Lima

Avaliação da atividade antiviral de peptídeos sintéticos contra o Zika vírus

São José do Rio Preto

2023

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Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Microbiologia, junto ao Programa de Pós-Graduação em Microbiologia, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus de São José do Rio Preto.

Financiadora: CAPES

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Dra. Mariana Nogueira Batista

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Financiadora: CAPES

Comissão Examinadora

Profa. Dra. Paula Rahal
UNESP – Câmpus de São José do Rio Preto
Orientadora

Prof. Dr. Bruno Moreira Carneiro
Universidade Federal de Rondonópolis

Profa. Dra. Rejane Maria Tommasini Grotto
UNESP – Câmpus de Botucatu

Profa. Dra. Paola Jocelan Scarin Provazzi Trabulsi
Instituto Municipal de Ensino Superior de Catanduva - IMES Catanduva

Prof. Dr. Guilherme Rodrigues Fernandes Campos
Faculdade de Medicina de São José do Rio Preto - FAMERP

São José do Rio Preto

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Dedico este trabalho aos meus pais, Maria Regina Duarte Lima e Celso Raimundo Lopes Lima, por serem meus maiores incentivadores, meus exemplos e que, com todos os seus esforços e ensinamentos, me permitiram chegar até aqui.

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“Por vezes sentimos que aquilo que fazemos não é senão uma gota de água no mar. Mas o mar seria menor se lhe faltasse uma gota”.

Madre Teresa de Calcutá

RESUMO

O Zika vírus (ZIKV) é um arbovírus pertencente à família *Flaviviridae*, genêro *Flavivirus*. Teve seu primeiro caso de transmissão autóctone relatado no Brasil em 2015 e, nesse mesmo período, espalhou-se para outros países e casou um grande surto nas Américas. A infecção por ZIKV é, em sua maioria, assintomática ou possui sintomas leves, porém esse vírus é capaz de causar complicações que envolvem alterações neurológicas, causando um impacto significativo na saúde pública. Uma delas é a Síndrome Congênita do Zika em bebês de mulheres infectadas durante à gestação. Além disso, esse vírus pode causar a Síndrome de Guillain-Barré em adultos. Atualmente, não há vacinas ou medicamentos específicos para o ZIKV, fazendo-se necessário a busca pelos mesmos. Na procura por novas terapêuticas, peptídeos tem se mostrado moléculas com grande potencial para essa finalidade, inclusive na busca por antivirais, já que tem sido observado que fazem inibição seletiva e apresentado relativas tolerabilidade e segurança. Nesse sentido, o objetivo deste trabalho foi avaliar o potencial antiviral dos peptídeos sintéticos AG-peptídeo (GA-KKALKKLKKALKKAL-$CONH_2$) e $(pBthTX\text{-}I)_2K$ [$(KKYRYHLKPFCKK)_2$] em células Vero infectadas pelo ZIKV. Para isso, primeiramente foi avaliada a citotoxicidade dos peptídeos nessas células por meio do ensaio de MTT, tendo sido determinada a máxima concentração não-tóxica de 25µM para ambos os peptídeos, e foi esta a concentração utilizada para os ensaios antivirais. Foi então realizado um ensaio de triagem a fim de avaliar se os peptídeos possuíam efeito antiviral contra o ZIKV. O AG-peptídeo demonstrou inibição de 88,57% dos níveis de RNA viral e para o $(pBthTX\text{-}I)_2K$ foi de 67,28%. Confirmada a atividade antiviral, foram realizados ensaios para compreender em que etapas do ciclo replicativo os peptídeos estão agindo. Ambos os peptídeos mostraram proteger as células Vero contra a infecção por ZIKV. O AG-peptídeo apresentou efeito virucida e também atuou inibindo a entrada de ZIKV nas células, especificamente as etapas de adsorção e internalização. Finalmente, tanto o AG-peptídeo quanto o $(pBthTX\text{-}I)_2K$ foram capazes de inibir o ZIKV em etapas pós-entrada nas células Vero. Em conclusão, ambos os peptídeos se mostraram potenciais antivirais contra o ZIKV *in vitro*, sendo que o AG-peptídeo teve ação em todas as etapas do ciclo replicativo viral analisadas no presente trabalho, enquanto o $(pBthTX\text{-}I)_2K$ foi capaz de proteger as células Vero da infecção por ZIKV e atuar nas etapas após a entrada viral nas células. Apesar de mais ensaios são necessários para determinar o mecanismo de ação desses peptídeos, o presente trabalho demonstrou moléculas com grande potencial para o desenvolvimento de antivirais contra o ZIKV.

Palavras-chave: Zika vírus. ZIKV. Antiviral. Peptídeo.

ABSTRACT

Zika virus (ZIKV) is an arbovirus with a positive-sense single-stranded RNA genome which belongs to the *Flaviviridae* family, genus *Flavivirus*. It had its first case of autochthonous transmission reported in Brazil in 2015, and in the same period, it spread to other countries and caused a large outbreak in the Americas. ZIKV infection is majority asymptomatic or has mild symptoms, but this virus is capable of causing complications involving neurological alterations, causing a significant impact on public health. Congenital Zika Syndrome is one of them and can occur in babies of women infected during pregnancy. Furthermore, this virus can cause Guillain-Barré Syndrome in adults. Currently, there are no vaccines or specific drugs for ZIKV, making it necessary to search for them. In the seeking for new therapies, peptides have shown to be molecules with great potential for this purpose, including the search for antivirals, since it has been observed that they perform selective inhibition and present relative tolerability and safety. In this context, this work aimed to evaluate the antiviral potential of the synthetic peptides GA-peptide (GA-KKALKKLKKALKKAL-$CONH_2$) and $(pBthTX\text{-}I)_2K$ [$(KKYRYHLKPFCKK)_2$] in Vero cells infected with ZIKV. For this, the cytotoxicity of the peptides in these cells was first evaluated using the MTT assay, and the maximum non-toxic concentration was 25µM for both peptides, which was the concentration used for antiviral assays. Then, a screening assay was performed to assess whether the peptides had an antiviral effect against ZIKV. GA-peptide demonstrated inhibition of 88.57% of viral RNA levels, and for $(pBthTX\text{-}I)_2K$ it was 67.28%. Once confirmed the antiviral activity some experiments were carried out to understand which stages of the replicative cycle the peptides are acting. Both peptides were capable to protect Vero cells against ZIKV infection. GA-peptide exhibited a virucidal effect and inhibited the entry of ZIKV into cells, specifically in attachment and internalization steps. Finally, both GA-peptide and $(pBthTX\text{-}I)_2K$ were able to inhibit ZIKV in post-entry steps in Vero cells. Concluding, both peptides exhibited antiviral potential against ZIKV in vitro. The GA-peptide had action in all stages of the viral replicative cycle analyzed in the present work, while $(pBthTX\text{-}I)_2K$ was able to protect Vero cells from ZIKV infection and act in the steps after viral entry into cells. Even though more assays are needed to determine the mechanism of action of these peptides, this work brought molecules with great potential to the development of antivirals against ZIKV.

Keywords: Zika virus. ZIKV. Antiviral. Peptide.

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LISTA DE ABREVIATURAS E SIGLAS

$(pBthTX\text{-}I)_2K$ - peptídeo $(KKYRYHLKPF)_2K$

+ssRNA - *positive-sense single-strand RNA*

7DMA - o 7-deaza-2'- *C* -metil-D-adenosina

AG-Hecate - peptídeo Hecate conjugado ao ácido gálico

AG-peptídeo - peptídeo (GA-KKALKKLKKALKKAL-$CONH_2$)

AmphB - *amphotericin B*

AMPs - *antimicrobial peptides*

BHK-21 - *Baby hamster kidney fibroblast cells*

BthTX-I - *Bothropstoxin-I*

C - proteína do capsídeo

CC_{50} - concentração citotóxica em 50% das células

cDNA - DNA complementar

CHIKV - vírus Chikungunya

CMC - *carboxymethylcellulose*

CMV - *human cytomegalovirus*

CPPs - *cell-penetrating peptides*

C_t - *cycle threshold*

CZS - *Congenital Zika Syndrome*

DENV - vírus da dengue

DMEM - *Dulbecco's Modified Eagle Medium*

DMSO - dimetilsulfóxido

E - proteína do envelope

EC_{50} - concentração efetiva em 50% de vírus

EEEV - vírus da encefalite equina oriental

EGCG - epigalocatequina-galato

FBS - *fetal bovine sérum*

GAGs - glicosaminoglicanos

GA-peptide - (GA-KKALKKLKKALKKAL-$CONH_2$) *peptide*

GB - síndrome de Guillain-Barré

H.p.i. - *hours post-infection*

HCV - vírus da hepatite C

HIV - vírus da imunodeficiência humana

HSV-1 - vírus herpes simplex tipo I

IC – intracelular

JEV – vírus da encefalite japonese

L15 - meio Leibovitz

M - proteína de membrana do vírus

MAYV - vírus Mayaro

MNTC - *maximum non-toxic concentration*

MOI - *multiplicity of infection*

MTase - domínio da metiltransferase

MTT - brometo de 3- (4,5-dimetiltiazol-2-il) -2,5-difenil tetrazólio

NLuc - NanoLuciferase

OMS - Organização Mundial da Saúde

ORF - *open reading frame*

OROV - vírus Oropouche

P/S - penicilina e estreptomicina

PAHO - Organização Pan-Americana da Saúde

PBS - *phosphate buffered saline*

PFU - *plaque forming units*

PHB1 - *type 1 prohibition*

prM - proteína pré-membrana

qRT-PCR- *real-time quantitative reverse transcription* PCR

RE - retículo endoplasmático

ROCV - vírus rocio

RpRd - RNA polimerase dependente de RNA

RT - *room temperature*

RVFV - *Rift Valley Fever Virus*

SARS-CoV-2 - coronavírus da síndrome respiratória aguda grave 2

SCZ - Síndrome Congênita do Zika

SD - *standard deviation*

SI - *selectivity index*

SLEV - vírus da encefalite de Saint Louis

SN - sobrenadante

SPPS - *solid-phase synthesis*

TIM-1 - *T-cell immunoglobulin and mucin domain protein 1*

VC - *vehicle control*

VEEV - vírus da encefalite equina venezuelana

WNV - vírus da febre do Oeste do Nilo

YFV - vírus da febre amarela

ZIKV - Zika vírus

ZIKV^{BR} - cepa brasileira de Zika vírus

SUMÁRIO

CAPÍTULO I –

Considerações gerais

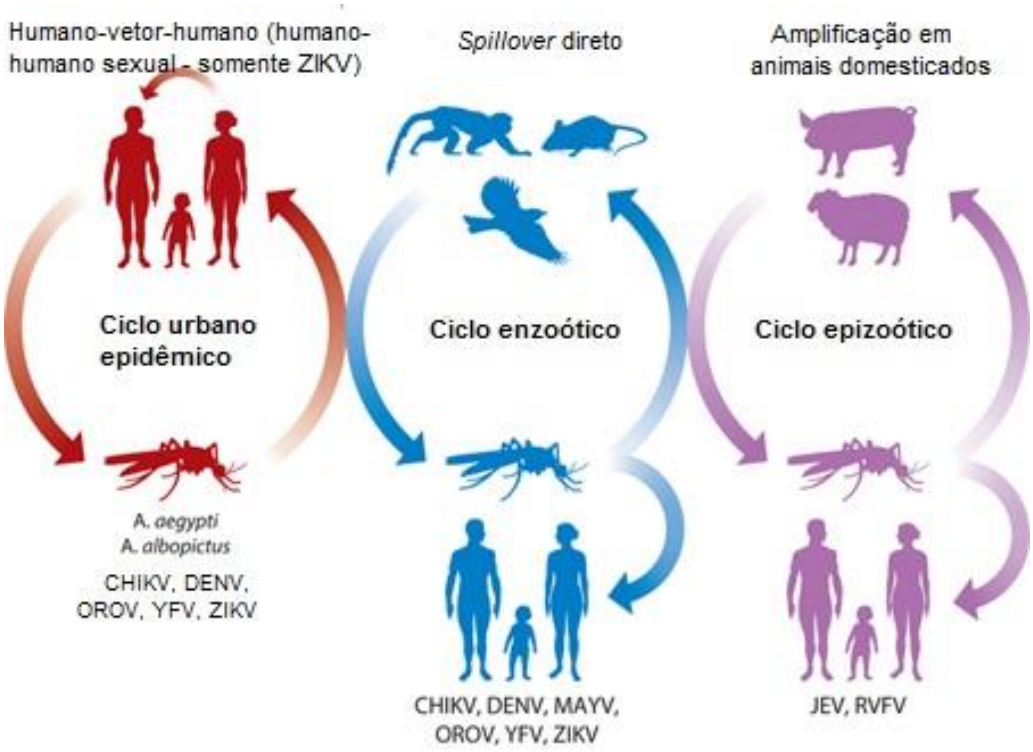
1. INTRODUÇÃO

1.1. Arbovírus

Os arbovírus são vírus cuja a transmissão ocorre por vetores artrópodes hematófagos, como mosquitos e carrapatos, para hospedeiros vertebrados (WEAVER; REISEN, 2010). Estes vírus se mantêm em um ciclo de transmissão em que eles se replicam em vetores hematófagos, que adquiriram o vírus ao se alimentar do sangue de um hospedeiro vertebrado infectado, e são capazes de transmitir a um novo hospedeiro pela saliva, durante o repasto sanguíneo (WEAVER; BARRETT, 2004; MUELLER; CAO-LORMEAU, 2018).

Inicialmente, esses vírus são mantidos em ciclo de transmissão silvestre enzoótico, tendo como reservatórios hospedeiros vertebrados como primatas não-humanos, aves e roedores (MUELLER; CAO-LORMEAU, 2018). Porém, pode ocorrer um transbordamento desse ciclo silvestre para os seres humanos, podendo causar doenças (WEAVER; BARRETT, 2004; WEAVER et al., 2018). Isso pode ocorrer por três mecanismos: o primeiro deles é através de transbordamento direto de um vetor enzoótico ou ponte que transmite o vírus de um hospedeiro silvestre para o homem. Já o segundo ocorre quando o arbovírus é amplificado em animais domésticos e depois ocorre o *spillover* para humanos. O terceiro ocorre quando há uma transição do ciclo enzoótico para um ciclo urbano, onde humanos infectados servem como hospedeiros desses vírus e mosquitos antropofílicos fazem a transmissão para outras pessoas (Figura 1) (WEAVER et al., 2018).

Figura 1 – Tipos de ciclo de arbovírus em que pode ocorrer transbordamento para humanos.

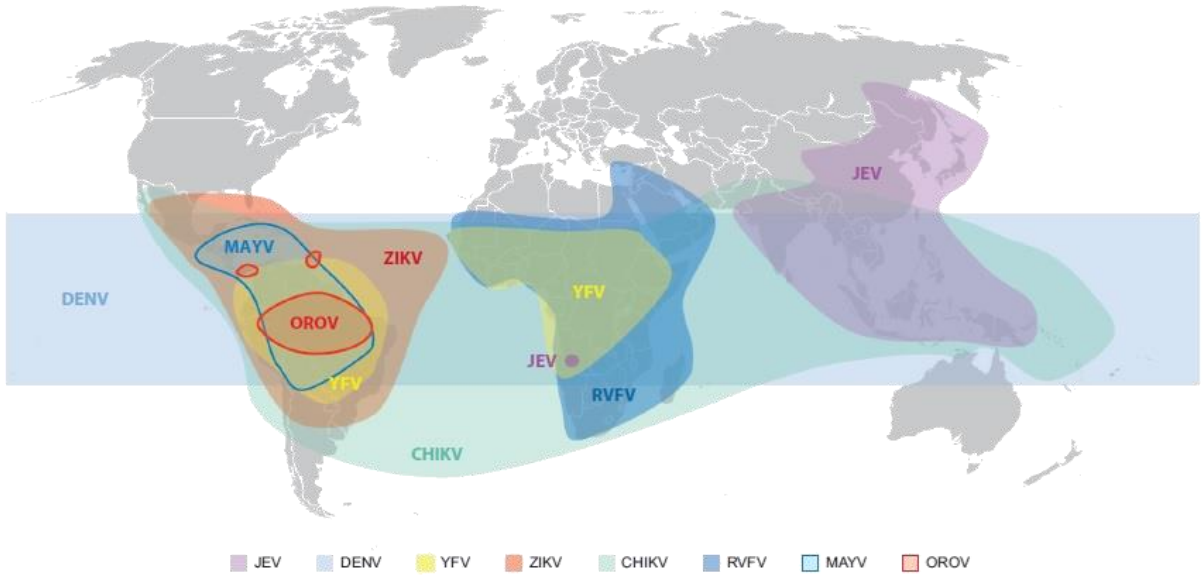


Fonte: Adaptado de WEAVER et al., 2018.

Os arbovírus possuem distribuição mundial, sendo encontrados principalmente em regiões tropicais (Figura 2). Parâmetros ecológicos restringem a distribuição geográfica de cada um desses vírus, sendo que tais parâmetros regem seu ciclo de transmissão (GUBLER, DUANE J., 2001). A vegetação, temperatura e precipitação são fatores que têm influência sobre a distribuição do vetor, bem como do hospedeiro reservatório, sendo este necessário para a manutenção viral (GUBLER, DUANE J., 2001).

Esses vírus são um grupo com diversidade taxonômica, sendo que a maioria desses arbovírus pertencem às famílias *Peribunyaviridae*, *Flaviviridade* e *Togaviridae*, mas também há representantes em menor número em outras famílias, como *Sedoreoviridae* e *Orthomyxoviridae* (KARABATSOS, 1985; GUBLER, DUANE J., 2001). Há mais de 540 espécies suspeitas de serem arbovírus e dessas, para mais de 150 há registros de infecções em humanos (KARABATSOS, 1985; GUBLER, DUANE J., 2001; CLETON et al., 2012).

Figura 2 – Distribuição de alguns arbovírus emergentes ao redor do mundo. JEV: vírus da encefalite japonesa; DENV: vírus da dengue; YFV: vírus da febre amarela; ZIKV: Zika vírus; CHIKV: vírus chikungunya; RVFV: vírus da febre do Vale do Rift; MAYV: vírus Mayaro; OROV: vírus Oropouche.



Fonte: (WEAVER et al., 2018).

1.2.Arbovírus no Brasil

O Brasil é um país de grande extensão territorial, predominantemente tropical, cujo clima, biodiversidade de fauna e vegetação, além número de habitantes e distribuição populacional, sendo que a maior parte vive em ambientes urbanos, fazem com que este país tenha grande potencial para ocorrência de arboviroses (FIGUEIREDO, 2000; FIGUEIREDO, 2007). O fato de grande parte da população brasileira viver em ambientes urbanos favorece a ocorrência de vetores de arbovírus adaptados a essas regiões, como as espécies de mosquitos *Culex* e *Aedes* (FIGUEIREDO, 2007).

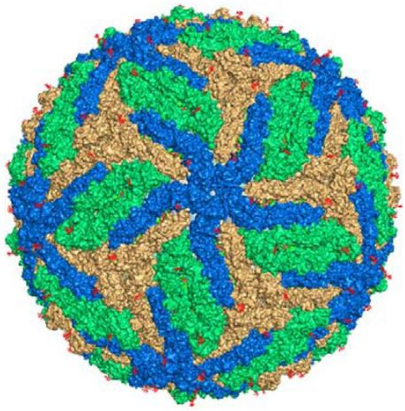
Há diversas arboviroses emergentes ou reermegentes responsáveis por causar infecções em humanas que circulam no país, sendo as principais distribuídas entre as famílias: *Togaviridae*, *Peribunyaviridae* e *Flaviviridae*. O vírus Mayaro (MAYV), Chikungunya (CHIKV), encefalite equina oriental (EEEV) e Encefalite equina venezuelana (VEEV) são vírus do gênero *Alphavirus* e representantes da família *Togaviridae*. Na família *Peribunyaviridae* está o vírus Oropouche (OROV), do gênero *Orthobunyavirus*. Com relação à família

Flaviviridae, os representantes pertencem ao gênero *Flavivirus* e são os vírus da dengue (DENV), da febre do Oeste do Nilo (WNV), da febre Amarela (YFV), encefalite de Saint Louis (SLEV), vírus Rocio (ROCV) e, o mais recente, o Zika vírus (ZIKV) (FIGUEIREDO, 2007; 2015). O primeiro caso de transmissão autóctone de ZIKV foi relatado no Brasil em 2015 (ZANLUCA et al., 2015).

1.3. O Zika vírus

O ZIKV (Figura 3) é um flavivírus que foi isolado pela primeira vez em 1947 em macacos rhesus sentinela na floresta Zika, na Uganda (DICK;KITCHEN; HADDOW, 1952). É um vírus envelopado, com genoma de RNA fita simples polaridade positiva com 10,8 kb, aproximadamente (CHAMBERS et al., 1990; KUNO; CHANG, 2007). O genoma possui um único sítio aberto de leitura (do inglês, *open reading frame* - ORF) com regiões não traduzíveis nas duas extremidades. Esta ORF codifica uma poliproteína que é clivada em três proteínas estruturais: C (capsídeo), prM (pré-membrana) e E (envelope); e sete não estruturais: NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5 (CHAMBERS et al., 1990; KNIPE; HOWLEY, 2013). Quanto à expressão proteica, o genoma se organiza da seguinte maneira: 5'-C-prM-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3' (KNIPE; HOWLEY, 2013; WHITE, MARTYN K. et al., 2016) (Figura 4a e 4b).

Figura 3 – Representação da partícula madura de Zika vírus.

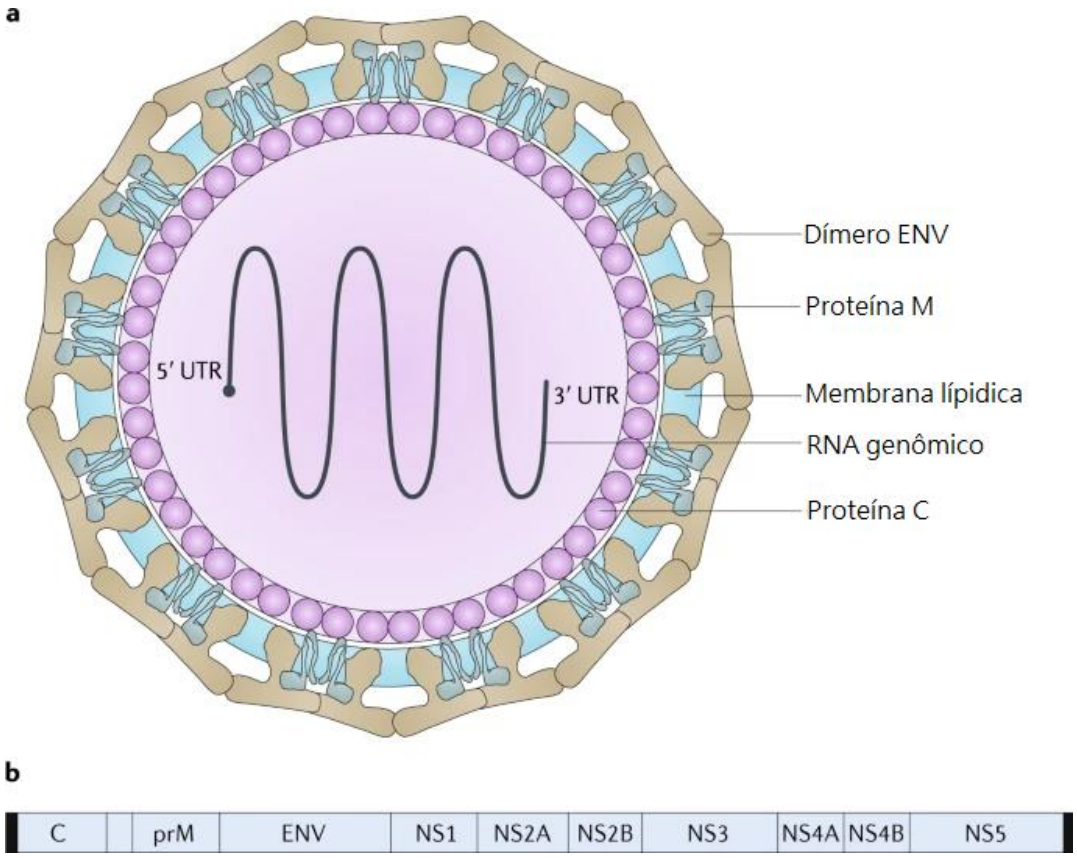


Fonte: Modificado de (SAIZ et al., 2017).

Com relação às proteínas estruturais, a proteína do capsídeo (C) é uma proteína com grande basicidade e se liga ao genoma viral para formar o nucleocapsídeo, já a membrana

externa do virion trata-se de uma bicamada lipídica composta pelas proteínas de membrana do vírus (M) e do envelope (E). A proteína M expressa-se como uma glicoproteína precursora de M (prM), sendo sintetizada durante a maturação dos virions e a E é a principal proteína de superfície dos virions (KNIPE; HOWLEY, 2013; WHITE, MARTYN K. et al., 2016).

Figura 4 – Proteínas de ZIKV e organização do genoma. (a) Representação esquemática do vírus Zika, com a organização de suas proteínas estruturais e seu genoma de RNA de fita simples polaridade positiva. (b) Organização das proteínas estruturais e não-estruturais no genoma de ZIKV.



Fonte: Adaptado de (ABBINK;STEPHENSON; BAROUCH, 2018).

As proteínas não-estruturais estão envolvidas no processo de replicação do genoma viral no citoplasma da célula hospedeira, bem como no empacotamento do genoma e também atua na modulação imune do hospedeiro (SIROHI; KUHN, 2017). A proteína NS1 faz parte do processo replicativo, além de possuir atividades imunomoduladoras (SAIZ et al., 2017). A proteína NS2A está envolvida no rearranjo da membrana do retículo endoplasmático (RE)

durante a replicação, parece ter relação com a evasão do sistema imunológico do hospedeiro, além de estar envolvida na montagem viral e síntese de RNA (FAJARDO-SÁNCHEZ;GALIANO; VILLALAÍN, 2017). A NS2B faz com que ocorra a ativação da NS3, levando a formação do complexo NS2B-NS3, exercendo atividade de protease, clivando a poliproteína viral nas proteínas que atuam na replicação. Além disso, a NS3 atua como helicase, sendo necessário para o que o intermediário de RNA de fita dupla, formada no processo de síntese do genoma, se desenrole. Além disso, ela também tem função de RNA triofosfatase e nucleosídeo. Essa proteína se envolve na formação de novas partículas, além da replicação (BYLER;OGUNGBE; SETZER, 2016; WHITE, MARTYN K. et al., 2016).

As proteínas não-estruturais NS4A e NS4B fazem parte do complexo replicativo associado ao RE. A NS5 possui o domínio da metiltransferase (MTase), cuja a função é metilar a estrutura cap do RNA do vírus e também possui o domínio RNA polimerase dependente de RNA (RpRd), nas quais dão início e catalisam a replicação (BYLER;OGUNGBE; SETZER, 2016; WANG, LILI et al., 2019).

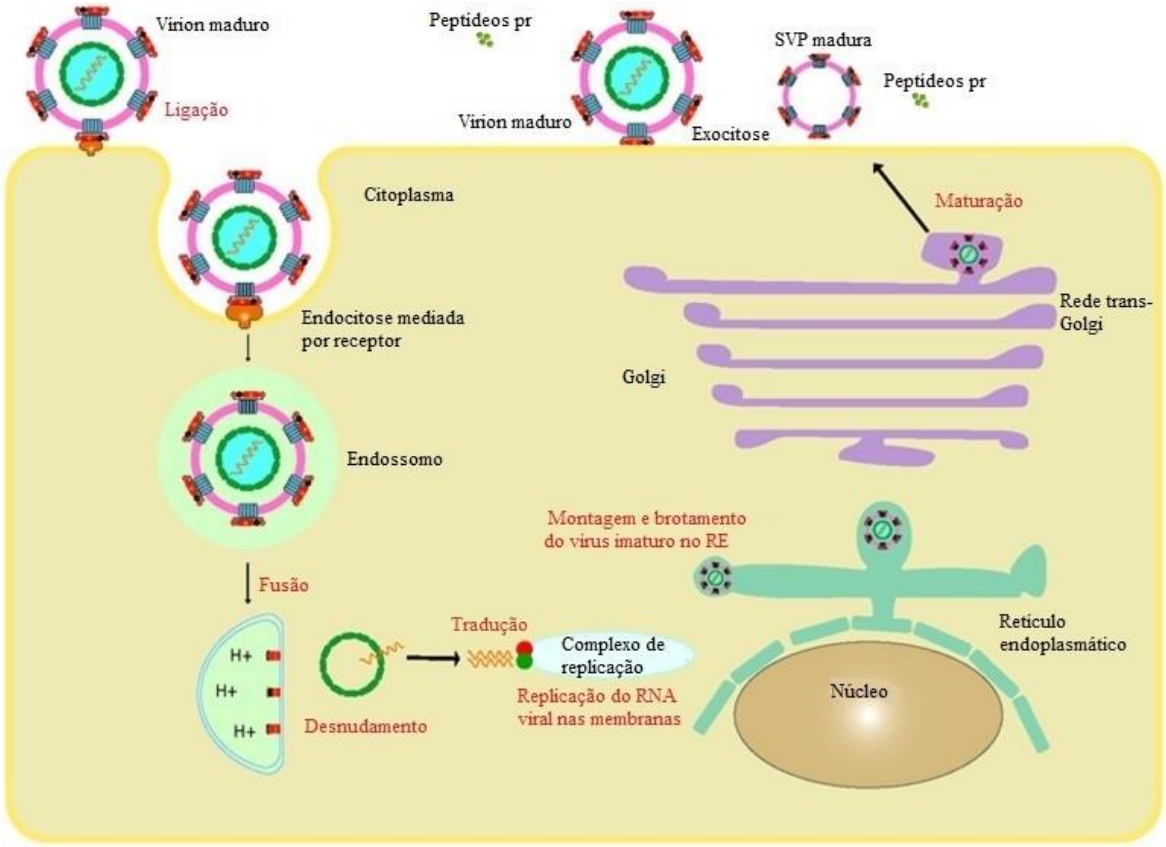
O ZIKV possui tropismo para vários tipos de tecidos e células. Hepatócitos, fibroblastos, células renais, trofoblastos, células endoteliais da placenta, células de Houfbauer, células do sistema genital masculino e feminino, células neuronais, astrócitos, vários tipos de células epiteliais, células dendríticas, entre outras, são alguns tipos celulares permissivos à infecção por ZIKV (NGONO; SHRESTA, 2018; SHAILY, S.; UPADHYA, A., 2019).

Para se replicar, o ZIKV se liga, por meio de sua glicoproteína E, à receptores específicos na membrana celular, tais como o AXL, DC-SIGN, TIM-1 e Tyro (SMIT et al., 2011; HAMEL, RODOLPHE et al., 2015; MUSSO; GUBLER, 2016). A partir disso, os vírions entram na célula por endocitose mediada por receptores, acessando o citoplasma celular por invaginações que formam vesículas revestidas por clatrina. Após isso, essas vesículas carregando os vírus são entregues a endossomos iniciais, que depois amadurecem para endossomos tardios (GOLLINS; PORTERFIELD, 1985; VAN DER SCHAAR et al., 2008; SMIT et al., 2011; KNIPE; HOWLEY, 2013). Com acidificação do endossomo, ocorrem mudanças conformacionais na proteína viral E, levando à fusão desta com a membrana endossomal, ao desnudamento da partícula e liberação do RNA do vírus no citoplasma da célula (SMIT et al., 2011; KNIPE; HOWLEY, 2013).

No citoplasma, tem-se início à tradução das proteínas virais a partir do único ORF presente no RNA de fita simples polaridade positiva, que atua como RNA mensageiro,

ocorrendo a formação de uma poliproteína. Esta é clivada co- e pós-traducionalmente por proteases virais e celulares, gerando, então, as três proteínas estruturais (C, prM e E) e a sete não-estruturais (NS1, NS2A, NS2B, NS3, NS4A, NS4B e NS5), levando à replicação do genoma viral (KNIPE; HOWLEY, 2013; WHITE, MARTYN K. et al., 2016). A fita negativa é sintetizada, direcionando para a síntese de novas fitas de RNA positivas e a replicação ocorre associada a uma rede membranosa induzida pelo vírus, provenientes do retículo endoplasmático. Algumas novas fitas de RNA positivas precisam ser empacotadas na progênie de vírions que brotam do RE, formando vírions envelopados imaturos, enquanto outras serão direcionadas ao início do ciclo, para a tradução de mais proteínas virais. Os vírions empacotados no RE seguem para a via secretora da célula, e, na rede trans-Golgi, ocorrerá a clivagem da proteína prM em M para a maturação dos vírions, com posterior liberação dos novos vírions da célula infectada, sendo estes liberados por exocitose (KNIPE; HOWLEY, 2013; ROBY et al., 2015; SAIZ et al., 2016; WHITE, MARTYN K. et al., 2016). O ciclo replicativo de Zika vírus está representado na Figura 5.

Figura 5 – Ciclo replicativo de Zika vírus.



Fonte: Adaptado de (SHANKAR;PATIL; SKARIYACHAN, 2017).

1.4. Histórico e epidemiologia

Após o isolamento em macacos rhesus em 1947 (DICK;KITCHEN; HADDOW, 1952), poucos casos de infecção em humanos foram relatados até 2007, quando, naquele ano, ocorreu uma epidemia nas Ilha Yap, Estados Federados da Micronésia. No ocorrido, aproximadamente três quartos da população local apresentaram infecção por ZIKV, que casou uma doença amena com duração de vários dias, mas que não levou a mortes ou hospitalizações das pessoas infectadas (DUFFY et al., 2009).

Em 2013-2014 ocorreu outro surto na Polinésia Francesa, espalhando-se para outras ilhas do Pacífico, tais como Nova Caledônia, Ilhas Cook, Vanatu, Ilhas de Salomão e Ilha de Páscoa (MUSSO;NILLES; CAO-LORMEAU, 2014; MUSSO, 2015). Em 2015 foi relatado o primeiro caso de transmissão autóctone de ZIKV no Brasil (ZANLUCA et al., 2015) e se espalhou para outros países da América do Sul e América do Norte e Central (HENNESSEY;FISCHER; STAPLES, 2016; SIKKA et al., 2016).

Análises retroativas de amostras coletadas em 2013, no Rio de Janeiro, em pacientes febris e negativos para a dengue, demonstraram que três delas foram positivas para o ZIKV (PASSOS et al., 2017). Além disso, um programa de vigilância epidemiológica de mosquitos também no Rio de Janeiro, realizado de 2014 a 2016, realizou análises filogenéticas e filogeográficas com os *pools* positivos para ZIKV e sugeriram que este vírus pode ter sido introduzido entre maio a novembro de 2013 neste estado brasileiro (AYLLÓN et al., 2017).

Outros estudos filogenéticos demostraram que a variante desse vírus que circula no Brasil pertence à linhagem asiática e assemelha-se em 99% com a sequência nucleotídica de uma amostra isolada de um paciente da Polinésia Francesa (CAMPOS;BANDEIRA; SARDI, 2015).

Segundo a Organização Pan-Americana da Saúde (PAHO)/Organização Mundial da Saúde (OMS), entre as semanas epidemiológicas 1 a 50 de 2022, foram registrados mais de 36 mil casos de ZIKV na região das Américas, sendo que quase 91% foram registrados somente no Brasil (ORGANIZATION, P. A. H., 2023b). Ainda segundo a OMS, até o momento foram relatadas evidências de transmissão vetorial em 89 países e territórios distribuídos pelas Américas, África, Sudeste Asiático e Pacífico Ocidental (ORGANIZATION, 2019; ORGANIZATION, W. H., 2023). Além disso, o ZIKV continua na lista de doenças prioritárias para pesquisa e desenvolvimento em contextos de emergência da OMS (ORGANIZATION, 2018).

1.5. Transmissão

A transmissão de ZIKV ocorre principalmente por vetores, sendo estas mosquitos fêmeas de espécies do gênero *Aedes*, principalmente as espécies *Aedes aegypt*i e *Aedes albopictus* (GRARD et al., 2014; VASCONCELOS; CALISHER, 2016). Esse vírus também pode ser transmitido por via vertical (materno-fetal) (BESNARD et al., 2014) e via transfusão sanguínea e de hemocomponentes (MUSSO et al., 2014).

A transmissão sexual de ZIKV também pode ocorrer e tem sido relatada por diversas vezes na literatura. Os relatos mostram casais que geralmente são de países onde não há a circulação do vetor, porém um deles viajou para um país onde há a transmissão autóctone, e o outro não havia viajado, mas ambos apresentaram sintomas e tiveram a infecção por ZIKV confirmada (MUSSO et al., 2015; ARSUAGA et al., 2016; D'ORTENZIO et al., 2016; DECKARD et al., 2016; TURMEL et al., 2016).

A transmissão sexual aumenta a problemática do ZIKV, pois pode expandir a transmissão deste vírus a países que não possuem o vetor (STASSEN et al., 2018). Além disso, estudos mostraram a ocorrência de alta persistência deste vírus no sêmen, sendo que o RNA viral foi encontrado no sêmen de um homem infectado por ZIKV por até 62 dias após o desaparecimento dos sintomas, enquanto que outro em até 370 dias, porém que a carga viral foi reduzida após 3 meses neste caso (NICASTRI et al., 2016; BARZON et al., 2018). Isso mostra que o trato genital masculino pode ser uma fonte de tal vírus por um longo período de tempo, o que aumenta a preocupação com relação à transmissão sexual (STASSEN et al., 2018).

1.6. Sintomas e complicações

A infecção por esse vírus geralmente é assintomática, e quando há sintomas, em uma pequena parcela de infectados, eles são inespecíficos, como febre e erupções cutâneas (YE et al., 2016). Além disso, a infecção pode causar cefaleia, conjuntivite não purulenta, mialgia, artralgia e astenia. Esse conjunto de sintomas é semelhante ao de outras arboviroses, como as de doenças causadas pelos DENV e CHIKV, podendo dificultar o diagnóstico (ARAUJO;FERREIRA; NASCIMENTO, 2016).

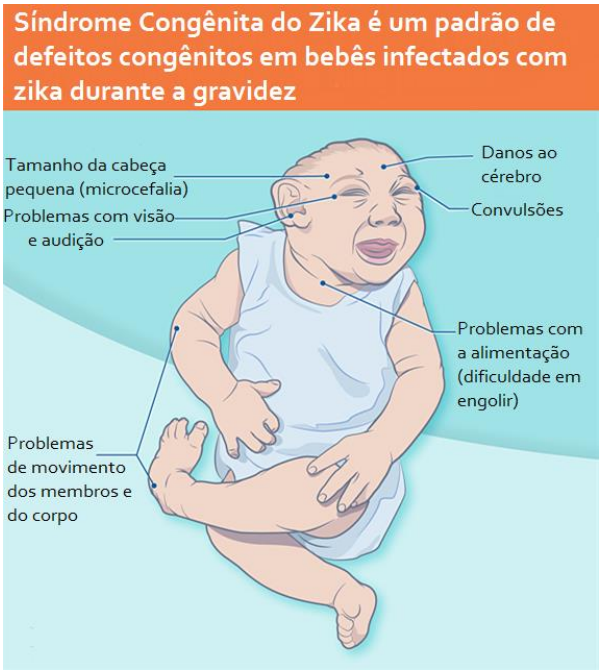
A infecção por ZIKV também tem sido relacionado a complicações neurológicas tanto em adultos quanto em fetos de gestantes infectadas por esse vírus durante a gravidez. Durante o surto que ocorreu na Polinésia Francesa em 2013/2014, observou-se um aumento de alterações neurológicas, citado anteriormente, como síndrome de Guillain-Barré (CONTROL,

2014). O mesmo foi observado no Brasil durante o surto deste vírus, onde houve claro aumento da ocorrência dessa síndrome (ARAUJO;FERREIRA; NASCIMENTO, 2016).

Ademais, no período de 2014 a 2015 ocorreu um aumento em cerca de vinte vezes nos casos de microcefalia no Brasil, período este que coincide com o surto de ZIKV no país. Este fato levou profissionais da saúde a acreditarem na possível correlação da infecção das mulheres grávidas com ZIKV ao desenvolvimento da microcefalia em seus bebês (FAUCI; MORENS, 2016). Alguns estudos obtiveram resultados que sustentam tal hipótese, como o de Calvet e colaboradores (2016) que fizeram o teste para ZIKV em duas gestantes cujo os bebês foram diagnosticados com microcefalia e, em ambas, o RNA deste vírus foi detectado (CALVET et al., 2016). Outro estudo verificou a presença de ZIKV no cérebro de um feto abortado que havia sido diagnosticado com microcefalia (MLAKAR et al., 2016).

Além da microcefalia, foram observados outros defeitos congênitos e esse conjunto da más-formações foi chamado de Síndrome Congênita do Zika (SCZ). Tal síndrome é descrita por cinco características: 1) grave microcefalia, na qual ocorreu parcial colapso do crânio; 2) redução do tecido cerebral com padrão específico de dano, o que inclui calcificações subcorticais; 3) cicatrizes maculares e manchas na retina pigmentar focal; 4) contraturas congênitas, tais como artrogripose (limitação dos movimentos de articulação) e pés tortos; 5) restrição do movimento do corpo logo após o nascimento, devido a hipertonia. Além disso, outras anormalidades foram relacionadas à SCZ, como tremores, convulsões, glaucoma, catarata, entre outras. Esta síndrome ocorre somente em fetos e bebês que foram infectados pelo Zika antes do nascimento (MOORE et al., 2017; PREVENTION, 2018) (Figura 6).

Figura 6 – Más-formações congênitas que podem ocorrer em bebês com a Síndrome Congênita do Zika.



Fonte: Adaptado de (PREVENTION, 2017).

Ademais, outras questões que merecem atenção com relação à infecção por ZIKV referem-se à transmissão sexual e ao fato de que o sistema genital masculino pode ser sítio de replicação e persistência deste vírus, podendo interferir na fertilidade masculina (STASSEN et al., 2018; KURSCHEIDT et al., 2019).

Para investigar como o ZIKV pode atuar nos órgãos do sistema genital masculino e os potenciais danos que ele pode causar, modelos animais têm sido utilizados. Um estudo utilizando camundongos machos verificou que os animais apresentaram inflamação nos testículos e epidídimos, podendo levar a infertilidade (MA et al., 2016). Outro trabalho utilizou primatas não-humanas para avaliar a infecção em testículos, epidídimos, próstata e vesícula seminal, e observaram que o RNA de ZIKV persistiu por até 60 dias (testículo), variando de acordo com o órgão analisado. Já o vírus infeccioso foi isolado de amostras de todos esses órgãos, tendo uma menor taxa de cultivo de ZIKV isolados de próstatas. Além disso, os macacos que estavam na maturidade sexual demonstraram inflamação no epidídimo grave e/ou na próstata (BALL et al., 2022). Estudos com esses utilizando modelos animais, em especial primatas não-humanos, podem ajudar a entender melhor o impacto do ZIKV sobre a fertilidade masculina e na transmissão sexual. Inclusive, foi relatado o nascimento de um bebê com

síndrome congênita do Zika como resultado de uma infecção via sexual da mãe durante a gestação (YARRINGTON et al., 2019).

Além desses danos neurológicos e no sistema genital masculino, o ZIKV se mostrou capaz de infectar também os olhos, podendo causar alterações oftalmológicas em bebês com SCZ, tais como anormalidades no nervo nervoso óptico, atrofia macular, glaucoma, catarata, como citadas anteriormente, dentre outras (DE PAULA FREITAS et al., 2017). Em adultos infectados por ZIKV também houveram relatos de alterações oftalmológicas, tais como conjuntivite não purulenta e uveíte (FURTADO et al., 2016; SUN et al., 2016; SHAILY, SANGYA; UPADHYA, ARCHANA, 2019). Por isso, apesar de muitos casos de ZIKV serem assintomáticos ou apresentarem doenças consideradas leves, esses agravamentos fazem desse vírus uma importante questão de saúde pública.

1.7. Tratamento e a busca por antivirais

Não há antivirais específicos para ZIKV. O tratamento feito é somente para o controle dos sintomas mais severos através do uso de analgésicos e antitérmicos. Por isso, diversos estudos têm sugerido possíveis antivirais contra esse vírus, porém a maioria se encontra na fase pré-clínica de avaliação (WANG, LILI et al., 2019; FONG; CHU, 2022b). Um exemplo é o 7-deaza-2'- *C* -metil-D-adenosina (7DMA), que demonstrou ser um potente inibidor da replicação deste vírus em cultura celular e retarda a progressão da doença *in vivo* (ZMURKO et al., 2016). Outro composto que apresentou atividade parecida ao 7DMA, tanto *in vitro*, quanto *in vivo*, é o NITD008, um análogo de adenosina, que também se mostrou um ótimo inibidor de ZIKV, além de prevenir a morte de camundongos infectados com este vírus (DENG et al., 2016; GARCIA;PADILLA; CASTANO, 2017).

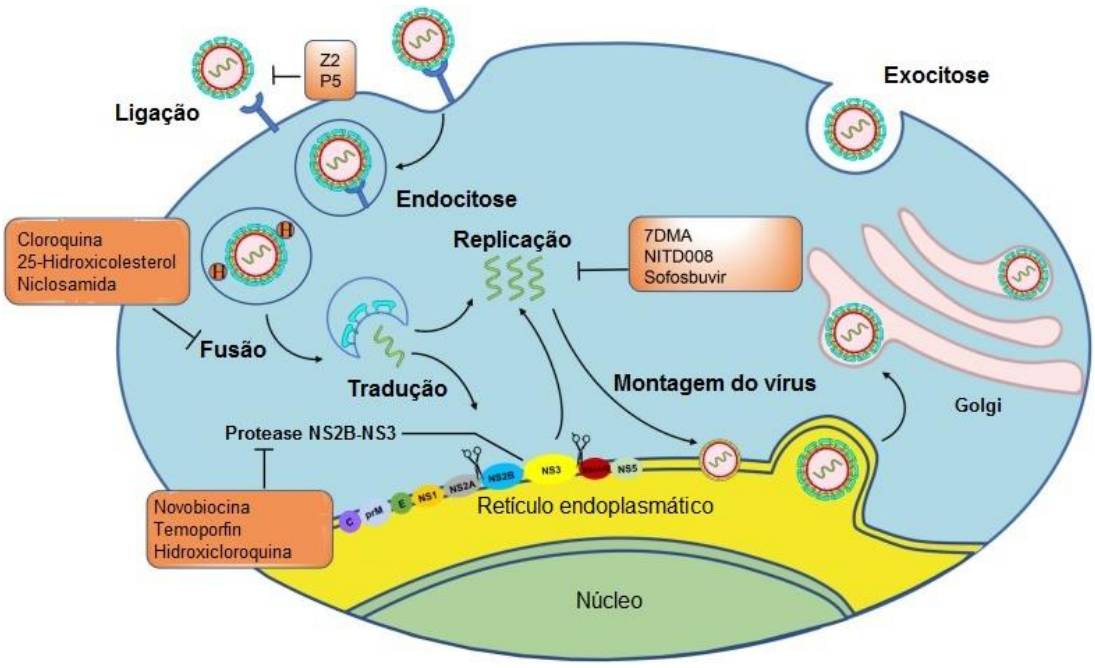
Compostos naturais também demonstraram efeito antiviral contra o ZIKV, como o epigalocatequina-galato (EGCG) que apresentou efeito na entrada de ZIKV *in vitro* (CARNEIRO et al., 2016). Batista e colaboradores (2019) relataram que a emodina e a berberina, isolados de ervas chinesas, demonstraram um grande efeito virucida *in vitro* contra o vírus Zika. O extrato de bagaço de cranberry, também demonstrou atividade anti-ZIKV e anti-DENV em linhagens celulares humanas (TAMKUTĖ et al., 2022).

Outros estudos mostraram que o Sofosbuvir, droga já aprovada para o tratamento contra o vírus da hepatite C (HCV), mostrou efeito de inibição na replicação de ZIKV *in vitro* (SACRAMENTO et al., 2017) e, *in vivo*, aumentou o percentual de sobrevivência dos camundongos e reduziu os níveis deste vírus no sangue e em alguns órgãos (FERREIRA et al.,

2017). A anidulafungina, um medicamento aprovado para tratar diversas formas de infecções pelo fungo *Candida*, demonstrou ter efeito virucida sobre o ZIKV (LU et al., 2021). Seu tratamento associado ao T-1105, um inibidor da polimerase viral em que já havia sido demonstrado possuir atividade anti-ZIKV, tiveram atividade sinérgica na inibição viral (CAI et al., 2017; LU et al., 2021).

No desenvolvimento de antivirais, muitos pesquisadores buscam moléculas capazes de inibir proteínas virais ou que tenham como alvo fatores do hospedeiro. Com relação ao ciclo replicativo viral, pretende-se interferir em uma ou mais etapas desses ciclo, podendo impedir a ligação viral a membrana da célula hospedeira e/ou sua entrada na célula, interferir no seu processo de replicação, montagem ou liberação, atuando sobre a maturação dos novos vírions que estão sendo formados (DE CLERCQ; LI, 2016; WANG, LILI et al., 2019). A Figura 7 demonstra algumas moléculas descritas na literatura por ter efeito anti-ZIKV e a etapa do ciclo replicativo nas quais cada uma delas atua. Alguns exemplos presentes na representação são os peptídeos Z2 e o P5 que interferiram na ligação de ZIKV à célula hospedeira, e outros como 7DMA, NITD008 e o sofosbuvir que atuaram sobre a replicação viral (Figura 7) (WANG, LILI et al., 2019).

Figura 7 – Moléculas já descritas na literatura por inibir o ZIKV e as etapas do ciclo replicativo nas quais elas atuam.



Fonte: Adaptado (WANG, LILI et al., 2019).

1.8. Peptídeos para fins terapêuticos

Peptídeos têm sido alvos promissores no desenvolvimento de novas terapêuticas. São moléculas que fazem sinalização seletiva, se ligando especificamente a receptores celulares, como àqueles acoplados à proteína G e canais iônicos, que levam a efeitos intracelulares. Além disso, são seguros e apresentam boa tolerância (FOSGERAU; HOFFMANN, 2015). Tais moléculas tem mostrado efeito no tratamento de diversas doenças, como Alzheimer, diabetes tipo II, câncer e doenças infecciosas (FUNKE; WILLBOLD, 2012; FOSGERAU; HOFFMANN, 2015). Mais de 80 drogas peptídicas chegaram ao mercado global e muitas outras estão em análise pré-clínica ou clínica (WANG, L. et al., 2022).

Além disso, com relação à terapia antiviral, peptídeos tem causada revolução nas busca desta área, já que eles têm se mostrado serem eficientes antagonistas e agonistas para receptores de ligação de vários agentes terapêuticos (KARWAL et al., 2020). Peptídeos com atividade antiviral já foram descritos contra o vírus Influenza, o WNV, vírus da imunodeficiência humana (HIV), HCV, entre outros (PORTAL-NUNEZ et al., 2003; BAI et al., 2007; JONES et al., 2011; NARUMI et al., 2012).

O peptídeo Hecate demonstrou efeito contra o vírus herpes simplex tipo I (HSV-1), atuando como inibidor da fusão celular que é induzida por sincícios de HSV-1, além da expressão de suas proteínas, em associação com alterações na permeabilidade na membrana da célula hospedeira (BAGHIAN et al., 1997a). Um estudo recente (2018) demonstrou a eficiência do peptídeo AG-Hecate, sendo este o Hecate modificado com ácido gálico acoplado na região N-terminal, contra o HCV, membro da família *Flaviviridae* (BATISTA, M. N. et al., 2018). Sanches e colaboradores (2015) verificaram que o peptídeo em baixas concentrações poderia atravessar as membranas celulares da célula hospedeira e atuar somente sobre os componentes intracelulares (SANCHES;CARNEIRO;BATISTA;BRAGA;LORENZÓN; et al., 2015).

A partir da Botropstoxina-I (BthTX-I) do veneno da serpente *Bothrops jararacussu* foi obtido o peptídeo $(p\text{-BthTX-I})_2$ [$(KKYRYHLKPFCKK)_2$], que demonstrou atividade antimicrobiana contra bactérias Gram-positivas e Gram-negativas (SANTOS-FILHO et al., 2015). Santos-Filho e colaboradores fizeram modificações no $(p\text{-BthTX-I})_2$, gerando o análogo peptídico $(p\text{-BthTX-I})_2K$ [$(KKYRYHLKPF)_2K$], que melhorou a atividade antibacteriana (SANTOS-FILHO et al., 2022). Além disso, o $(p\text{-BthTX-I})_2K$, demonstrou atividade antiviral contra o coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2), atuando sobre a protease PL^{pro} desse vírus (FREIRE et al., 2021).

Peptídeos com atividade anti-ZIKV também foram descritos, como o peptídeo Yodha, um peptídeo de defesa de anfíbios, que demonstrou grande atividade virucida contra diversas cepas de Zika vírus (LEE et al., 2021). O peptídeo sintético Z2, extraído da proteína de envelope do ZIKV, inibiu a infecção por esse mesmo vírus *in vitro*, além de proteger fetos de camundongos C57BL/6 prenhas da transmissão vertical do vírus Zika (YU et al., 2017). Outro exemplo é um peptídeo catelidicina, do veneno da cobra marinha *Hydrophis cyanocinctus*, o Hc-CATH, que foi capaz de inibir a infecção por ZIKV *in vitro*, e também demonstrou proteção contra a infecção por ZIKV em camundongos previamente tratados, além de apresentar efeito terapêutico em animais infectados. Esse peptídeo causou diminuição na expressão de AXL, um dos tipos de receptores celulares nas quais o ZIKV se liga para entrar na célula hospedeira e também foi capaz de perturbar a membrana viral, atuando diretamente sobre a partícula viral (WANG, JING et al., 2022).

Dada toda problemática do ZIKV, especialmente em relação à da Síndrome Congênita do ZIKV, bem como o risco de complicações neurológicas, oculares, entre outras, em adultos infectados, faz-se necessário a busca de inibidores efetivos contra o ZIKV, visto que ainda não existem fármacos específicos aprovados para tratar a infecção por esse vírus. Neste contexto, baseando-se nos benefícios e potencial de peptídeos na busca de novos agentes terapêuticos, a análise de peptídeos sintéticos como potenciais inibidores é importante e pode colaborar com avanços na área terapêutica contra esse vírus, bem como para outros flavivírus.

2. OBJETIVOS

2.1. Objetivo geral

Este trabalho teve como objetivo avaliar o potencial inibitório dos peptídeos sintéticos AG-peptídeo e $(pBthTX\text{-}I)_2K$ contra a infecção de Zika vírus *in vitro*.

2.2. Objetivos específicos

- Avaliar a capacidade dos peptídeos AG-peptídeo (GA-KKALKKLKKALKKAL-$CONH_2$) e $(pBthTX\text{-}I)_2K$ $[(KKYRYHLKPFCKK)_2]$ em inibir o ZIKV em células Vero (ATT CCL-81) infectadas;

- Avaliar a capacidade virucida dos peptídeos AG-peptídeo e $(pBthTX\text{-}I)_2K$;

- Analisar a atividade antiviral dos peptídeos AG-peptídeo e $(pBthTX\text{-}I)_2K$ em diferentes etapas do ciclo viral: entrada, replicação e liberação.

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CAPÍTULO II –

Artigos científicos

Manuscrito I

Manuscrito I

The synthetic bioconjugate peptide GA-KKALKKLKKALKKAL-$CONH_2$ has antiviral activity in several steps of ZIKV replicative cycle *in vitro*

Maria Letícia Duarte Lima[1#]; Paulo Ricardo da Silva Sanches[2#]; Gabriela Miranda Ayusso[1]; Pâmela Jóyce Previdelli da Conceição[1]; Tamara Carvalho[1]; Giovanna André Antoniucci[3]; Natasha Marques Cassani[3]; Mariana Nogueira Batista[4]; Ana Carolina Gomes Jardim[3]; Cintia Bittar[1,4]; Eduardo Maffud Cilli [5]; Marilia de Freitas Calmon[1]; Paula Rahal[1*].

[1] *Universidade Estadual Paulista, Instituto de Biociências, Letras e Ciências Exatas*, São José do Rio Preto, SP, Brazil

[2] *Universidade Estadual Paulista, Faculdade de Ciências Farmacêuticas*, Araraquara, SP, Brazil.

[3] Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, MG, Brazil.

[4] The Rockefeller University, Nova York, United States.

[5] *Universidade Estadual Paulista, Instituto de Química*, Araraquara, SP, Brazil.

[#]These authors contributed equally to this work.

***Corresponding author**

E-mail address*:* p.rahal@unesp.br

ABSTRACT

Zika virus (ZIKV) is an emergent arbovirus that caused an outbreak in the Americas in about 2015. Even though ZIKV infection is an asymptomatic or mild disease in most cases, can cause several neurological disorders and become a public health issue. These complications can be Guillain-Barré Syndrome (GB) and Congenital Zika Syndrome (CZS), and the last one affects fetuses in which the mother is infected during the pregnancy. Since there are no approved treatments or vaccines against ZIKV, it is necessary to search for possible therapeutics, and peptides have been shown to have excellent potential in new drug development. Here we presented an unedited synthetic peptide, and its effect against ZIKV infection *in vitro*. The peptide was able to inhibit the virus in a dose-dependent way and acted in several steps of the ZIKV replicative cycle. The GA-peptide (GA-KKALKKLKKALKKAL-$CONH_2$) showed virucidal activity besides protecting Vero cells against ZIKV. It also impaired the viral entry, specifically on attachment and internalization steps, and postentry stages of infection. With this, the new synthetic GA-peptide has shown a great potential against ZIKV, highlighting the importance of this molecule to the studies of new antiviral molecules.

Keywords: Zika virus; ZIKV; antiviral; peptides; bioconjugate.

INTRODUCTION

Zika virus (ZIKV) is an arbovirus included in the family *Flaviviridae*, genus *Flavivirus* [1]. It is an enveloped virus that has a positive-sense single-strand RNA genome (+ssRNA), with about 10.8 kilobases [2,3]. The ZIKV genome has a single open reading frame (ORF), that codifies a polyprotein that is cleaved into three structural proteins, capsid (C), envelope (E), pre-membrane (prM) proteins, and seven non-structural, which are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [1].

Most cases of ZIKV infection are asymptomatic, and when the symptoms appear, they are nonspecific, usually mild, and similar to other arboviruses, such as Dengue virus (DENV) and Chikungunya virus (CHIKV) [1,4]. But the infection by this virus is a public health concern due to the cases of neurological complications, especially because of congenital Zika syndrome (CZS), a set of neurological disorders that can occur in fetuses of mothers infected during the pregnancy [5,6]. Furthermore, ZIKV infection was related to Guillain-Barré and other neurological alterations in adults [7]. Nowadays, there are no vaccines or specific antivirals approved against ZIKV, being necessary to look for new therapeutics [8].

Peptides are molecules that do selective signalization, binding specifically to receptors on the cell surface, leading to intracellular effects with high affinity. These molecules usually have good tolerability, efficacy profile, and safety [9]. They have an excellent potential for new drug development, and due to this, several peptide drugs are in clinical or preclinic stages of development, and more than 80 are already in the global market [9,10]. Regarding the peptides with antiviral activity, some examples are currently approved such as enfurvitidae, against HIV [11], and boceprevir [12] and telaprevir [13] for HCV. Besides that, much data in the literature showed several peptides with antiviral activity, including against ZIKV and other flavivirus[14-17].

The synthetic peptide Hecate, which is amphipathic and α-helical, showed antiviral activity against the Herpes Simplex Virus-1 (HSV-1), inhibiting the viral-induced cells fusion cells and Hsv-1 propagation [18]. Later, Batista and collaborators showed that the Gallic acid conjugated with the Hecate peptide (GA-Hecate/GA-FALALKALKKALKKLKKALKKAL-CONH2), has a potent antiviral effect against the HCV, which belongs to *Flaviviridae* family, the same as ZIKV, acting in the main steps of the HCV replicative cycle [19]. When the Hecate is cleaved, six metabolites are generated (data in publication process), and one of these metabolites, conjugated to Gallic acid, is the GA-KKALKKLKKALKKAL-CONH2 (GA-

peptide). Here we show that this new synthetic bioconjugate peptide is capable of inhibiting ZIKV in different steps of the replicative cycle, presenting promising antiviral activity *in vitro*.

MATERIALS AND METHODS

Cell culture

Vero cells (ATCC CCL-81^{TM}) from the kidney of an African green monkey were grown in Dulbecco's Modified Eagle Medium (DMEM) (Cultilab, Brazil), supplemented with 10% fetal bovine serum (FBS) (Gibco – Thermo Fisher Scientific, USA), and 1000 U/mL penicillin-streptomycin (P/S) (Thermo Scientific, USA). They were kept in a humidified incubator at 37ºC with 5% CO_2 enrichment.

C6/36 cells (ATCC CRL-1660) from *Aedes albopictus* mosquitoes were cultured in Leibovitz L-15 medium (Cultilab, Brazil), with supplementation of 10% FBS and 1000 U/mL of P/S.

Viral stock preparation

The virus used was the Zika virus Brazilian strain (ZIKV^{BR} - BeH815744)[20], provided by Dr. Pedro Fernando da Costa Vasconcelos from Evandro Chagas Institute, Brazil. To obtain viral stocks, C6/36 cells were infected and kept for 48 – 96 hours until the cytopathic effects appeared. Then, the supernatant of infected cells was collected, filtered, and stored at -80°C.

The viral titration was performed by plaque assay. For it, 1x10^5 Vero/wells were seeded in a 24-well plate (TPP, SWI) 24 hours before the assay. Then, the virus was 10-fold serially diluted in DMEM, and the cells were infected for 1 hour at 37ºC. After this, the viral dilutions were removed, and was added a semi-solid medium, with the composition of 1x DMEM, 2% carboxymethylcellulose (CMC) (Sigma-Aldrich, USA) (1:1) supplemented with 1% FBS and 1% P/S). The plate was incubated at 37ºC for 96 hours, and then, the medium was substituted by 10% formaldehyde (Merck, DE), followed by staining with 1% crystal violet (Merck, DE). The plaques were counted, and the viral titer was expressed in plaque-forming units/milliliters (PFU/mL).

Peptide synthesis

The GA-peptide was synthesized via solid-phase synthesis (SPPS) in the automatic synthesizer TRIBUTE-UV, following the standard protocol Fmoc (9-

fluorenylmethyloxycarbonyl) on a rink-MBHA resin. To confirm the identity of the peptide, electrospray mass spectrometry was performed and the compound was tested after obtaining at least 95% purity [21]. The amino acid sequence, and molecular weight of the GA-peptide are in table 1.

Table 1. Amino acid sequence, and molecular weight (M.W.) of the peptide bioconjugate.

	Amino acid sequence	**M.W. (g/mol)**
	*GA-KKALKKLKKALKKAL-$CONH_2$	1,860.37

*GA- Gallic Acid

Cell viability assay

To evaluate the cytotoxicity profile of the bioconjugate peptide in Vero cells and to determine the maximum non-toxic concentration (MNTC), the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) method was used. For this, $5x10^3$ cells/well were seeded in a 96-well culture plate (TPP, SWI). From the next day, the cells were treated with different concentrations of GA-peptide: 6.25, 12.5, 25, 50, and 100 µM and incubated for 48 hours. Then, the media was removed and it was added 100 µL/well of MTT (Sigma-Aldrich, USA) diluted in DMEM. The cells were incubated at 37ºC for 30 minutes and this medium was replaced by 100 µL dimethylsulfoxide (DMSO) (Synth, BR) per well. The plate was incubated for 5 min with the agitation of 200 rpm and then the absorbance was measured at 572 nm in a spectrophotometer (FLUOstar Omega/BMG LABTECH, DE). Cell viability was calculated by comparation with the vehicle control (VC), that is sterile ultrapure water (peptide diluent), and the results were expressed in percentage. An arbitrary concentration of equal or more than 80% was established to determine the MNTC. The cell viability data was used to calculate the CC_{50} (The cytotoxic concentration that reduces the cell viability to 50%).

RNA extraction, cDNA synthesis, and quantitative reverse transcription PCR (qRT-PCR)

Viral RNA levels of the samples obtained from the experiments were quantified by qRT-PCR. First, the RNA was extracted with TRIzol (Invitrogen, USA), according to the manufacturer's instructions. Subsequently, the cDNA was synthesized using a High Capacity cDNA Archive kit (Applied Biosystems, USA), following the guidelines of the manufacturer.

The real-time quantitative reverse transcription PCR (qRT-PCR) was performed based on a standard curve expressed on a log_{10} scale. To prepare the reaction, TaqMan Universal PCR Master Mix, No AmpErase® UNG (Thermo Fisher Scientific, USA), was used along with the specific ZIKV 1086, ZIKV 1162c, and 1107-FAM primers [22], and 1µL of cDNA. To normalize the intracellular RNA from the postentry assay were submitted to a relative quantification using β-actin as an endogenous control (F: 5' CAGCACAATGAAGATCAAGAT; R: 5' – CTAGAAGCATTTGCGGTGGAC, 5' – [6FAM]ACCTTCCAGCAGATGTGGATC[BHQ1]). They were carried out in QuantStudio™ 12 K Flex Real-Time PCR System (Applied Biosystems, USA). RNA viral loads were normalized with vehicle control (VC) and expressed in percentage.

Evaluation of the inhibitory potential of the GA-peptide against the ZIKV

To verify if the GA-peptide had antiviral activity against ZIKV, $2x10^4$ Vero cells per well in a 24-well culture plate were seeded a day before the assay. The peptide at 25 µM (MNTC) and the ZIKV MOI 0.1 were simultaneously added, and the plate was incubated for 48 hours at 37ºC with 5% CO_2. After this period, the supernatant was collected and the RNA levels of the samples were obtained by qRT-PCR.

Dose-dependence assay

The dose-dependence assay was performed to observe if the peptides inhibited ZIKV in a dose-dependent manner, $2x10^4$ cells were seeded in a 24-well plate, 24 hours before the experiment. On the day, different concentrations of the peptide were incubated with ZIKV MOI 0.1. The peptide concentrations were 6.25, 12.5, 25, 50, and 100 µM. The cells were incubated at 37ºC with 5% CO_2 for 48 hours. Then, the levels of viral RNA in the supernatant were determined by qRT-PCR. The data obtained was used to calculate the EC_{50} (effective concentration of the peptide that decreased 50% of viral RNA).

Virucidal assay

The virucidal assay was performed to verify if the GA-peptide had direct action on the ZIKV particles. One day after seeding the cells ($2x10^4$ cells/well in a 24-well plate), the ZIKV MOI 0.1 was incubated with 25 µM of the GA-peptide for 1 hour at RT, cell-free. Then, the medium was removed, and the mix of ZIKV and peptide was added and incubated for 1 hour. The supernatant was aspirated and DMEM supplemented with 2% FBS and 1% P/S was added. The plate was incubated for 48 hours at 37ºC, the supernatant was collected, and the RNA levels were quantified by qRT-PCR.

Pretreatment assay

To analyze if the peptide could protect the Vero cells against the ZIKV infection, one day after the Vero cells ($2x10^4$ cells/well) were seeded in a 24-well culture plate, they were treated with 25 µM of the GA-peptide for 1 hour at 37ºC. After this period, the peptide was removed, the cells were washed three times with phosphate-buffered saline (PBS), and ZIKV MOI 0.1 was added. After 1 hour of infection, the virus was removed and the cells were incubated with DMEM supplemented with 2% FBS and 1% P/S for 48 hours at 37ºC. Then, the supernatant was collected, and the ZIKV RNA levels were determined by qRT-PCR.

Entry assay

To evaluate the effect of the GA-peptide in the entry step of the ZIKV replicative cycle,the entry assay was performed. For this, $2x10^4$ Vero cells/well were cultured in a 24-well plate 24 hours before the assay. Then, the cells were incubated with 25 µM of the GA-peptide and ZIKV MOI 0.1 for 1 hour at 37ºC. Next, they were removed, the cells were washed with PBS twice, and DMEM was added supplemented with 2% FBS and 1% P/S. After 48 hours, the supernatant was collected and the ZIKV RNA levels were determined by qRT-PCR.

Attachment assay

To analyze if the peptide was acting specifically in the attachment of the virus to cell receptors, $2x10^4$ Vero cells/well were cultured a day before the assay. Then, the culture plate was kept at 4ºC for 15 minutes, followed by the incubation with the ZIKV MOI 0.1 and GA-peptide at 25 µM for 1 hour at 4ºC. Subsequently, the mix was removed, the cells were washed twice with PBS, DMEM with 2% FBS, and 1% P/S was added to the cells and incubated for 48 hours at 37ºC. The RNA levels in the supernatant were determined by qRT-PCR.

Internalization assay

This assay was performed to analyze if the GA-peptide interfered in the internalization step of the ZIKV in Vero cells. The cells were seeded ($2x10^4$ cells/well) 24 hours before the experiment. Then, the cells were incubated at 4ºC for 15 minutes, infected with ZIKV MOI 0.1, and kept for 1 hour at 4ºC. Subsequently, the virus was removed, the cells were washed two times with PBS, and the peptide (25 µM) was added. The plate was incubated for 1 hour at 37ºC, then the peptide was removed, the cells were washed twice with PBS, and DMEM with 2% FBS and 1% P/S was added. After 48 hours, the supernatant was collected, and RNA levels were measured by qRT-PCR.

Postentry assay

The postentry assay was performed to evaluate if the peptide was acting in steps after the virus entry in the cells. For this, $2x10^4$ Vero cells/well were cultured in a 24-well plate a day before infection. The cells were infected with ZIKV MOI 0.1, and incubated for 1 hour at 37ºC. Subsequently, the virus was removed, and the cells were treated with 25 µM of the GA-peptide, and incubated for 48 hours at 37ºC. The viral RNA levels in the supernatant (SN) and intracellular (IC) were quantified by qRT-PCR.

Statistical analysis

MTT assay was performed in triplicate, the antiviral trials in duplicate, and the qRT-PCR in triplicate of each sample using one replicate/event at least. All experiments were carried out in three independent assays. All data was normalized with the vehicle control (ultrapure water) and expressed in percentage. The statistical analysis was done by paired test T for parametric data and the Mann-Whitney test for non-parametric data. P values <0.05 were considered significant. The CC_{50} and EC_{50} were calculated by non-linear regression of the dose-response curves (Log[peptide] x response) to cell viability assay and dose-dependence assay, respectively. The selectivity index (SI) was calculated by the relation of the CC_{50}/ EC_{50}. Statistical analysis and graphs were executed in GraphPad Prism 5 software (GraphPad Software, USA) and Microsoft Excel (Microsoft, USA) helped in the analyses.

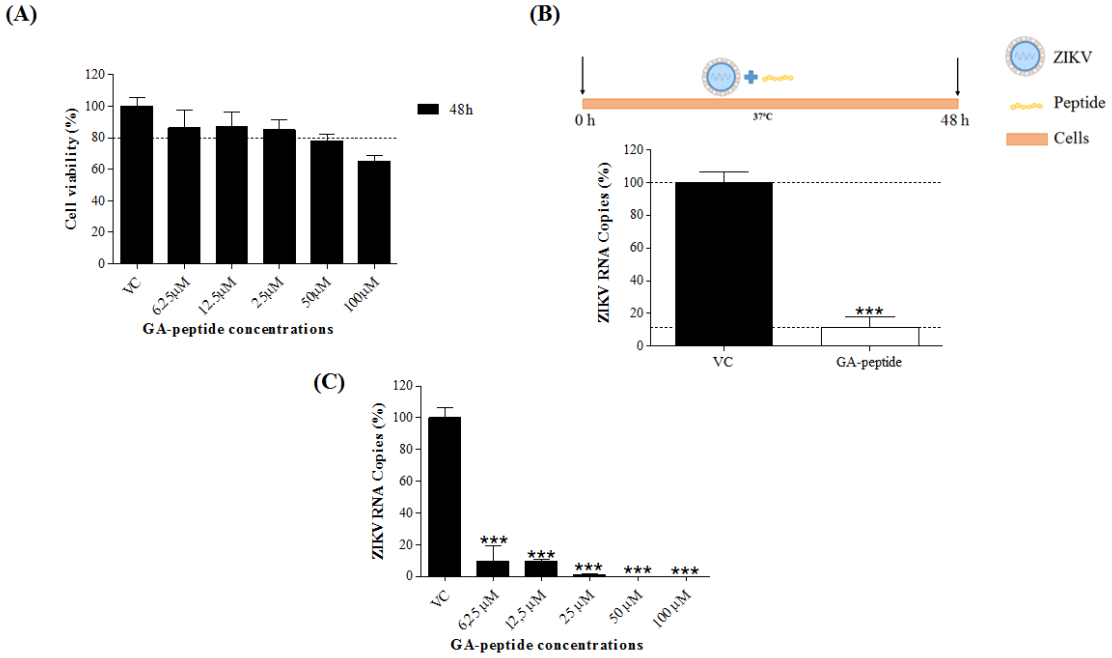
RESULTS

The synthetic GA-peptide has antiviral activity against ZIKV

The cytotoxicity profile of the GA-peptide in Vero cells was determined by MTT (Figure 1A). To define the maximum non-cytotoxic concentration, an arbitrary limit of 80% or more of the cell viability has been established. Then, the 25 µM concentration was chosen to perform the antiviral assay. The CC_{50} was 17,64 µM. After the definition of the MNTC, the inhibitory potential assay was carried out to evaluate the antiviral activity of the peptide against ZIKV. We observed that the RNA levels of ZIKV in cells treated with the GA-peptide decreased by 88.57% at 48 hours post-infection (h.p.i.), showing an inhibition statistically significant (p<0.0001) (Figure 1B). We also performed a dose-dependence assay to calculate the EC_{50}. The GA-peptide was capable to inhibit ZIKV in a dose-dependent manner (Figure 1C), and the EC_{50} was 3.3 µM. Therefore, we calculated the selectivity index (SI) and the result

was 5.35 µM. Then, we decided to investigate the specific steps in which the compound was acting in the ZIKV replicative cycle.

Figure 1. GA-peptide cytotoxicity, inhibitory potential against ZIKV, and dose-dependence analysis. **(A)** Cell viability of Vero cells treated with different concentrations of GA-peptide at 48 h post-treatment. **(B)** Schematic representation and graph of the inhibitory potential assay, where ZIKV (MOI 0.1) and GA-peptide at 25µM were added simultaneously in Vero cells and kept for 48 h. **(C)** Dose-dependent inhibition of the ZIKV by GA-peptide in different concentrations of the compound. ***: p<0.0001. Data were presented as mean and ± bars showed standard deviation (SD). **VC**: vehicle control (sterile water).



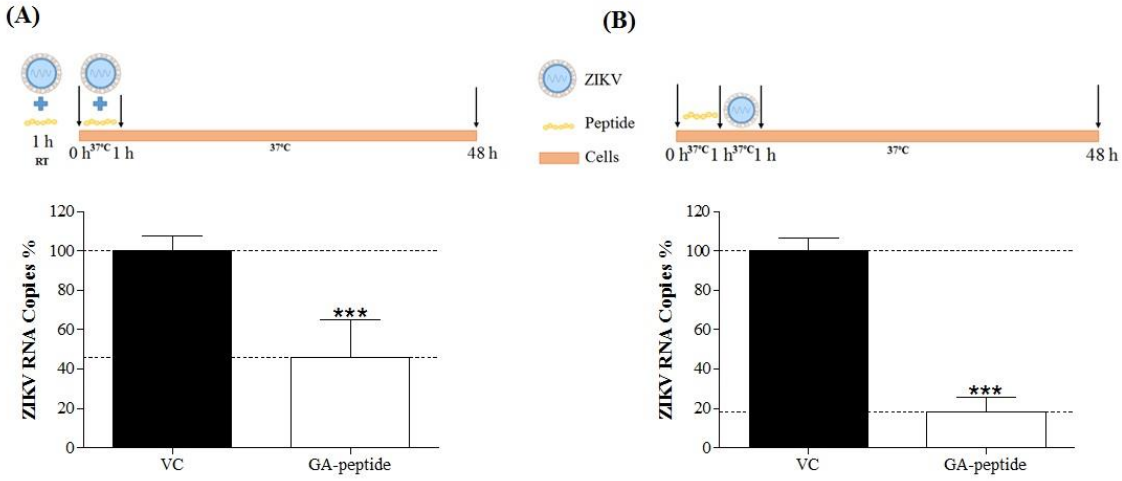
GA-peptide showed virucidal activity and protects the cells against ZIKV infection

To evaluate the virucidal activity of the compound GA-peptide and ZIKV were incubated at room temperature for one hour, and then they were added to Vero cells for 1 hour at 37ºC. In this assay the compound reduced 53.97% (p<0.0001) of the viral RNA in Vero cells, compared to VC (Figure 2A).

We also analyze if the GA-peptide could protect the Vero cells against ZIKV infection, first, the cells were treated with the peptide for 1 hour, and then, after the peptide was removed,

the cells were infected with ZIKV for 1 hour. The qRT-PCR results showed that the ZIKV RNA levels decreased by 81.9% (p<0.0001) compared to VC (Figure 2B).

Figure 2. Virucidal and protective effect of the GA-peptide against ZIKV. **(A)** For the virucidal assay, the compound at 25µM was incubated with ZIKV (MOI 0.1) for 1 hour at room temperature (RT), then both were added simultaneously, and they were removed after 1 h. At 48 h.p.i, the supernatant was collected and quantified by qRT-PCR. **(B)** For the pretreatment assay, the Vero cells were treated with 25 µM of the GA-peptide for 1 h, at 37°C, and then the peptide was removed, the cells were washed with PBS and infected with ZIKV MOI 0.1, which was removed 1 h.p.i. The viral RNA levels of the supernatant were quantified at 48 h.p.i. ***: p<0.0001. Data were presented as mean, and ± bars showed standard deviation (SD). **VC**: vehicle control (sterile water). The schematic representations over the graphs illustrate how the assays were performed.



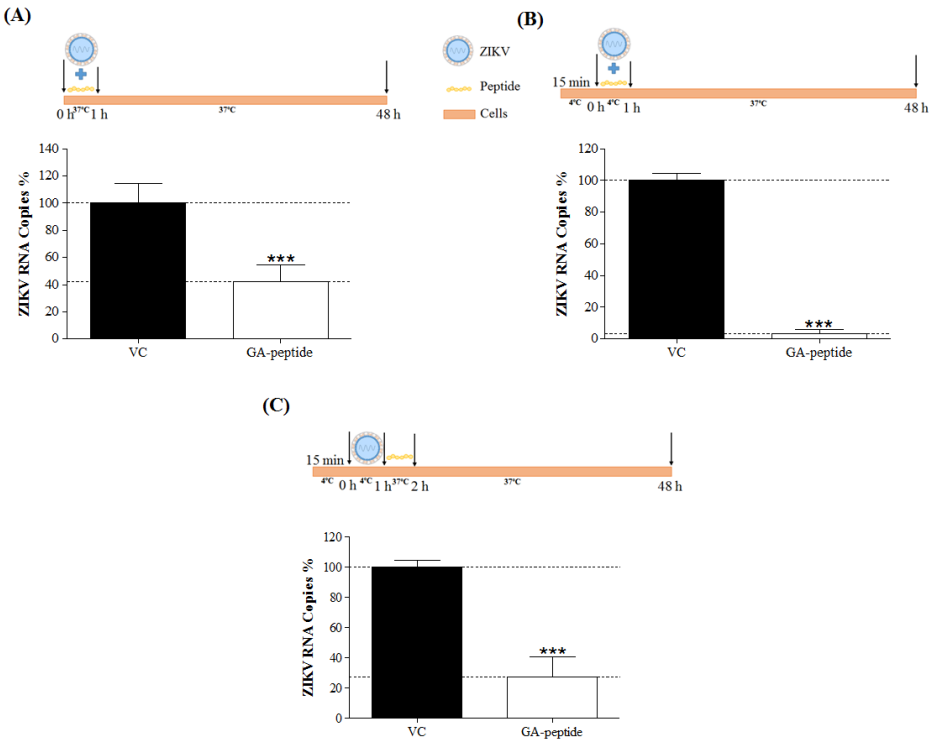
The GA-peptide inhibits the initial steps of the ZIKV replicative cycle

The entry assay was performed by simultaneous incubation of the virus and the peptide at 37ºC for 1 hour. We observed that the GA-peptide reduced 57.62% (p<0.0001) of the viral RNA levels, showing the capability to act in the entry step of the ZIKV replicative cycle (Figure 3A).

Due to this, we decided to investigate which step of entry specifically the compound was acting on, evaluating if the GA-peptide could interfere in the attachment and/or internalization of the virus. In the attachment assay, the GA-peptide decreased by 96.52%

(p<0.0001) of the viral RNA levels compared to the VC (Figure 3B). And to internalization assay, the compound also showed an effect in this step and inhibited 72.67% (p<0.0001) of the ZIKV RNA levels (Figure 3B). In conclusion, the peptide was capable to act in the initial steps of the ZIKV replicative cycle.

Figure 3. GA-peptide inhibited the early stages of ZIKV infection in Vero cells. **(A)** To assess the effect in the entry steps, 25 µM of the peptide was incubated with Vero cells infected ZIKV MOI 0.1 for 1 h at 37ºC. The peptide and virus were removed, and the cells were incubated 48 h. The supernatant was collected for quantification by qRT-PCR. **(B)** To evaluate the action of the peptide in the attachment step, the cells were first incubated at 4ºC for 15 min, followed by addition of GA-peptide (25 µM) and ZIKV (MOI 0.1) for 1 h and kept at 4ºC. They were removed, and the cells were incubated at 37ºC for 48 h. **(C)** To verify the impact in internalization steps, the Vero cells were kept at 4ºC for 15 min. Then, they were infected with the virus MOI 0.1 and incubated for 1 h at 4ºC. After ZIKV was removed, the GA-peptide (25 µM) was added and kept for 1 h at 37ºC. After 48 h, the supernatant of the cells was collected and quantified by qRT-PCR. *****:** p<0.0001. Data were presented as mean, and ± bars showed standard deviation (SD). **VC**: vehicle control (sterile water). The schematic representations over the graphs illustrate how the assays were performed.

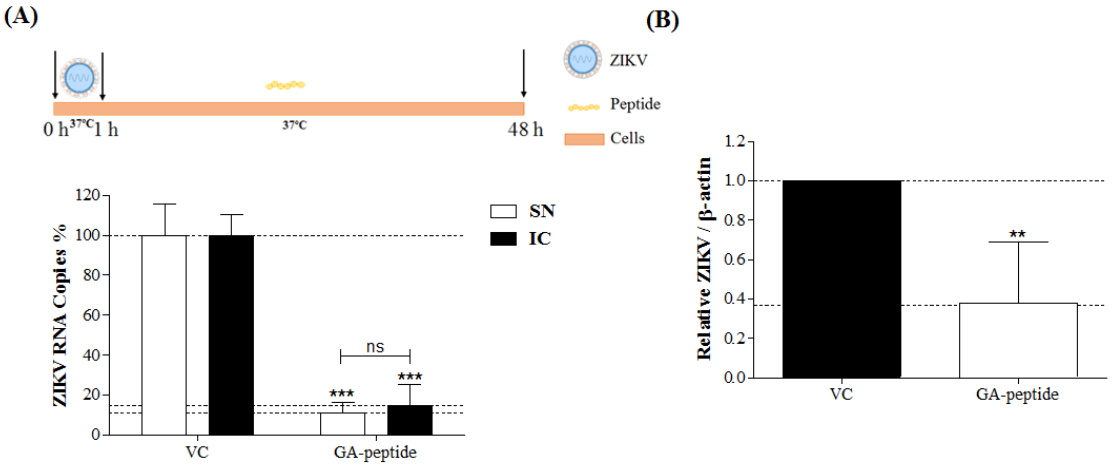


The GA-peptide also interferes with the postentry steps of ZIKV

Aimed to verify if the peptide could affect the postentry steps of the ZIKV replicative cycle, Vero cells were infected with the virus MOI 0.1 for 1 hour at 37ºC. Then, the ZIKV was removed and the cells were treated with 25 µM of the GA-peptide, and kept for 48 hours. After quantification by qRT-PCR, the data showed that the peptide decreased 88.94% (p<0.0001) (Figure 4A) of ZIKV RNA levels in the supernatant and 85.12% (p<0.0001) (Figure 4A) in the intracellular compartment. Besides that, we performed a relative qRT-PCR to normalize the total RNA levels in the intracellular samples using β-actin mRNA as standard, and we confirmed that the relative levels also were significant (p = 0.0084) (Figure 4B).

We observed if the peptide was interfering in the release step by analyzing if the levels of ZIKV RNA in supernatant decreased in relation to intracellular, such as described by Batista and coworkers [19], but there is no significant difference between intracellular and supernatant ZIKV RNA levels (Figure 4A). In summary, the GA-peptide has anti-ZIKV properties in postentry steps, not related to the release step.

Figure 4. GA-peptide decreased the levels of ZIKV RNA in steps postentry in Vero cells. **(A)** To evaluate the effect in viral postentry steps, Vero cells were infected with ZIKV MOI 0.1 for 1 h. The virus was removed, and the cells were treated with 25 µM of the peptide for 1 h. After 48 h, supernatant and intracellular samples were collected and quantified by qRT-PCR. The schematic representation over the graph illustrate how the assay was performed. **(B)** Normalization of the intracellular ZIKV RNAs of postentry assay using the β-actin mRNA as endogenous control. *****:** p<0.0001; ****:** p = 0.0084; **ns**: non-significant. Data were presented as mean, and ± bars showed standard deviation (SD). **VC**: vehicle control (sterile water).



DISCUSSION

Due to problematic of ZIKV involving neurological alterations, such as Congenital Zika Syndrome and Guillain-Barré syndrome, and the fact that there are no specific antiviral or vaccines approved against this virus, it is necessary to search for prophylactic and therapeutics options [6-8]. Peptides have great potential in this context, and many studies are showing their effect on several diseases, including antiviral activity [9]. Besides that, dozens of peptide drugs are already being used thought the world[10].

In this work, we presented a new synthetic peptide, GA-KKALKKLKKALKKAL-$CONH_2$, which is one metabolite of Hecate peptide conjugated with gallic acid, and its antiviral activity against ZIKV. This compound inhibited the virus in a dose-dependent manner, presenting a SI of around 5.35 µM**,** and affected several steps of the ZIKV replicative cycle. This peptide was capable to protect Vero cells from the ZIKV infection. It also acted in ZIKV particle, showing a virucidal effect. Furthermore, the GA-peptide interfered with the entry of the virus, specifically by inhibiting both attachment and internalization steps. The postentry steps were also affected by the GA-peptide. In this way, this compound could be a good option for antiviral against ZIKV because it acts in different steps of the viral replicative cycle.

In brief, for ZIKV to enter cells, the viral glycoprotein E bindings to specific cell receptors, like AXL, DC-SIGN, Tyro, TIM-1, and glycosaminoglycans (GAGs) [23,24]. Then, the virus suffers receptor-mediated endocytosis and is internalized in clathrin-coated vesicles, the endosomes. Due to acidification in these vesicles, the protein E undergoes conformational changes and fuses with the endosomal membrane, releasing the viral RNA to the cytoplasm of the cell [25,26].

Analyzing the effects of the GA-peptide under ZIKV, we can suppose some possible modes of action, and they may be related to direct action to the virus and/or host factors that are important to viral replication. Based on the virucidal effect, we observed that the peptide might be capable of binding directly to the viral particle, preventing the attachment to the host cell. The peptide could also be disrupting the virus particle itself. However, the virucidal results had a viral RNA inhibition at 53.97%, and they were modest when compared to the pretreatment assay, which decreased 81.9% of the RNA levels. It is possible then that the effect observed in the virucidal assay is related to the effect the peptide has on the cell alone, evidenced by the pretreatment assay. The protective effect of the peptide may occur because it can be capable of binding to cell receptors that are necessary for ZIKV entry therefore blocking the virus attachment. Cell receptors with a negative charge, such as GAGs, interact electrostatically with

positively charged regions of ZIKV E protein to facilitate viral entry [24,27]. In this way, being GA-peptide positively charged, we can hypothesize it interacts with cell receptors by charge interactivities. A promising class of antiviral peptides is precisely that the one that infers in the membrane fusion process of the viral entry into the cells [28]. These peptides have been called fusion inhibitor peptides, and this process may occur by the involvement of them with membrane lipids and/or glycoproteins of the fusion process through hydrophobic and electrostatic interactivities [28,29].

These effects are likely related to the inhibition of the initial steps of the replicative cycle, also observed in this work. The viral entry was affected specifically by the attachment and internalization stages. The attachment step showed 96.52% of inhibition of RNA levels, which was higher than the inhibition of 72.67% in the internalization step. These results reinforce the hypothesis raised for the protective effect that the peptide may capable of binding the cell receptors and blocking them to ZIKV attachment.

Besides that, this effect in the internalization step may be interfering with the endosomal acidification, disturbing the conformational changes of the glycoprotein E of the ZIKV and the fusion of the viral envelope with the endosome membrane [30]. There are some peptides capable to cross the membranes and access the interior of the cells [31]. They are named cell-penetrating peptides (CPPs), and one of the ways that they may cross the membrane is by endocytosis [31], which is the same way that ZIKV internalizes in host cells. Then, we hypothesized that the GA-peptide may be internalized in the same endosome of the virus and affected the acidification of these vesicles.

Furthermore, the peptide also inhibited the postentry stage of the ZIKV replicative cycle, decreasing 88.94% of the viral RNA in the SN and 85.12% in the IC. After entry into the cells, the viral RNA is released in the cytoplasm, and the translation of structural and non-structural proteins begins from the ORF present in the +ssRNA, followed by the replication process. Negative-sense single-strands RNA is synthesized and new +ssRNA from them [25,26]. Then, these RNA strands are packaged with the viral progeny which is assembled in association with the endoplasmic reticulum. The immature virion formed passes by the secretory pathway in the trans-Golgi where the cleavage of prM protein in M occurs for the virus maturation, and finally, the released happens, being the new virions take out of the cells by exocytosis [25,26].

Based on this, the postentry effect of the GA-peptide may be occurring in replication, assembly, maturation, and/or release. GA-peptide is a peptide with a net positive-charge, and one hypothesis is that the peptide could be interacting with the viral RNA, because nucleic acids

have a negative charge [32], and interfering in the replication. Other possibilities to explain this effect are the action under the ZIKV protease, the complex NS2B-NS3, and under the NS5 non-structural protein, which has two domains, a methyltransferase, and the RNA-polymerase RNA-dependent domain. These factors are essential to viral replication and may be affected by the GA-peptide [33]. Besides the replication, the effect could be happening in assembly and/or release, but this action did not seem to be related to release because when comparing the viral RNA levels in the SN and IC, there is no significant difference [19].

In addition, the effect of the GA-peptide can involve host cell factors. With our results, we can observe that the interaction with lipid membranes is a possibility. The Hecate peptide has a property well-established that is the incorporation into the lipid bilayer of the cells membrane [18,34]. Besides that, Batista and coworkers verified that the GA-Hecate, which was effective against HCV, acting in several steps of viral infection, had as the main effect the action in lipids droplets, affecting the HCV replicative cycle [19]. The peptide presented here is a metabolite of the Hecate conjugated to Gallic acid, so the interaction with lipids of the host cells may be one way that the peptide is acting and disturbing the ZIKV infection in host cells.

The replicative cycle of flaviviruses is related to the lipids of host cells because they are important for viral entry, both in attachment and internalization, for replication, and assembly [35]. Peruzzu and collaborators showed that ZIKV explored lipid rafts in its entry into Vero cells, and when the cells were treated with amphotericin B (AmphB), which is a disruptor of lipid rafts, the viral infection was inhibited. Besides this, AmphB did not have virucidal activity, showing that the action was direct to host cells [36]. Lipid rafts in the cell membrane are a location for several receptors, and some of them are important to flaviviruses binding and entry, then the interference in these lipids may disturb the initial stages of the viral infection [35,37]. The lipidic composition also is necessary for the internalization of the flaviviruses, in the fusion of the viral E protein with the endosomal membrane, as reviewed by Martín-Acebes, Vázquez-Calvo, and Saiz [35]. The possible interaction of GA-peptide with lipids present in membranes could explain the effect of this peptide in the early steps of ZIKV replicative cycle.

Regarding the postentry stages of the infection, the replication of the flaviviruses occurs associated with the endoplasmic reticulum membrane, where the virus remodels these membranes to form a replication complex [38]. Analyzes show that ZIKV is capable to regulate the lipid metabolism of the host cell, and this is important to the replication, package, and assembly of the viral RNA [38,39]. GA-peptide could have interacted with these lipids, and this may be one reason of the postentry effect.

More studies are necessary to better clarify the action mechanisms of the GA-KKALKKLKKALKKAL-$CONH_2$ against ZIKV. But here, we demonstrated a new synthetic bioconjugate peptide that was able to inhibit important steps of ZIKV infection in *vitro*. In this way, the tested compound may represent a potential drug for ZIKV treatment and another flaviviruses.

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CAPÍTULO II –

Artigos científicos

Manuscrito II*

*__Nota:__ O artigo científico a seguir é fruto de duas teses de doutorado distintas, nas quais os resultados foram combinados para uma única publicação, porém a parte desenvolvida no presente trabalho corresponde aos resultados de Zika vírus.

Manuscrito II

The dimeric peptide $(KKYRYHLKPF)_2K$ has a broad-spectrum antiviral activity inhibiting different steps of infection of the Chikungunya and Zika viruses

Gabriela Miranda Ayusso [1#], Maria Letícia Duarte Lima [1#], Paulo Ricardo da Silva Sanches [2#], Igor Andrade Santos [3], Daniel Oliveira Silva Martins [1,3], Pâmela Jóyce Previdelli da Conceição [1], Tamara Carvalho [1], Cíntia Bittar [1,4], Andres Merits [5], Norival Alves dos Santos-Filho [6], Eduardo Maffud Cilli [6], Ana Carolina Gomes Jardim [1,3], Marilia de Freitas Calmon [1], Paula Rahal [1*]

[1] *Universidade Estadual Paulista (UNESP), Instituto de Biociências, Letras e Ciências Exatas*, São José do Rio Preto, SP, *Brasil*.

[2] *Universidade Estadual Paulista (UNESP), Faculdade de Ciências Farmacêuticas*, Araraquara, SP, *Brasil*.

[3] *Universidade Federal de Uberlândia (UFU), Instituto de Ciências Biomédicas*, Uberlândia, MG, *Brasil*.

[4] Laboratory of Molecular Immunology, The Rockefeller University, New York, NY, USA.

[5] University of Tartu, Tartu, EE.

[6] *Universidade Estadual Paulista (UNESP), Instituto de Química*, Araraquara, SP, *Brasil*.

[#] These authors contributed equally to this work.

*** Corresponding author**

E-mail address*:* p.rahal@unesp.br

ABSTRACT

Chikungunya virus (CHIKV) and Zika virus (ZIKV) are important concerns in global health. Currently, there are no antiviral drugs and vaccines approved against these viruses. In this context, peptides have great potential for new drugs development. A recent study described that the (p-BthTX-I)$_2$K [(KKYRYHLKPF)$_2$K], a peptide derived from the Bothropstoxin-I toxin of the venom of the *Bothrops jararacussu* snake, showed antiviral activity against SARS-CoV-2. Here, we assessed the activity of this peptide against CHIKV and ZIKV and its antiviral action in the different stages of the viral replicative cycle *in vitro*. We observed that the (p-BthTX-I)$_2$K impaired the CHIKV infection by interfering with the early steps of the viral replication cycle, reducing the CHIKV entry into BHK-21 cells by 20.3% when compared to the control. Besides that, this peptide inhibited the viral entry by reducing both attachment and internalization of the CHIKV in BHK-21 cells in about 44% and 34%, respectively. (p-BthTX-I)$_2$K also inhibited the replication cycle of ZIKV in Vero cells. The peptide protected the cells against ZIKV infection, and was also capable to decrease the viral RNA levels in the virus postentry steps, reducing genome copy number by 86% in the supernatant of the cell cultures and by 73.6% of the intracellular virus when compared to the control. In conclusion, this study highlighs the potential of the (p-BthTX-I)$_2$K peptide as a novel broad-spectrum antiviral candidate, targeting different steps of the replication cycle of both CHIKV and ZIKV.

Keywords: CHIKV; ZIKV; antiviral; peptide.

INTRODUCTION

Arboviruses are a group of viruses that are transmitted between vertebrate hosts by hematophagous arthropods vectors, such as ticks and mosquitoes [1]. Currently, more than 500 species of arboviruses belonging to 14 different families have been reported, with more than 100 species being human or animal pathogens [2]. Arboviruses that cause disease in humans or animals belong to four main families: *Peribunyaviridae*, *Flaviviridae*, *Togaviridae,* and *Sedoreoviridae* [2,3]. The temperature, climate, and vegetation are ecological parameters that interfere with both vectors and host distribution [4]. Brazil is a large and tropical country which have many characteristics that favor the arboviruses occurrence, such as climate, vegetation, and biodiversity [5]. Among the main arboviruses which circulate in Brazil are Zika virus (ZIKV) and Chikungunya virus (CHIKV) [6,7].

The CHIKV is an enveloped virus with a single-stranded, positive-sense RNA genome that belongs to the *Togaviridae* family and *Alphavirus* genus. Currently, this virus has a worldwide distribution and is considered a serious public health problem. According to the Pan American Health Organization (PAHO), more than 250,000 cases were reported in 12 countries and territories of the Americas in 2022. Brazil was the most affected country with 98.8% of the cases [8]. The CHIKV infection triggers symptoms in 72 to 95% of the patients [9] and symptoms associated with pain and swelling, particularly in the wrists, hands, ankles, and feet can persist for years [10]. As of now, no specific antiviral therapy or licensed vaccine is available for CHIKV and only palliative care is recommended to alleviate the symptoms [9,11].

ZIKV is also an enveloped virus with a positive-sense single-strand RNA genome. It belongs to the *Flaviviridae* family, genus *Flavivirus* [12]. ZIKV caused an epidemic in the Americas around 2015 and, in this period, the infection in pregnant women was related to a set of neurological alterations in their fetuses, named Congenital Zika Syndrome (CZS) [13-15]. ZIKV infection may also cause Guillain-Barré syndrome and other neurological complications in adults [16]. Thus, albeit the majority of infections by ZIKV being asymptomatic, this virus is an important concern of public health, especially because of CZS [15]. In 2022, more than 31,400 ZIKV cases were reported in the Region of the Americas, according to the PAHO. Most of these cases were reported in Brazil, representing 92.6% of them[8]. Such as CHIKV, there are no vaccines or antivirals approved against the ZIKV [17]. Therefore, there is a significant need for treatments or vaccines that are safe and effective to reduce the viral spread and limit the disease burden caused by CHIKV and ZIKV.

In the last decades, the discovery of the therapeutic potential of several peptides with different biological targets increased the interest of the scientific community in this compound class [18,19]. The peptides have cellular biocompatibility, demonstrating high affinity and specificity, which makes them potential drug candidates [18-20]. The antimicrobial peptides (AMPs) have a natural or synthetic origin. Among the synthetic peptides, there are analogues based on the structures of natural peptides [18]. The peptide (p-BthTX-I)$_2$ [(KKYRYHLKPFCKK)$_2$], derived from the Bothropstoxin-I (BthTX-I) toxin of the *Bothrops jararacussu* snake venom, was described with antibacterial activity against Gram-positive and Gram-negative bacteria [21]. Santos-Filho and coworkers demonstrated that the removal of four lysines located in the C-terminal region of the peptide (p-BthTX-I)$_2$ [des-Lys^{12},Lys^{13}-(p-BthTX-I)$_2$] [(KKYRYHLKPFC)$_2$] did not promote lack of the antibacterial activity [22]. Another work developed by the same group reported that the absence of Lys^{12} and Lys^{13}, the removal of two cysteines, and the insertion of lysine residue as a branch point at the C-terminal region [des-Cys^{11},Lys^{12},Lys^{13}-(p-BthTX-I)$_2$K – [KKYRYHLKPF)$_2$K] [(p-BthTX-I)$_2$K peptide] resulted in greater antibacterial activity than the wild type peptide (p-BthTX-I)$_2$ [23]. This peptide also showed antiviral activity against the Severe Acute Respiratory Syndrome virus 2 (SARS-CoV-2), an enveloped virus that belongs to the *Coronaviridae* family [24].

This study aimed to evaluate the anti-CHIKV and anti-ZIKV activity of the (p-BthTX-I)$_2$K peptide and investigate its effects on different steps of the viral infection cycles. These data are the first report of the antiviral activity of the (p-BthTX-I)$_2$K peptide against *Alphavirus* and *Flavivirus* infections.

MATERIAL AND METHODS

Peptide

The (p-BthTX-I)$_2$K peptide [(KKYRYHLKPF)$_2$K] (molecular weight = 2869.45 g/mol) was synthesized according to Santos-Filho and coworkers [25]. In brief, the peptide was prepared on TRIBUTE-UV automatic synthesizer (Protein Technologies, AZ, USA) via solid phase peptide synthesis (SPPS), using the standard Fmoc protocol (9-fluorenylmethyloxycarbonyl) on a Rink-MBHA resin. To obtain the dimer, Fmoc-Lys(Fmoc)-OH was used at C-terminus as a branch point, allowing for the growth of peptide chains from the α-amino and ε-amino groups. The peptide identity was confirmed by electrospray ionization mass spectrometry. The purity of the (p-BthTX-I)$_2$K peptide was higher than 95%.

Cells

The Baby hamster kidney fibroblast cells (BHK-21; ATCC CCL-10) and African green monkey kidney cells (Vero; ATCC CCL-81) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Cultilab, São Paulo, BR) supplemented with 10% fetal bovine serum (FBS, Gibco – Thermo Fisher Scientific, Massachusetts, USA) and 1% penicillin (10,000 IU/mL)/streptomycin (P/S) (10 mg/mL) (Cultilab, São Paulo, BR). The cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

C6/36 cells (ATCC CRL-1660) from *Aedes albopictus* mosquitoes were cultured in Leibovitz L-15 medium (Cultilab, São Paulo, Brazil), with supplementation of 10% FBS (Gibco – Thermo Fisher Scientific, Massachusetts, USA), and 1% P/S (Cultilab, São Paulo, BR). These cells were kept in an incubator at 28 ºC.

Viruses

The recombinant CHIKV-NLuc, used here, was based on the CHIKV isolate LR2006OPY1 (East/Central/South African genotype). The infectious cDNA plasmid of this virus contains the human cytomegalovirus (CMV) promoter upstream of sequence corresponding to the virus genome; sequence of reporter gene that encodes the NanoLuciferase (NLuc) protein, is inserted into region encoding for nsP3 protein of CHIKV [26]. The $ZIKV^{BR}$ - BeH815744, isolated from a febrile case in Paraíba state, Brazil was used for the experiements with ZIKV [27].

Viral stock preparation

To produce infectious viral particles of the CHIKV-NLuc, BHK-21 cells seeded in 24-well culture plates (TPP, Trasadingen, SWI) (1×10^5 cells/well) were transfected with 1 µg of CMV-CHIKV-NLuc plasmid, using Lipofectamine 2000 (Thermo Fisher Scientific, Massachusetts, USA) and OPTI-MEM (Reduced Serum Medium) (Gibco – Thermo Fisher Scientific, Massachusetts, USA), following the previously described protocol with modifications [28]. Seventy-two hours post-transfection, the supernatant was collected and stored at – 80 °C.

For ZIKV stock, C6/36 cells were infected and incubated for 48 – 96 hours, until cytopathic effect was observed. The supernatant of infected cells was collected, filtered, and stored at – 80°C.

Viral stocks were plaque-titered, according method used by Santos and coworkers with modifications [28]. BHK-21 cells were used for CHIKV while Vero cells were used for ZIKV. These cells were cultured in 24-well culture plates (TPP, Trasadingen, SWI) (1×10^5 cells/well) and infected with 10-fold serially dilutions of the viruses for 1 hour at 37 ºC. Then, the viral inoculum was removed and a semi-solid medium, consisting DMEM (Cultilab, São Paulo, BR) and 2% carboxymethylcellulose (CMC) (Sigma-Aldrich, Missouri, USA) supplemented with 1% FBS (Gibco – Thermo Fisher Scientific, Massachusetts, USA) and 1% P/S (Cultilab, São Paulo, BR) was immediately added. After incubation of the plate for 48 hours (for CHIKV) and 96 hours (for ZIKV), the cells were fixed with 10% formaldehyde (Merck, Darmestádio, DE) and stained with 1% crystal violet (Merck, Darmestádio, DE). The viral foci were counted to determine the infectivity titer of the viruses, which was expressed in plaque-forming units per milliliter (PFU/mL).

Cytotoxicity analysis

The toxicity of the (p-BthTX-I)$_2$K peptide in BHK-21 and Vero cells was determined by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, as previously described [29] with modifications. The cells were seeded in 96-well culture plates (TPP, Trasadingen, SWI) (5×10^3 cells/well) for 24 hours. Then, the cells were incubated with the (p-BthTX-I)$_2$K peptide at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50, and 100 µM. After 24 hours (BHK-21) or 48 hours (Vero), the medium containing the peptide was aspirated and 100 µL of MTT (Sigma-Aldrich, Missouri, USA) diluted in DMEM (Cultilab, São Paulo, BR) (final concentration: 1 mg/mL) was added to cells. After 30 minutes of incubation at 37 °C, the medium containing MTT (Sigma-Aldrich, Missouri, USA) was removed and 100 µL of dimethylsulfoxide (DMSO) (Synth, São Paulo, BR) was added to each well of the plate, which was agitated at 200 rpm for 5 minutes. The absorbance was measured at a wavelength of 572 nm on a plate reader (FLUOstar Omega/BMG LABTECH, Ortenberg, DE).

Analysis of activity of CHIKV-NLuc encoded reporter

The antiviral activity of the (p-BthTX-I)$_2$K peptide against CHIKV-NLuc was determined by measurement of activity of virus-encoded NLuc reporter. After the incubation period, the supernatant was removed, the cells were washed with PBS (phosphate buffered saline) solution and 30 µL of Renilla Luciferase Assay Lysis Buffer (Renilla Luciferase Assay Lysis Buffer, Promega, Wisconsin, USA) was immediately added to each well. After 30 minutes, the plates containing the cell lysates were placed in the plate reader (FLUOstar

Omega/BMG LABTECH, Ortenberg, DE), where 50 µL of substrate for Renilla Luciferase (Renilla Luciferase Assay Reagent, Promega, Wisconsin, USA) was automatically injected in the wells. The light intensity reading was performed and the values obtained were expressed as a percentage of expression compared to the vehicle control (sterile water).

ZIKV RNA yield inhibition assay using reverse transcription quantitative RT-PCR (qRT-PCR)

Briefly, (p-BthTX-I)$_2$K antiviral activity was evaluated based on the detection of ZIKV RNA copy numbers using a qRT-PCR assay. Supernatants from each well were collected at the end of every experiment and the total RNA was extracted using TRIzol reagent (Invitrogen, Massachusetts, USA) following the manufacturer's instructions. Virus load was then quantified using a two-step qRT-PCR. The cDNA was synthesized with a High Capacity cDNA Archive kit (Applied Biosystems, Massachusetts, USA), according to the guidelines of the manufacturer. Quantification of the synthesized cDNA was then carried out using QuantStudio™ 12 K Flex Real-Time PCR System (Applied Biosystems, Massachusetts, USA) utilizing ZIKV 1086, ZIKV 1162c, and 1107-FAM primers[30] and TaqMan Universal PCR Master Mix, No AmpErase® UNG (Thermo Fisher Scientific, USA) using 1µL of cDNA/reaction. The thermal cycler parameters included initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The cycle threshold (C_t) was analyzed and ZIKV RNA copy numbers of each sample were determined based on the standard curve, expressed on a $\log_{10}$ scale. For the postentry assay total RNA was isolated from infected cells. To analyze the abundance of ZIKV RNA in these samples its amounts were normalized to the mRNA of β-actin; the abundance of the latter was analyzed using primers (F: 5' CAGCACAATGAAGATCAAGAT; R: 5' – CTAGAAGCATTTGCGGTGGAC, 5' – [6FAM]ACCTTCCAGCAGATGTGGATC[BHQ1]), ΔCt method and the QuantStudio™ 12K Flex software v1.4.

Primary antiviral screening of (p-BthTX-I)$_2$K against CHIKV and ZIKV

The antiviral activity of the (p-BthTX-I)$_2$K peptide on the CHIKV and ZIKV infection was analyzed according to previously described Oliveira and coworkers [31] and Silva and collaborators [32] with modifications, respectively. For CHIKV screening, 1×10^4 BHK-21 cells were seeded per well of 96-well white culture plates (Greiner Bio-one, São Paulo, BR). For ZIKV screening, 2×10^4 Vero cells were seeded per well of 24-well culture plate (TPP, Trasadingen, SWI). Twenty-four hours later, the CHIKV-NLuc or ZIKV^{BR} at a multiplicity of

infection (MOI) of 0.1 and the (p-BthTX-I)$_2$K peptide at the maximum non-toxic concentration (MNTC), previously established in the MTT assay, were simultaneously added to cells. The plate was incubated at 37 °C for 16 hours (CHIKV) or 48 hours (ZIKV). The inhibitory effect of (p-BthTX-I)$_2$K on CHIKV was evaluated by measurement of NLuc activity and of the ZIKV by qRT-PCR as described above.

Analysis of the protective action of (p-BthTX-I)$_2$K against CHIKV and ZIKV infection

The protective action of the (p-BthTX-I)$_2$K peptide against the CHIKV and ZIKV infection was evaluated according to Oliveira and coworkers [31] and Carneiro and collaborators [33] with modifications, respectively. The cells seeded and incubated, as described above, were treated with the (p-BthTX-I)$_2$K at MNTC. After 1 hour of incubation at 37 °C, the supernatant was aspirated, the wells were washed twice with PBS, and the CHIKV-NLuc or ZIKV (MOI 0.1) was added to cells. The plate was incubated at 37 °C for 16 hours (CHIKV) or 48 hours (ZIKV). The inhibitory effect of (p-BthTX-I)$_2$K on CHIKV was evaluated by measurement of NLuc activity and of the ZIKV by qRT-PCR as described above.

Investigation of the virucidal effect of (p-BthTX-I)$_2$K

The investigation of the virucidal action of the (p-BthTX-I)$_2$K for the CHIKV was performed following the previously described [28,31] with modifications. For ZIKV, this assay was adapted from a protocol of Carneiro and collaborators [33]. For CHIKV, 1×10^4 BHK-21 cells were seeded per well of 96-well white culture plates while for ZIKV, 2×10^4 Vero cells were seeded per well of 24-well culture plate (TPP, Trasadingen, SWI). The virions of CHIKV-NLuc taken in amount to achive infection at a MOI 5 or ZIKV taken in amount to achive infection at a MOI 0.1 were incubated with the (p-BthTX-I)$_2$K peptide at MNTC at 37 °C (CHIKV) or room temperature (ZIKV) for 1 hour. Then, the viral inoculum was added to the cells. After 1 hour of incubation, the supernatant was removed, the cells were washed twice with PBS and DMEM (Cultilab, São Paulo, BR) supplemented with 2% FBS (Gibco – Thermo Fisher Scientific, Massachusetts, USA) was added to each well. The cells were incubated at 37 °C for the specific period for each virus. Sixteen hours post-infection (h.p.i.) the CHIKV replication was quantified by measuring NLuc activity. For ZIKV, 48 h.p.i. the viral RNA in the supernatant of the cells was quantified by qRT-PCR.

Evaluation of the antiviral activity of (p-BthTX-I)$_2$K on the CHIKV and ZIKV entry into the cells

The antiviral activity of the (p-BthTX-I)$_2$K peptide on the viral entry into the cells was analyzed according to previously described [28,31] with modifications. BHK-21 cells (for CHIKV) and Vero cells (for ZIKV) were plated as described above. After 24 hours of culturing the cells were infected with the CHIKV-NLuc at a MOI of 0.1 or ZIKV^{BR} at a MOI 0.1 in the presence of the (p-BthTX-I)$_2$K peptide at MNTC. After 1 hour of incubation at 37 °C, the supernatant was aspirated, the wells were washed twice with PBS, and DMEM (Cultilab, São Paulo, BR) with 2% FBS (Gibco – Thermo Fisher Scientific, Massachusetts, USA) was added to cells. The plate was incubated at 37 °C for 16 h.p.i (CHIKV) or 48 h.p.i (ZIKV). (p-BthTX-I)$_2$K inhibitory effects on CHIKV were evaluated by measurement of NLuc activity and effect on ZIKV was evaluated by qRT-PCR as described above.

Analysis of an impact of (p-BthTX-I)$_2$K on the CHIKV attachment to the cells

The antiviral effect of the (p-BthTX-I)$_2$K peptide on the CHIKV attachment to cell receptors was evaluated according to previously described [28,31,34] with modifications. For this, 1×10^4 BHK-21 cells were plated per well of 96-well white culture plate and cultured for 24 hours. After this, the plate was incubated at 4 °C for 15 minutes and CHIKV-NLuc (MOI 0.1) and the (p-BthTX-I)$_2$K peptide at MNTC were simultaneously added. The plate was again incubated at 4 °C. At this temperature, there is an interaction of viral particles with cell receptors, but the virus is not able to enter cell [28]. After 1 hour, the supernatant was removed, the cells were washed twice with PBS and DMEM (Cultilab, São Paulo, BR) supplemented with 2% FBS (Gibco – Thermo Fisher Scientific, Massachusetts, USA) was added. The cells were incubated at 37 °C for 16 h.p.i. (p-BthTX-I)$_2$K inhibitory effect on CHIKV attachment to host cells was evaluated by measurement of NLuc activity.

Evaluation of the antiviral action of (p-BthTX-I)$_2$K on the CHIKV internalization in cells

The antiviral action of the (p-BthTX-I)$_2$K peptide on the CHIKV internalization in cells was analyzed according to previously described [34] with modifications. BHK-21 cells (1×10^4 cells/well of 96- well white culture plate) were seeded 24 hours before the experiment; then the plate was incubated at 4 °C for 15 minutes. The cells were infected with the CHIKV-NLuc (MOI 0.1). After 1 hour of incubation at 4 °C, the supernatant was aspirated, the wells were washed twice with PBS, and supplemented with culture medium containing the (p-BthTX-I)$_2$K peptide at MNTC. After 1 hour of incubation at 37 °C, the supernatant was removed, the wells

were again washed twice with PBS, and DMEM (Cultilab, São Paulo, BR) with 2% FBS (Gibco – Thermo Fisher Scientific, Massachusetts, USA) was added to the cells. The plate was incubated at 37 °C for 16 hours h.p.i., and the CHIKV inhibition was evaluated by measurement of NLuc activity.

Analysis of the antiviral activity of (p-BthTX-I)$_2$K on the postentry steps of the CHIKV and ZIKV infection

The antiviral activity of the (p-BthTX-I)$_2$K peptide on the postentry stages of the infection of viruses was evaluated according to previously described [28,31] with modifications. BHK-21 and Vero cells plated and cultured, as described above, were incubated with the CHIKV-NLuc or ZIKV^{BR} (MOI 0.1) for 1 hour at 37 °C. After this period, the supernatant was aspirated, the cells were washed twice with PBS, and the (p-BthTX-I)$_2$K peptide at MNTC was added to each well. The cells were incubated at 37 °C for the specific period for each virus. Sixteen h.p.i. the CHIKV replication was quantified by measuring NLuc activity. For ZIKV^{BR}, 48 h.p.i. the viral RNA in the supernatant of the cells as well as intracellular viral RNA was quantified by qRT-PCR.

Statistical analysis

The cytotoxicity and antiviral activity experiments were performed in three independent assays. For CHIKV, each biological replicate was performed in three (cytotoxicity) or four (antiviral activity) technical replicates; for ZIKV, three (cytotoxicity) or two (antiviral activity) technical replicates were used. The statistical significance of observed cytotoxic and antiviral effects of the peptide were determined using the GraphPad Prism 5.0 software (GraphPad Software, California, USA). The statistical analyses were performed using the paired Student's T-test for parametric data and the Mann-Whitney test for non-parametric results. p value < 0.05 was considered statistically significant. The data obtained were normalized with the vehicle control and expressed in percentage.

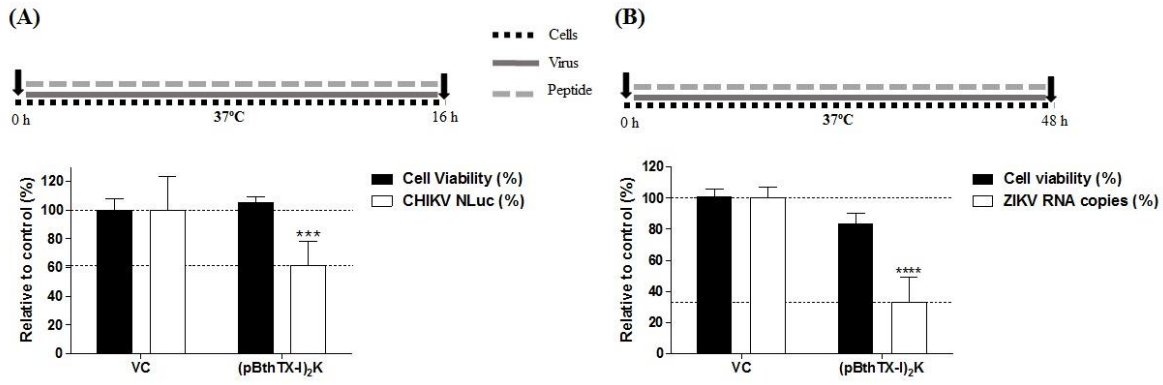
RESULTS

p-Bth peptide has antiviral activity against CHIKV and ZIKV^{BR}

The (p-BthTX-I)$_2$K peptide reached its MNTC in BHK-21 and Vero cells at 12.5 µM and 25 µM, respectively (Figure S1A and S1B, Supplementary Material). When applied at the maximum non-toxic concentration (MNTC), the (p-BthTX-I)$_2$K peptide inhibited 38.3% ($p \leq$

0.001) of the CHIKV replication compared to the vehicle control (VC) (Figure 1A); ZIKV^{BR} RNA levels were decreased about 67.3% ($p \leq 0.0001$) compared to the VC (Figure 1B). Thus, peptide caused a significant inhibition against of these two arboviruses warranting subsequent analysis of the effects on different stages of the CHIKV and ZIKV infection cycles.

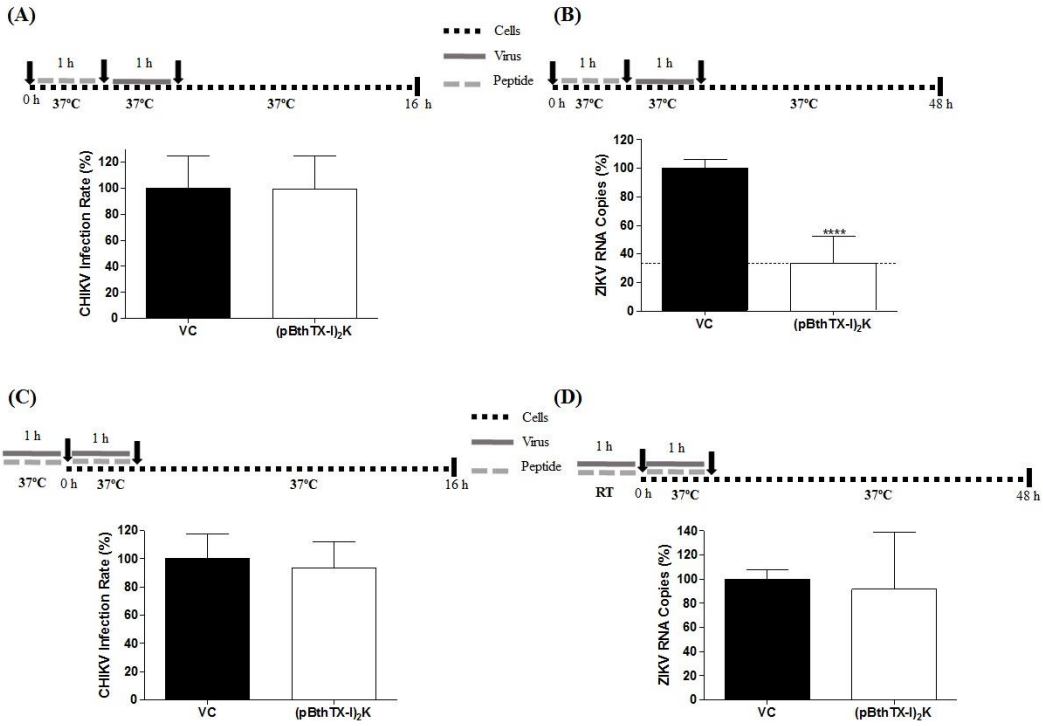
Figure 1. (p-BthTX-I)$_2$K inhibits CHIKV and ZIKV^{BR} infection. **(A)** BHK-21 cells were incubated with (p-BthTX-I)$_2$K at 12.5 µM and infected with CHIKV-NLuc at and MOI 0.1. CHIKV replication was analyzed measuring NLuc activity at 16 h.p.i. Mean values ± standard deviation (SD) were calculated from data from minimum of three independent experiments each performed in quadruplicate. **(B)** Vero cells were incubated with the (p-BthTX-I)$_2$K at 25 µM and infected with ZIKV^{BR} at a MOI 0.1. The RNA levels of ZIKV^{BR} in cell culture supernatant at 48 h.p.i. were quantified by qRT-PCR. Mean values ±SD represent data from three independent experiments, and each of which was performed in duplicate. *****:** $p \leq 0.001$; ******:** $p \leq 0.0001$. **VC:** vehicle control (sterile water). The schematic representation of the respective experiment is shown over each graph.



Prophylactic effect of the (p-BthTX-I)$_2$K against CHIKV and ZIKV^{BR} infection

To assess the protective effect of treatment of cells with (p-BthTX-I)$_2$K against CHIKV and ZIKV infection, the cells were pretreated with the peptide at the MNTC. After 1 hour of incubation, the cells were washed with PBS to remove the peptide and infected with the CHIKV-NLuc or ZIKV^{BR}. It was found that the pretreatment with (p-BthTX-I)$_2$K peptide was not able to protect the BHK-21 cells against CHIKV infection (Figure 2A). However, pretreatment with this peptide was capable to protect Vero cells against ZIKV as it is eveident from significant inhibition of 66.5% ($p \leq 0.0001$) in the ZIKV RNA levels for treated cultures compared to the cultures treated with VC (Figure 2B).

Figure 2. Prophylactic and virucidal activity of the (p-BthTX-I)$_2$K peptide. **(A)** BHK-21 cells pretreated with peptide at 12.5 µM had no protection against CHIKV-NLuc infection (MOI 0.1). **(B)** Treatment of Vero cells with peptide at 25 µM protects cells against infection of ZIKV^{BR} (MOI 0.1). **(C)** The peptide at 12.5 µM has no impact on CHIKV virions. **(D)** The peptide at 25 µM has no impact on ZIKV^{BR} virions. CHIKV replication was analyzed measuring NLuc activity at 16 h.p.i. The RNA levels of ZIKV^{BR} in cell culture supernatant were quantified by qRT-PCR at 48 h.p.i. Mean values ± SD represent data from a minimum of three independent experiments each performed in quadruplicate for CHIKV and in duplicate for ZIKV. ******:** $p \leq 0.0001$. **VC:** vehicle control (sterile water). The schematic representation of the respective experiment is shown over each graph.



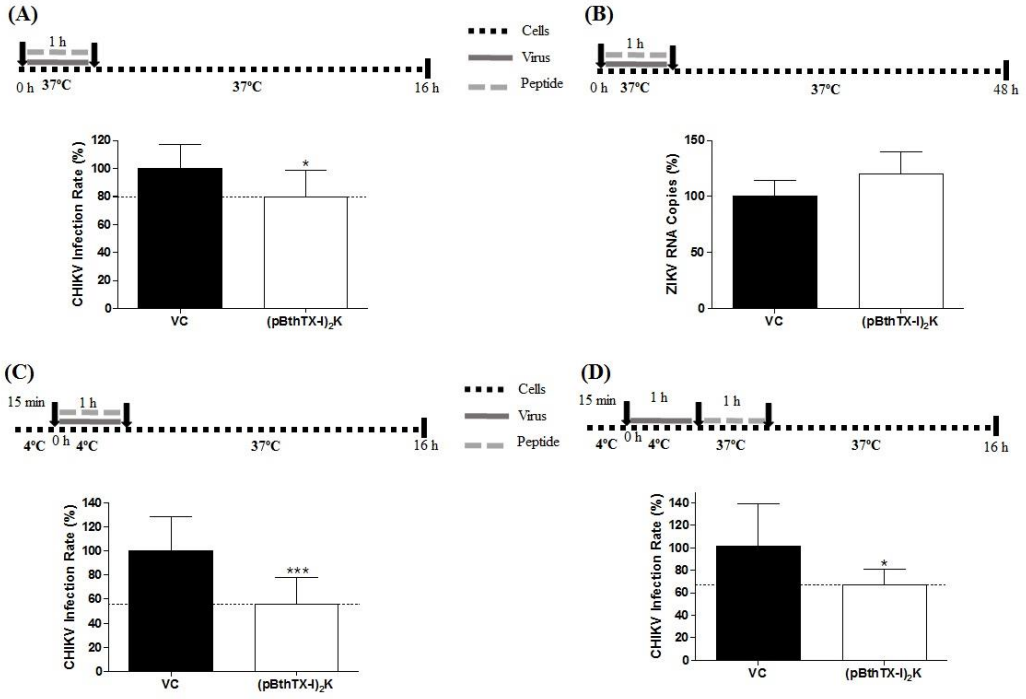
Virucidal effect of the (p-BthTX-I)$_2$K peptide on the CHIKV and ZIKV extracellular particles

To analyze if the (p-BthTX-I)$_2$K peptide has a virucidal effect, CHIKV-NLuc or ZIKV virions were treated with the peptide at MNTC for 1 hour and then used to infect the cells. In this assay, the (p-BthTX-I)$_2$K peptide did not demonstrate the ability to act on CHIKV and ZIKV virions as no reduction of NLuc levels in CHIKV-NLuc infected cells or in ZIKV RNA levels was observed (Figures 2C and D).

(p-BthTX-I)$_2$K peptide inhibits the early stages of the CHIKV infection, but not of the early stages of ZIKV infection

The action of the (p-BthTX-I)$_2$K peptide on the early stages of the CHIKV and ZIKV infection was evaluated. It was found that the peptide impaired the CHIKV-NLuc entry into the BHK-21 cells, resulting in a modest (20.3%) but significant ($p \leq 0.05$) reduction of the viral replication levels in relation to the VC (Figure 3A). In contrast, to ZIKV, (p-BthTX-I)$_2$K did not inhibit the entry step (Figure 3B).

Figure 3. Effect of the (p-BthTX-I)$_2$K peptide on the early stages of the CHIKV and ZIKV^{BR} infections. **(A)** The peptide has significant inhibition action at 12.5 µM on the CHIKV-NLuc (MOI 0.1) entry into BHK-21 cells. **(B)** The peptide at 25 µM does not inhibit entry of ZIKV (MOI 0.1) into Vero cells. **(C)** The (p-BthTX-I)$_2$K has a significant inhibition effect at 12.5 µM on the CHIKV-NLuc attachment to BHK-21 cells. **(D)** The peptide has significant inhibition activity at 12.5 µM on the CHIKV-NLuc internalization into BHK-21 cells. CHIKV replication was analyzed by measuring NLuc activity at 16 h.p.i. The RNA levels of ZIKV were quantified by qRT-PCR at 48 h.p.i. Error bars represent ± SD. Mean values ± SD are calculated from data from a minimum of three independent experiments each performed in quadruplicate for CHIKV and in duplicate for ZIKV. ***:** $p \leq 0.05$; *****:** $p \leq 0.001$. **VC:** vehicle control (sterile water). The schematic representation of the respective experiment is shown over each graph.



MR1903 peptide affects both the attachment and internalization of the CHIKV

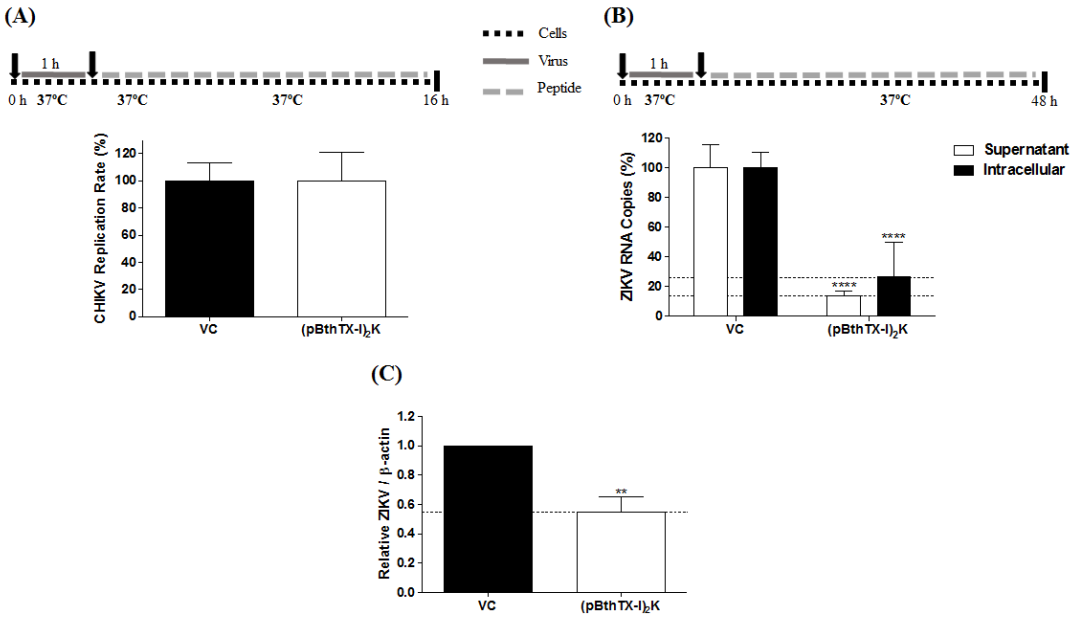
The early stages of the viral infection include attachment and internalization of the virus in host cells [35]. Thus, the antiviral effect of the (p-BthTX-I)$_2$K peptide on these specific steps of the CHIKV entry was investigated to determine how this peptide acts in the early stages of the viral infection.

It was observed that the (p-BthTX-I)$_2$K peptide significantly inhibited the CHIKV entry by reducing the virus attachment in BHK-21 cells by 44% ($p \leq 0.001$) when compared to the VC (Figure 3C). Furthermore, the (p-BthTX-I)$_2$K was also able to inhibit significantly CHIKV on the internalization stage. At this step, the CHIKV inhibition was 34.4% ($p \leq 0.05$) compared to the VC (Figure 3D). The fact that these inhibitory effects are more prominent than the one observed in entry assay (Figure 3A) probably originates from different experimental conditions (such as use of low temperature step in attachment and internalization assays).

(p-BthTX-I)$_2$K peptide inhibits postentry stages of the ZIKV, but not of the CHIKV infection

Finally, we assessed the antiviral action of the (p-BthTX-I)$_2$K peptide on the postentry steps of the CHIKV and ZIKV infection cycles. It was found that the peptide did not exhibit an antiviral effect on the processes that occur after the CHIKV entry into BHK-21 cells (Figure 4A). In contrast, the peptide was capable to inhibit postentry steps of the ZIKV infection in Vero cells: a significant difference between peptide treated and VC treated cells was observed. The effect was also prominent: levels of ZIKV RNA in the supernatant were reduced by 86% ($p \leq 0.0001$) while 73.6% ($p \leq 0.0001$) of reduction was observed for the intracellular ZIKV RNAs (Figure 4B). qRT-PCR analysis using β-actin mRNA as standard confirmed that not only absolute levels of intracellular ZIKV RNAs but also their relative levels (normalized to mRNA of β-actin) were significantly ($p \leq 0.01$) reduced in (p-BthTX-I)$_2$K treated cells (Figure 4C). Based on the study of Batista and collaborators, we also verified if the effect in postentry against ZIKV could be related to the release step, calculating the difference between intracellular and supernatant [36]. We observed that there is no significant reduction of virus RNA in supernatant compared to intracellular RNAs indicating that the (p-BthTX-I)$_2$K did not act in the release step.

Figure 4. Analysis of the effect of the (p-BthTX-I)$_2$K peptide on postentry stages of the CHIKV and ZIKV infection. **(A)** Effect of the peptide at 12.5 µM on the postentry steps of the CHIKV-NLuc in BHK-21 cells. **(B)** Effect of the peptide at 25 µM on the postentry stages of ZIKV in the Vero cells measured by amount of virus RNA in supernatant and in infected cells. **(C)** Normalization of ZIKV RNA levels in total intracellular RNA to the mRNA of β-actin. CHIKV replication was analyzed by measuring NLuc activity at 16 h.p.i. The levels of ZIKV^{BR} RNAs were quantified by qRT-PCR at 48 h.p.i. Error bars represents ± SD. Mean values ± SD represent data from a minimum of three independent experiments each performed in quadruplicate for CHIKV and in duplicate for ZIKV. ****:** $p \leq 0.01$; ******:** $p \leq 0.0001$. **VC:** vehicle control (sterile water). The schematic representation of the respective experiment is shown over each graph.



DISCUSSION

The CHIKV and ZIKV arboviruses currently represent an emerging health threat. Although the mortality of the disease caused by these viruses is considered low, the morbidity associated with the CHIKV infection is high, causing social and economic impacts [37]. The ZIKV infection is also a concern, due to neurological alterations, such as Congenital Zika Syndrome and Guillain-Barré syndrome [38]. Since there are no antivirals and vaccines approved against these viruses, it is necessary to search for potential therapeutics [11,17].

Peptides exhibit characteristics that favor their use in therapeutic approaches, such as cellular biocompatibility and low toxicity [18-20]. The peptide (p-BthTX-I)$_2$K is a synthetic peptide whose structure was based on another peptide [(p-BthTX-I)$_2$] derived from the BthTX-I toxin of snake venom. This peptide demonstrated activity against several Gram-positive and Gram-negative bacteria [23,39], and antiviral activity against SARS-CoV-2 [24], which is an enveloped positive-sense single-strand RNA virus such as ZIKV and CHIKV. Here, we assessed the antiviral activity of the (p-BthTX-I)$_2$K peptide against CHIKV and ZIKV infection, as well as the steps of viral replicative cycle that this peptide acted.

Our results demonstrated that this peptide impaired the infection of these viruses, being the antiviral effect against ZIKV more pronounced. However, the antiviral compounds can act at different stages of the replication cycles [40,41]. Somewhat unexpectedly it was found that the steps of the infection that the (p-BthTX-I)$_2$K peptide inhibited were completely different for these viruses. The peptide inhibited the CHIKV infection by interfering with the early steps of the viral replication cycle, preventing the CHIKV entry into the BHK-21 cells. In contrast, pre-treatment of cells with (p-BthTX-I)$_2$K was protective against ZIKV and the peptide also inhibited postentry steps of ZIKV infection.

The entry stage of the CHIKV into host cells is divided into two steps. Firstly, the viral particles attach to the cell's surface. There includes a non-specific interaction between cell and virus that is followed by specific binding between the E2 glycoprotein, present on the surface of the viral particle, and specific receptors on the host cells, such as the cell adhesion molecule Mxra8, the main receptor of CHIKV, as well as the mucin 1 (TIM-1), the type 1 prohibition (PHB1) and glycosaminoglycans (GAGs). This interaction triggers the CHIKV internalization into cells through clathrin-mediated endocytosis. After endosome acidification, there is a conformational change in the E1 glycoprotein present in the viral envelope, resulting in the exposure of the hydrophobic fusion peptide and consequently fusion of the viral and endosomal membranes. After this process occurs, the nucleocapsid is released into cell cytoplasm, being then disassembled for the release of viral RNA [35,42]. Thus, the viral entry process involves different factors, making this step a target widely used by several antiviral compounds [43].

We have shown that the (p-BthTX-I)$_2$K peptide inhibited the CHIKV entry by interfering in both attachment and internalization of the virus in BHK-21 cells. Furthermore, we observed that the viral attachment was affected to larger extent than the virus internalization in the cells. We also demonstrated that the peptide does not exert its inhibitory action on the CHIKV entry into BHK-21 cells by protecting the cells against viral infection or by damaging

the viral particle since this peptide did not exhibit virucidal effect and pre-treatment of cells provided no protection against subsequent CHIKV infection. It is not easy to explain these finding, as most compounds with an inhibition effect on the viral entry act through two main mechanisms: (1) direct action on the virus structure, inactivating the virion and consequently preventing its interaction with the host cell; and/or (2) interference with host cell components that are crucial to occur the viral infection [28,44].

In summary, our data showed that the (p-BthTX-I)$_2$K peptide exerts its activity against CHIKV when used as a co-treatment. The inhibition effect on the viral attachment suggests that one of the antiviral action modes of this peptide is characterized by interference in the binding of the viral glycoprotein E2 with cell receptors. One of the characteristics of the peptides that favors their therapeutic approach is cellular biocompatibility. The peptides are natural ligands for several cell surface receptors, demonstrating high affinity and specificity [18-20]. Besides, the (p-BthTX-I)$_2$K peptide falls into the category of cell-penetrating peptides (CPPs) [21]. This peptide class enters the eukaryotic cells without damaging the plasma membrane. Neundorf (2019) reported that GAGs, indicated as cellular factor important for CHIKV binding, are essential for the interaction of CPPs with cells [45]. Therefore, as CPP's and CHIKV have cell receptors in common, competition may occur between peptides and virus for binding sites on the cells. Probably, the (p-BthTX-I)$_2$K peptide has a greater affinity than the E2 viral glycoprotein to the cell receptors, such as GAGs, reducing the number of viral particles that enter the cells.

The antiviral compounds that prevent the CHIKV internalization in host cells mainly target clathrin-mediated endocytosis and endosome acidification [43]. As the main entry mechanism of the CPPs into eukaryotic cells is by endocytosis [45], we hypothesized that, when added concomitantly to BHK-21 cells, the virus and the (p-BthTX-I)$_2$K peptide can internalize cells in the same endosome. Thus, the (p-BthTX-I)$_2$K peptide can perform its antiviral action on the CHIKV internalization by (1) blocking the reduction of pH in the endosomal environment, preventing the conformational change of the E1 viral glycoprotein and consequent fusion of the viral and endosomal membranes; and/or (2) interacting with the E1 viral glycoprotein, preventing the exposure of the hydrophobic fusion peptide and consequent fusion of the viral envelope and cell membrane [40]. Finally, it is plausible that presence of CPP causes premature or delayed release of virus genome to the cytoplasm that may hamper subsequent events in virus infection.

Our work also demonstrated that the (p-BthTX-I)$_2$K peptide does not cause an antiviral effect on the later stages of the CHIKV replication cycle. Thus, this peptide did not affect the steps that follow the CHIKV entry into BHK-21 cells, such as the synthesis of viral RNA, production of non-structural and structural proteins of the virus, and assembly and release of the viral particle [40].

Regarding ZIKV, the (p-BthTX-I)$_2$K peptide showed a protective effect of Vero against ZIKV infection but did not act in the entry step. As CHIKV, the GAGs, required for the interaction of the CPPs with the cells, also may be attachment factors to ZIKV entry into the cells. However, even if ZIKV and CPPs have common attachment factors [45,46], the impact of (p-BthTX-I)$_2$K treatment is clearlt different. For the ZIKV, the protection recuires pre-treatment of cells i.e. peptide needs to be present before the viral infection. In the co-treatment case, the peptide was not capable to inhibit the viral entry, and ZIKV seems to have had more success when competing with cell receptors. In addition, considering that endocytosis is the main mechanism to CPPs entering the cells [45], we hypothesized that after pre-treatment of the Vero cells with (p-BthTX-I)$_2$K, the peptide was internalized by endocytosis, being loaded for sites into the cells where postentry stages of ZIKV replication will occur, and acting in these stages after the ZIKV infection of the cells. If this is indeed the case, the protective effect of pre-treatment may be related to mechanism used for inhibition of postentry stages of ZIKV infection.

The postentry stages of positive-strand RNA virus infection are genome translation, RNA replication, virion assembly, and release [47]. In summary, after the entry into the cells and the internalization step, ZIKV RNA is released in the cytoplasm and is translated into viral proteins from the only open reading frame (ORF) in the viral RNA takes place; the translation product represents a polyprotein which is cleaved into structural and non-structural proteins. RNA replicase is formed by non-structral proteins, associated to intracellular membranes [48,49] and binds viral RNA to initiate RNA replication. First, negative-sense RNA is synthesized, followed by the synthesis of new positive-sense single-strand RNAs. Then, the new viral RNAs are packaged by structural proteins, forming immature enveloped virions budding in the endoplasmic reticulum. Then, these virions go to the secretory pathway in the trans-Golgi network, maturing by the cleaved prM protein in M protein, and the new virions are released from the cell by exocytosis [48,49].

The significant reduction of RNA levels in the intracellular and extracellular environment of treated Vero cells may indicate that (p-BthTX-I)$_2$K is acting in the RNA

replication steps [50]. Freire and collaborators verified that (p-BthTX-I)$_2$K showed an inhibitory potential of the protease PL^{pro} of SARS-CoV-2, and the authors suggest that peptides derived from BthTX- I, such as (p-BthTX-I)$_2$K, may be attractive to inhibit enzymatic activities of viral and cellular proteins [24]. The inhibition of these activities can be related to the effect in ZIKV postentry steps. SARS-Cov-2 has a positive-sense single-strand RNA, which works as mRNA, ready for translation of the viral polyprotein, and viral proteases act cleaving it into viral proteins [51], similar to the case of ZIKV. The potential of (p-BthTX-I)$_2$K to inhibit enzymatic activities, as seen to SARS-CoV-2, may be related to this effect in postentry steps of the ZIKV, potential target being the NS2B-NS3 complex (protease) or NS5 (RNA polymerase) of ZIKV [47]. However, the effect may also be more indirect – flavivirus RNA replication occurs on membranes of endoplasmatic reticulum and if the (p-BthTX-I)$_2$K is capable for altering the structure/composition/properties of this cellular compartment it may have a negative impact for ZIKV RNA replication as well. Other possibilities are that the peptide is interfering in the viral assembly and/or maturation of the virions. In relation to the viral release, our results show that this step seems not to be affected by (p-BthTX-I)$_2$K as it causes a similar reduction of the RNA levels in the extracellular and intracellular. But more studies are necessary to better clarify the effect of the (p-BthTX-I)$_2$K in the ZIKV replication cycle.

In conclusion, the obtained data evidenced that the (p-BthTX-I)$_2$K peptide has antiviral activity against CHIKV by interfering in the early stages of the viral infection. The peptide was able to inhibit the virus entry into BHK-21 cells by impairing the attachment and internalization in the host cell. In addition, our study demonstrated that the inhibitory action of the (p-BthTX-I)$_2$K peptide on the CHIKV entry was more significant on the viral attachment than on the internalization of the virus in the BHK-21 cells. For ZIKV, this peptide caused a protective effect in Vero cells against the infection by this virus and was capable to decrease the ZIKV RNA levels by inhibiting postentry steps of the viral infection cycle. Therefore, this work suggests that the (p-BthTX-I)$_2$K peptide may be a promising antiviral drug candidate against CHIKV and ZIKV and, based on the antiviral properties of this peptide, highlights the use of this structure to the development of new broad-spectrum antivirals derivatives.

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Supplementary Material

The Dimeric Peptide $(KKYRYHLKPF)_2K$ has a Broad-spectrum Antiviral Activity Inhibiting Different Steps of Infection of the Chikungunya and Zika Viruses

Gabriela Miranda Ayusso [1#], Maria Letícia Duarte Lima [1#], Paulo Ricardo da Silva Sanches [2#], Igor Andrade Santos [3], Daniel Oliveira Silva Martins [1,3], Pâmela Jóyce Previdelli da Conceição [1], Tamara Carvalho [1], Cíntia Bittar [1,4], Andres Merits [5], Norival Alves dos Santos-Filho [6], Eduardo Maffud Cilli [6], Ana Carolina Gomes Jardim [1,3], Marilia de Freitas Calmon [1], Paula Rahal [1*]

[1] *Universidade Estadual Paulista (UNESP), Instituto de Biociências, Letras e Ciências Exatas*, São José do Rio Preto, SP, *Brasil*.

[2] *Universidade Estadual Paulista (UNESP), Faculdade de Ciências Farmacêuticas*, Araraquara, SP, *Brasil*.

[3] *Universidade Federal de Uberlândia (UFU), Instituto de Ciências Biomédicas*, Uberlândia, MG, *Brasil*.

[4] Laboratory of Molecular Immunology, The Rockefeller University, New York, NY, USA.

[5] University of Tartu, Tartu, EE.

[6] *Universidade Estadual Paulista (UNESP), Instituto de Química*, Araraquara, SP, *Brasil*.

[#] These authors contributed equally to this work.

*** Corresponding author**

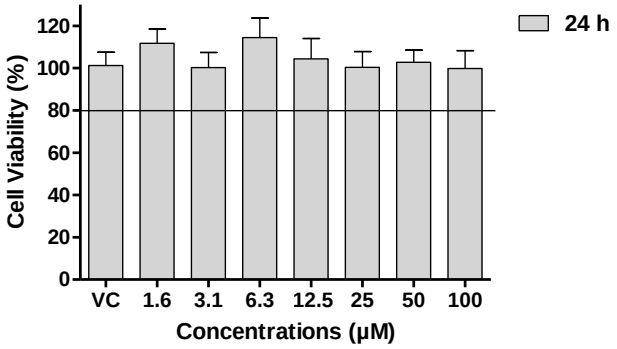
E-mail address: p.rahal@unesp.br

Determination of the maximum non-toxic concentration (MNTC) in BHK-21 and Vero CCL-81 cells

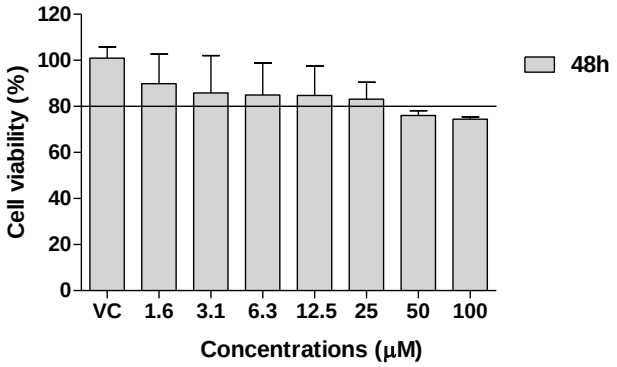
The cytotoxicity of the $(pBthTX\text{-}I)_2K$ peptide was analyzed in BHK-21 cells at 24 hours or 48 hours in Vero cells by the MTT (3(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) method. The peptide was tested at concentrations of 1.6, 3.1, 6.3, 12.5, 25, 50 and 100 µM in BHK-21 and Vero cells. The maximum non-toxic concentration (MNTC), expressed in µM, was defined as the highest concentration of the peptide that maintained at least 80% of the cells viable within 24 hours or 48 hours. Considering the arbitrary threshold of 80% cell viability, the $(pBthTX\text{-}I)_2K$ peptide reached its MNTC in BHK-21 cells at 12.5 µM at 24 hours (Figure S1A) and 25 µM in Vero cells at 48 hours (Figure S1B).

Figure S1. Cytotoxic effect of the $(pBthTX\text{-}I)_2K$ peptide in **(A)** BHK-21 cells and **(B)** Vero cells. Black lines represent the arbitrary limit of 80% cell viability. **VC:** vehicle control (sterile water). Error bars represents ± standard deviation (SD).

(A)



(B)



CAPÍTULO III –

Conclusão

1. CONCLUSÃO

Os peptídeos sintéticos AG-peptídeo e $(p\text{-BthTX-I})_2$ mostraram-se potenciais antivirais contra o ZIKV pela análise feitas em células Vero, sendo que o primeiro apresentou inibição de mais etapas do ciclo replicativo viral do que o segundo.

O peptídeo inédito AG-peptídeo teve atividade virucida, mostrando capacidade de agir diretamente na partícula viral e também efeito protetivo das células Vero contra a infecção por ZIKV. Esse peptídeo também inibiu a entrada viral, mais especificamente as etapas de adsorção e internalização, apresentando ação mais acentuada na primeira. O AG-peptídeo também teve ação sobre os estágios pós-entrada do ZIKV nas células hospedeiras, esse feito pode ter sido sobre a replicação, montagem e/ou liberação, mas não parece estar relacionado a última etapa, devido ao fato de não ter ocorrido diferenças significativas nos níveis de RNA viral intracelulares e do sobrenadante dessas células.

Com relação ao peptídeo $(p\text{-BthTX-I})_2$, este apresentou efeito protetivo contra o ZIKV e também inibiu as etapas pós-entrada, mas, mais uma vez, o efeito parece não estar relacionado a liberação pelo mesmo motivo que o peptídeo AG-peptídeo.

Conclui-se então que, apesar de mais estudos serem necessários para determinar os mecanismos de ação de ambos os peptídeos, o presente trabalho apresentou dois peptídeos com potencial para desenvolvimento de antivirais contra o ZIKV. Um desses peptídeos, o AG-peptídeo, é inédito e teve atuação em praticamente todas as etapas do ciclo replicativo analisadas no trabalho e, futuramente, e pode ser testado contra outros flavívirus. O $(p\text{-BthTX-I})_2$ teve efeito em menos etapas da infecção viral comparado ao AG-peptídeo, mas também surge como uma alternativa ao tratamento contra o ZIKV, e de vírus pertencentes à outras famílias, como o CHIKV e o SARS-CoV-2, tendo potencial para o desenvolvimento de um antiviral de amplo espectro.

CAPÍTULO IV –

Artigos publicados e patente

1. ARTIGOS PUBLICADOS

Brazilian Journal of Microbiology (2022) 53:1279–1287
https://doi.org/10.1007/s42770-022-00761-x

CLINICAL MICROBIOLOGY - RESEARCH PAPER

Early infection of Zika virus in the male reproductive system of AG129 mice: molecular and immunohistochemical evaluation

Maria Letícia Duarte Lima[1] · Ágata Silva Cabral[1] · Cintia Bittar[1] · Luiz Roberto Falleiros Junior[1] · Luiz Henrique Alves Guerra[1] · Bruno Moreira Carneiro[1,2] · Luis Carlos de Souza Ferreira[3] · Maurício Lacerda Nogueira[4] · Sebastião Roberto Taboga[1] · Marília Freitas Calmon[1] · Paula Rahal[1]

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Abstract
Sexual transmission of Zika virus (ZIKV), an important arbovirus, and the virus persistence in semen raise several questions about how and where it circulates in the male reproductive system (MRS). Several studies reported detection of the virus in testes, epididymis, and prostate at 5 days post-infection (dpi) or more in animal models. In the present study, we investigated the interactions of ZIKV with mouse MRS using the AG129 strain, a ZIKV permissive immunodeficient mouse strain, at two dpi. Viral RNA was detected in blood, testes, epididymis, and prostatic complexes (prostate and seminal vesicles). Immunohistochemical (IHC) analyses, based on the envelope protein, showed an early infection in organs of MRS since ZIKV positive antigens were detected in cells within or surrounding blood vessels, Sertoli, and germ cells in testes and epithelial cells in epididymis and prostate. Positive antigens for NS5 protein, the virus RNA-dependent RNA polymerase, were also detected by IHC in these organs and circulating leukocytes, suggesting that the virus replicates in these sites as early as 2 days post-infection. Analysis of the early stages of ZIKV infection in MRS may improve the current knowledge about this issue and contribute to the development of therapies directed to the infection at this site.

Keywords ZIKV · Zika virus · Male reproductive system · Mice · AG129

Cell Biology International

Research Article

Postnatal exposure to finasteride causes different effects on the prostate of male and female gerbils

Juliana S. Maldarine, Bruno D. A. Sanches, Vitória A. Santos, Ágata S. Cabral, Maria L. D. Lima, Carolina M. Bedolo, Marília F. Calmon, Paula Rahal, Rejane M. Góes, Patricia S. L. Vilamaior, Sebastião R. Taboga ... See fewer authors

First published: 26 February 2020 | https://doi.org/10.1002/cbin.11328

Cell Biology International

RESEARCH ARTICLE

Explant culture: A relevant tool for the study of telocytes

Bruno D. A. Sanches, Juliana D. S. Maldarine, Guilherme H. Tamarindo, Alana D. T. Da Silva, Maria L. D. Lima, Paula Rahal, Rejane M. Góes, Sebastião R. Taboga, Hernandes F. Carvalho

First published: 19 August 2020 | https://doi.org/10.1002/cbin.11446 | Citations: 4

RESEARCH ARTICLE Previous Next

Prenatal exposure to finasteride promotes sex-specific changes in gerbil prostate development

Juliana S. Maldarine [A], Bruno D. A. Sanches [A], Ágata S. Cabral [B], Maria L. D. Lima [B], Luiz H. A. Guerra [C], Carolina M. B. Baraldi [C], Marília F. Calmon [B], Paula Rahal [B], Rejane M. Góes [C], Patricia S. L. Vilamaior [C] and Sebastião R. Taboga [A C D]

+ Author Affiliations

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2. PATENTE

- CARNEIRO, B. M.; SANCHES, P. R. S.; BITTAR, C.; CILLI, E. M.; AYUSSO, G. M.; **LIMA, M. L. D.**; BATISTA, M. N.; CALMON, M. F.; RAHAL, P. COMPOSTO COM PROPRIEDADES ANTIVIRAIS CONTRA VÍRUS ZIKA. 2021, Brasil. Patente: Privilégio de Inovação. Número do registro: BR1020210147377, título: "COMPOSTO COM PROPRIEDADES ANTIVIRAIS CONTRA VÍRUS ZIKA", Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial. Depósito: 27/07/2021